UFRRJ

INSTITUTO DE TECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

TESE DE DOUTORADO

FORMAÇÃO DE COMPLEXOS COACERVADOS A PARTIR DAS PROTEÍNAS DA CLARA DE OVO E POLISSACARÍDEOS

Clitor Junior Fernandes de Souza

2015

UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
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Sob a Orientação do Professor

Dr. Edwin Elard Garcia Rojas

Tese submetida como requisito parcial para obtenção do grau de **Doutor em Ciência e Tecnologia de alimentos**, no Programa de Pós Graduação em Ciência e Tecnologia de Alimentos, Área de Concentração Tecnologia de Alimentos.

Seropédica, RJ
Novembro, 2015

664
S729f Souza, Clitor Junior Fernandes
T de, 1982-
Formação de complexos coacervados a partir das proteínas da clara de ovo e polissacarídeos / Clitor Junior Fernandes de Souza – 2015.
161 f.: il.

Orientador: Edwin Elard Garcia Rojas.
Tese (doutorado) – Universidade Federal Rural do Rio de Janeiro, Curso de Pós-Graduação em Ciência e Tecnologia de Alimentos.
Inclui bibliografias.

1. Tecnologia de alimentos – Teses. 2. Ovos – Teses. 3. Proteínas – Teses. 4. Pectina – Teses. 5. Polissacarídeos – Teses. 6. Alimentos – Indústria – Teses. I. Garcia Rojas, Edwin Elard, 1972-. II. Universidade Federal Rural do Rio de Janeiro. Curso de Pós-Graduação em Ciência e Tecnologia de Alimentos. III. Título.

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TESE APROVADA EM 02/10/2015

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DEDICATÓRIA

Dedico esta Tese a Deus, aos meus pais e as minhas irmãs, pelo apoio e principalmente pelo carinho que sempre me foi dado ao longo de toda minha vida.

AGRADECIMENTOS

A DEUS em primeiro lugar por me orientar, por me dar motivação, perseverança, saúde e alegria de estudar.

Ao Dr. Edwin Elard Garcia Rojas por aceitar me orientar. "Eu queria te agradecer por tudo mais não sei como, você fez tanta coisa por mim, me ajudou quando mais precisei. O senhor é daquelas pessoas que nos ensinaram muito mais que teorias, que nos preparam também para vida. Faço registrar todo o meu carinho e gratidão".

A Dra. Arlene que tanto me ajudou no momento mais crítico do início desta jornada. Muito obrigado.

Agradeço à UFF pela permissão do uso de seus laboratórios e aos meus grandes amigos "Angélica, Juarez e Luciano". Meus amigos, vocês foram primordiais para o sucesso deste trabalho. "Muito obrigado". Agradeço muitíssimo a Capes pela bolsa de estudo que me manteve durante toda a jornada.

Agradeço a Université Laval pelo suporte e disponibilidade dos laboratórios. Em especial gostaria de agradecer a professora Sylvie Turgeon pelos ensinamentos e tempo despendido com nosso projeto.

Agradeço a UFRRJ pelo conhecimento que adquirir durante esses dois anos. Agradeço a uma amizade em especial construída durante a minha estadia no alojamento. "Juan muito obrigado pelo carinho e apoio durante toda esta jornada". Agradeço a minha amiga de classe Elaine pela amizade duradoura que desenvolvemos.

Devo meus sinceros agradecimentos ao CNPEM e ao LNNano pelo apoio a este projeto especificamente, pelas imagens de AFM contidas nos capítulos III e IV.

Agradeço aos amigos Julia, Andressa. Obrigado pela paciência e apoio durante todo este percurso da minha vida.

Agradeço a um casal muito especial "André e Kelly". A amizade de vocês e essencial para minha vida. Agradeço em especial a minha família "Mãe, Pai, Clis e Elinha". Sem vocês eu não chegaria até aqui. "Eu os amo muito". Agradeço a alguns amigos em especial que torcem por mim: Geise (Sister), Deise, Paulina e Julio (Pastor).

MENSAGEM

"Escolhe um trabalho de que gostes, e não terás que trabalhar nem um dia na tua vida. "

Confúcio

RESUMO

SOUZA, Clitor Junior Fernandes. **Formação de complexos coacervados a partir das proteínas da clara de ovo e polissacarídeos.** 2015. 162p. Tese de Doutorado (Doutorado em Ciência e Tecnologia de Alimentos). Instituto de Tecnologia de Alimentos. Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2015.

Proteínas e polissacarídeos são os hidrocolóides mais comumente utilizadas na indústria de alimentos, e a sua interação pode fornecer desenvolver produtos tais como complexos coacervados. Complexos coacervados ovoalbumina-pectina foram analisados em várias concentrações de NaCl com diferentes concentrações de proteína/polissacarídeo através potencial-ζ, e por difração de raios-X. Alterações nas concentrações de proteínas alteraram a região de formação de complexos insolúveis (no ponto isoelétrico). Quando as concentrações de NaCl foram aumentadas 0,1-0,4 M, a dissociação do complexo foi suprimida. Difração de raios-X do complexo coacervado ovoalbumina-pectina mostrou uma região parcialmente cristalina definida 27-20° sugerindo que a estrutura do complexo é mais organizada do que os polímeros amorfos individuais. A complexação entre lisozima (Lyso) e pectina cítrica (Pec) foi estudado *in situ* por acidificação (12,0 - 1,0) usando medições de turvação e potencial zeta. Os complexos foram analisados em diferentes concentrações de NaCl, com diferentes razões de proteínas: polissacárido. Na proporção de 1: 1 com 0,01 mol/L de NaCl a região de formação de complexo insolúvel correspondeu uma gama de pH de 7,0 até 2,0, o que representa um grande intervalo para aplicação destes complexos em diferentes matrizes alimentícias. Quando a concentração de NaCl foi aumentada de 0,01 mol/L a 0,2 mol/L foi possível observar uma redução progressiva da turbidez e a da gama de formação do complexo. As imagens de microscopia de amostras revelaram que complexo apresenta uma aparência esferoide como com a estrutura heterogênea contendo uma única fase polimérico de núcleo. O complexo Lyso / pectina representa um grande potencial em diversas aplicações comerciais da biotecnologia. A complexação entre lisozima / κ-carragena e ovoalbumina / κ-carragena foi estudada *in situ* por acidificação (12,0 - 1,0) usando potencial zeta, turbidez e medições reológicas. Os complexos foram analisados em diferentes concentrações de NaCl com diferentes proporções de proteína / polissacarídeo. À medida que a proporção de proteína / polissacarídeo aumentou de 1: 1 a 10: 1, os pHs críticos se deslocaram para valores de pH mais elevados para os complexos de ovoalbumina / κ-carragena, seguido pela diminuição dos valores de G' nas proporções 5: 1 e 10: 1. O aumento da razão nos complexos lisozima / κ-carragena suprimiu os pHs críticos, porém, os valores de G aumentaram com o aumento da razão. A adição de sal suprimiu a interação eletrostática entre proteínas e κ-carragena. Os dados reológicos associados com as imagens de microscopia demonstram a formação de complexos inter-poliméricos e nós sugerimos que estes complexos representam um grande potencial para incrementar a textura, a estabilidade mecânica e a consistência de produtos alimentícios. A complexação entre lisozima (Lyso) / goma xantana (XG) e ovoalbumina (Ova) / goma xantana foi estudada *in situ* por acidificação (12,0 - 1,0) usando potencial zeta, turbidez, (FTIR), e medidas reológicas. Os complexos foram analisados em diferentes concentrações de NaCl com diferentes proporções de proteína / polissacarídeo. O

aumento da proporção de 2: 1 a 10: 1 nos complexos Lyso/XG suprimiu a formação de pHφ, contudo, os valores de G' também diminuíram na razão 5: 1 e 10: 1, indicando que o excesso de proteína impactou na compactação de estruturas de rede. A adição de sal nos sistemas Ova/XG e Lyso/XG suprimiu a interação eletrostática entre proteínas e XG. Os dados reológicos associados com imagens de microscopia demonstraram que uma estrutura da rede do tipo gel foi formada em ambos os complexos, e sugerimos que os complexos representam um grande potencial para melhorar as propriedades sensoriais dos produtos alimentícios. Géis de lyso/XG foram estudados com o objetivo de identificar o papel dos biopolímeros na formação, bem como a influência da sua proporção sobre o mecanismo de gelificação, das propriedades de textura, no diâmetro dos poros e na sinérese dos géis. O excesso de proteína afetou a compactação do gel, o que resultou em geleis com zonas de junção densas e baixa capacidade de retenção de água. A força do gel dependeu principalmente do teor XG, e por este motivo, na proporção de 1:2 a dureza e a retenção de água foram mantidos elevados e o G' foi quase cinco vezes mais forte do que a razão de 1: 1. Géis liso-XG produzidos sem tratamento térmico e com baixo teor de água representam um grande potencial de aplicação para a indústria alimentícia e farmacêutica. Por fim, a influência da proteína (lisozima e β-lactoglobulina) sobre a formação de hidrogéis com a goma de xantana foi estudada em função de três concentrações de sólidos totais. Em geral, a tensão crítica se estendeu à medida que a concentração de sólidos totais foi aumentada. Comparando o valor de G' e G" entre géis produzidos com lisozima, e β-lactoglobulina notamos que os géis Lyso-XG são menos fortes do que BLG-XG. Este estudo experimental elucidou aspectos fundamentais sobre a formação de textura de hidrogéis e sugerimos estes resultados podem ser utilizados por pela indústria biomédica, farmacêutica e de alimentos para desenvolver novos produtos semi-sólidos funcionais com alto teor de água e baixa concentração de proteína.

Palavras Chave: Proteínas da clara, Pectina, Carragena, Goma xantana, Complexo coacervado

ABSTRACT

SOUZA, Clitor Junior Fernandes. **Formation of complex coacervates from egg white proteins and polysaccharides.** 2015. 162p. Ph.D Theses (Doctorate in Science and Food Technology). Instituto de Tecnologia de Alimentos. Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2015.

Proteins and polysaccharides are the most frequently used hydrocolloids in the food industry, and their interaction can provide products such as complexes coacervates. Ovalbumin-pectin coacervate complexes was analyzed in various NaCl concentrations with different protein:polysaccharide ratios by measuring zeta (ζ)-potentials, and by X-ray diffraction. Changes in protein concentrations led to shifts in the region of insoluble complex formation (at the isoelectric point). When NaCl concentrations increased from 0.1 to 0.4 M, complex dissociation were suppressed. X-ray diffraction of the ovalbumin-pectin coacervate complex showed a partially defined crystalline region from 27-20° suggesting that the structure of the complex is more organized than the individual amorphous polymers. The complexation between lysozyme (Lyso) and citrus pectin (Pec) were studied in situ by acidification (12.0-1.0) using zeta potential and turbidity measurements. The complexes were analyzed in different NaCl concentrations with different protein:polysaccharide ratios. In the ratio 1:1 with 0.01 mol/L of NaCl the region of the formation of insoluble complex corresponded a pH range 7.0 to 2.0 which represent a wide range to apply this complex on different matrix food. When NaCl concentration was increased from 0.01 mol/L to 0.2 mol/L was possible to see a progressive reduction of turbidity and the pH range of complex formation achieving in a total suppression of complex in 0.4 mol/L. The microscopy images of samples revealed that complexes presented spheroid like appearance with the heterogeneous structure containing a single core polymeric phase. The Lyso/Pectin complex particles described here may represents a great potential in various commercial applications in the biotechnological The complexation between lysozyme/κ-carrageenan and ovalbumin/κ-carrageenan was studied in situ by acidification (12.0-1.0) using zeta potential, turbidity and rheological measurements. The complexes were analyzed in different NaCl concentrations with different protein/polysaccharide ratios. As the protein:polysaccharide ratio increased from 1:1 to 10:1, critical pHs shifted to higher pHs with ovalbumin/κ-carrageenan complexes followed by a decrease of G' values of the ratios 5:1 and 10:1. The increase of ratio with lysozyme/κ-carrageenan complexes suppressed the critical pH transitions points forming large insoluble complexes from pH 12.0 to 1.0 and the values of G' increased simultaneously reaching the higher value of the ratio 10:1. Addition of salt to the ovalbumin/κ-carrageenan and lysozyme/κ-carrageenan mixtures suppressed the electrostatic interaction between proteins and κ-carrageenan. The rheological data associated with microscopy images showed that intrapolymer complexes with heterogeneous structure were formed for both complex, and we suggest that complexes represent a great potential to improving texture, mechanical stability, consistency, and taste of food products. The complexation between lysozyme (Lyso)/xanthan gum (XG) and ovalbumin (Ova)/xanthan gum was studied in situ by acidification (12.0-1.0) using zeta potential, turbidity, Fourier transform infrared spectroscopy (FTIR), and rheological measurements. The complexes were analyzed in different NaCl concentrations with different protein/polysaccharide ratios. The increase of the ratio from 2:1 to 10:1 for

Lyso/XG complexes suppressed the pH_{φ} forming insoluble at pH 12.0, however, the values of G' decreased at ratio 5:1 and 10:1 indicating that excess of protein impact on compacted the network structures. Addition of salt in the Ova/XG and Lyso/XG mixtures suppressed the electrostatic interaction between proteins and XG shifting to lowering pHs the critical pH transitions points. The rheological data associated with microscopy images showed that interconnected gel-like network structure with heterogeneous structure were formed for both complex, and it is suggested that complexes represent a great potential to improve sensorial properties of food products. Formation of Lysozyme-Xanthan Gum (Lyso-XG) gels were studied with the objective to identify the role of individual biopolymer as well as the influence of their mixing ratio on gelation mechanism, texture properties, pores, and syneresis. The excess of protein affected the compaction of gel, which resulted in gels with dense clusters, with poorer water-bolding capacity. The gel strength mainly depended on the XG content, hence, at the ratio 1:2 hardness and held-water increased and final G' was almost five times stronger than ratio 1:1. Lyso-XG gels produced without heat treatment and with low water content presented a great potential for application in the food and pharmaceutical industry. Finally, the influence of protein (lysozyme and β-lactoglobulin) on hydrogels formation with xanthan gum was studied as a function of three different solid total concentrations. In general, the critical strain enlarged as far as the solid total of the system was increased, and gels became more stronger and rigid. Comparing the value of G' and G" between gels produced with lysozyme, and β-Lactoglobulin, it was noted that Lyso-XG gels were stronger than BLG-XG. This experimental study elucidated fundamental aspects about texture formation of hydrogels and it is also suggested that results can be used by biomedical, pharmaceutical, and food industries to develop new functional semi-solid products with high water and low protein content.

Keywords: Egg white**,** pectin, carrageenan, xanthan gum, complex coacervate

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NOMENCLATURA / ABREVIAÇÕES

CA carragena
BSA Albumina do soro bovino
E' elasticidade da superfície de dilatação
E_a elasticidade aparente
G´ Módulo de armazenamento
G´´ Módulo de elasticidade
GA Goma árabica
GAL glutaraldeído
GDL Glucono-δ-lactone
GX goma xantana
IEC Comissão Internacional do Ovo
IgY imunoglobulina Y
IP índice de peróxidos
ITC Calorimetria de titulação isotérmica
KI iodeto de potássio
Lyso Lisozima
NaCl Cloreto de Sódio
OVA/Ova Ovoalbumina
PDADMAC poli (dialildimetilamônio)
PDI Índice de polidispersão
PEC Pectina de alto grau de metoxilação
pH Potencial de hidrogênio
pH_c Região de ligeiro aumento de turbidez
pH_ϕ Região de aumento abrupto de turbidez
$pH_{\phi 2}$ Região de dissociação do complexado
p*I* Ponto isoelétrico da proteína
pK_a (- log Ka)
T- Turbidez
$T\Delta S$ Entropia
β-lg β-Lactoglobulina
ΔG Total de energia livre de Gibbs
ΔH Entálpia
η* viscosidade complexa

SUMÁRIO

INTRODUÇÃO GERAL

O interesse na aplicação de tecnologias envolvendo a utilização de polissacarídeos vem aumentando na última década. Grandes investimentos têm sido realizados por este ramo da indústria para fabricar produtos com alto valor agregado. Como exemplo pode-se citar a goma xantana, polissacarídeo que se tornou desde a introdução no mercado, o espessante mais escolhido em muitas aplicações. Isto é devido ao fato de ter comportamento reológico único, pois forma soluções altamente viscosas em concentrações muito baixas, sem grandes alterações por mudanças no pH, presença de sais e temperatura do meio. Assim como a goma xantana, a pectina e a carragena também apresentam excelentes propriedades funcionais, tendo grande aplicabilidade nas indústrias alimentícias.

Aliado a esse fato está à necessidade de estudos na área de formação de complexos coacervados de tais polissacarídeos com proteínas já bastante úteis à indústria, como é o caso das proteínas da clara do ovo, que devido às suas importantes propriedades funcionais (coagulação, gelificação e formação de espuma), ação antimicrobiana e propriedades nutricionais, tem sido largamente empregada como ingrediente em alimentos processados. Porém, durante o processamento e o armazenamento, as proteínas da clara podem sofrer significativa perda de algumas de suas propriedades funcionais ou nutricionais devido a diversos fatores, como as altas temperaturas e pHs extremos aos quais podem ser submetidas.

Os complexos coacervados formados, após a mistura de polissacarídeos com proteínas, têm demonstrado grande potencial para melhorar as características funcionais dos biopolímeros de origem. A potencialidade de aplicação decorre do fato que certas propriedades do complexo se apresentam superiores em relação aos materiais de partida isolados, tornando-se assim uma ferramenta tecnológica importante nas indústrias de alimentos ou farmacêuticas para a proteção de agentes ativos, como as proteínas da clara de ovo.

No capítulo II e III foi estudado o processo de formação dos complexos coacervados entre ovoalbumina/pectina e lisozima/pectina em função da razão de proteína e NaCl em diferentes pHs e este estudo foi desenvolvido na UFF/RJ sendo o capítulo II publicado na revista *Food Hydrocolloids* no ano de 2015. No capítulo VI foi estudado a influência da proteína, pH, força iónica e da razão proteína/polissacarídeo na cinética da formação de complexos inter-poliméricos entre ovoalbumina/κ-carragena e lisozima/κ-carragena assim como as propriedades reológicas e este estudo foi realizado na UFF/RJ. No capítulo V estudou-se a influência da proteína, pH, força iónica e da razão proteína / polissacarídeo na cinética da formação de complexos inter-poliméricos entre ovoalbumina/goma xantana e lisozima/ goma xantana, assim como as propriedades reológicas e este estudo foi desenvolvido na UFF/RJ. No capítulo VI foi estudado o papel da lisozima e da goma de xantana na formação de gel, bem como a influência da proporção de ambos no mecanismo de gelificação e das propriedades de textura e sinérese dos géis e este estudo foi desenvolvido na *Université Laval*CA. Finalmente no capítulo VII foi estudado a influência da lisozima e β-lactoglobulina sobre a formação de rede induzido pela interação eletrostática comparando as propriedades mecânicas, de textura, de capacidade de retenção de água, e o tamanho dos poros entre os géis este estudo foi desenvolvido na *Université Laval*CA.

OBJETIVO GERAL

Estudar a formação de complexos coacervados a partir das proteínas da clara de ovo (Lisozima e ovoalbumina) com diferentes polissacarídeos (pectina de alto grau de metoxilação, goma xantana e carragena).

OBJETIVOS ESPECÍFICOS

- Capítulo II e III. Estudar o processo de formação dos complexos coacervados entre ovoalbumina/pectina e lisozima/pectina em função da razão de proteína e NaCl em diferentes pHs.
- Capítulo IV. Elucidar a influência da proteína, pH, força iónica e da razão proteína/polissacarídeo na cinética da formação de complexos inter-poliméricos entre ovoalbumina/κ-carragena e lisozima/κ-carragena assim como as propriedades reológicas e o rendimento de formação dos mesmos.
- Capítulo V. Estudar a influência da proteína, pH, força iónica e da razão proteína / polissacarídeo na cinética da formação de complexos inter-poliméricos entre ovoalbumina/goma xantana e lisozima/ goma xantana, assim como as propriedades reológicas
- Capítulo VI. Estudar o papel da lisozima e da goma de xantana na formação de gel, bem como a influência da proporção de ambos no mecanismo de gelificação e das propriedades de textura e sinérese dos geis.
- Capítulo VII. Estudar a influência da lisozima e β-lactoglobulina sobre a formação de rede induzido pela interação eletrostática comparando as propriedades mecânicas, de textura, de capacidade de retenção de água, e o tamanho dos poros entre os géis

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CAPÍTULO I

REVISÃO DE LITERATURA

1 HISTÓRICO SOBRE O ESTUDO DA FORMAÇÃO DOS COMPLEXADOS

A primeira explicação teórica sobre o fenômeno de coacervação foi proposta por BUNGENBERG DE JONG e KRUYT (1929), onde estes afirmam que a estabilidade dos hidrocolóides era influenciada por dois fatores sendo estes a carga elétrica capilar e a hidratação. A coacervação seria o resultado da remoção destes dois fatores, pois sugeria-se que isto iria provocar uma dessolvatação entorno das partículas, que então seriam fundidas formando assim os coacervados. Esta explicação foi usada inicialmente para o fenômeno de coacervação simples, contudo posteriormente, BUNGENBERG DE JONG (1949), ao estudar a formação de coacervação complexa entre gelatina/goma árabica (GA), este pode perceber que esta interação ocorria devido à alteração das cargas eletrostáticas ao redor dos biopolímeros (GA carga –; gelatina carga +) promovida pela alteração do pH e dos teores de NaCl.

OVERBEEK e VOORN (1957) com base nos resultados experimentais de Bungenberg de Jong desenvolveram a primeira teoria quantitativa sobre coacervação complexa tendo como modelo a coacervação entre gelatina/GA. Eles puderam interpretar que a coacervação ocorria por uma competição ente as forças eletrostáticas e os efeitos entrópicos que tendiam formar interação entre as moleculas carregadas ou como o caso dos efeitos entrópicos tendiam a dispersar a interação. Pode-se perceber que a presença de determinados solventes contribuiam para o aumento da entropia permitindo assim que houvesse um certo rearranjo das moléculas. Esta teoria foi baseada nas seguintes suposições: (1) as moléculas têm uma configuração aleatória da cadeia, (2) interações solvente-soluto são menos significantes, (3) as forças interativas são distribuidas conforme a orientação da sua natureza do biopolímero, portanto estas cargas são livres para se moverem (4) não existe qualquer interação local específico entre as moléculas.

O tratamento teórico da coacervação complexa foi colocado sobre uma base quantitativa por meio das equações de Debye-Hückel para as interações eletrostáticas e através da teoria de Flory-Huggins para a entropia. De acordo com esta teoria, para um sistema de dois componentes constituído por um sal poliônico e água as condições críticas satisfatórias para a coacervação são alcançadas quando $Ó^3r \geq 0{,}53$, isto é, quando a densidade de carga (ó) ou a massa molar (r) são suficientemente grandes. Este modelo foi aumentado para três ou quatro componentes de sistemas e Overbeek e Voorn explicaram que a supressão da coacervação causada pelo excesso de sal ocorreu devido a um aumento da solubilidade dos polions em detrimento da quantidade de polions do coacervado.

Veis e Aranyi desenvolveram uma teoria onde $Ó^3r < 0{,}53$, teoria esta que não se aplicava ao modelo de Voorn-Overbeek (VEIS e ARANYI, 1960; VEIS, 1961, 1963; VEIS et al., 1967). Esta teoria foi baseada em um caso prático de coacervação que ocorria com a redução da temperatura com duas gelatinas de cargas opostas. Veis modificou a teoria de Overbeek incluindo o parâmetro de interação Huggins, correspondente a interação solvente-soluto, pois este parâmetro aumentava com a diminuição da temperatura. Atraves desta nova forma de avaliação a coacervação foi considerada como um processo de dois passos ao invez de uma interação apenas espontâneo. Primeiro, as gelatinas formavam agregados espontaneamente por interação eletrostática até que estes agregados adquirisem entropia configuracional baixa e em seguida, estes agregados eram lentamente rearranjados para formar a fase coacervada. Este comportamento era impulsionado pelo ganho na entropia configuracional resultante da formação de uma fase de coacervado misturada aleatoriamente, pois Veis e Aranyi consideravam que as moléculas não eram distribuídas de forma aleatória em ambas as fases, mas que os agregados de ions emparelhavam-se na fase diluída.

A teoria Tainaka é o modelo mais recente desenvolvido para o entedimento da formação da coacervacão complexa e este modelo foi adaptada da teoria de Veis e Aranyi, porém a principal diferença era que os agregados presentes tanto na fase diluída quanto an fase concentrada eram formados sem o emparelhamento de íons específicos (TAINAKA, 1979; TAINAKA, 1980). Os agregados biopoliméricos presentes na fase inicial se condensam para formar um coacervado e de acordo com Tainaka, as forças motrizes para a separação de fases são a electrostática e a força de atração entre os agregados, pois estas se tornam mais fortes quando a massa molar e a densidade de carga dos polímeros aumentam. A densidade de carga e a massa molar dos polímeros devem cair dentro de um intervalo crítico para que a coacervação possa ocorrer, pois se a densidade de carga ou a massa molar do polímero aumentar acima de um ponto crítico, haverá a formação de um gel concentrado ou um precipitado induzido pelas forças de longo alcance entre os agregados. Por outro lado se a densidade de carga, ou a massa molar estiver abaixo do ponto crítico as forças repulsivas de curto alcance irão estabilizar a solução diluída e não haverá a formação de coacervado. A teoria proposta por Tainaka é mais abrangente do que todas as teorias anteriores e é aplicável a ambos os sistemas de alta e baixa densidade de carga fornecendo uma explicação adequada do processo de coacervação complexa para um grande número de sistemas.

2 COMPLEXAÇÃO

Macromoléculas são os principais componentes de produtos alimentícios formulados e o controle das propriedades estruturais de proteínas e polissacarídeos são um amplo tema de investigação, pois este está diretamente ligado às características físico-químicas e sensoriais do alimento (TOLSTOGUZOV, 2002). As interações entre macromoléculas do alimentos podem ser repulsivas ou atrativas, demonstrando assim dois fenômenos opostos que são a incompatibilidade biopolímerica e a formação do complexo (TOLSTOGUZOV, 2007). Tal separação pode ocorrer por meio de dois caminhos principais, além da miscibilidade entre os componentes (Figura 1), dependendo principalmente da carga elétrica das duas macromoléculas e, portanto, dos fatores que as afetam, como pH e a força iônica. Em um dos caminhos ocorre a incompatibilidade termodinâmica (segregação), também conhecida como separação segregativa de fases. Outra possível situação é a complexação (coacervacão complexa), também conhecida por separação de fases associativa (KASAPIS, 2009).

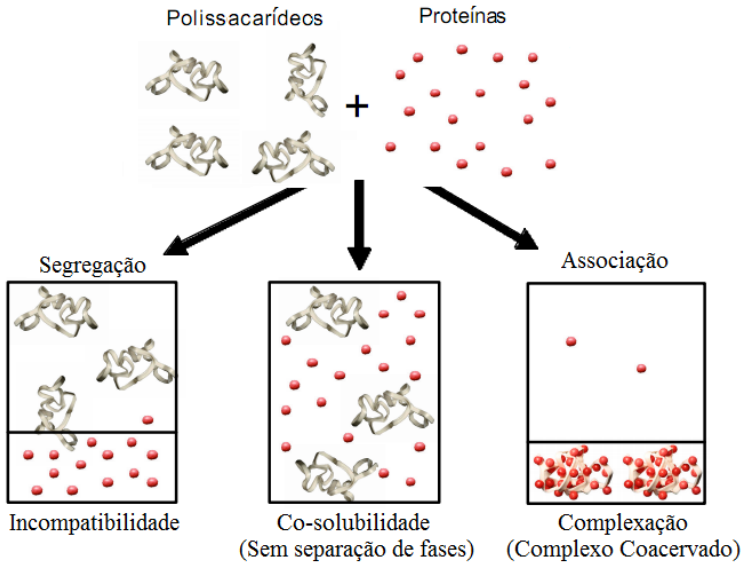

Figura 1: Principais tendências no comportamento de misturas de proteínas-polissacarídeos (adaptado de WEINBRECK, 2004).

A separação de fase segregativa ocorre quando há incompatibilidade termodinâmica entre os polímeros, predominando a repulsão eletrostática entre as moléculas. Com isso, são formadas duas fases aquosas imiscíveis, sendo uma rica em proteína e a outra em polissacarídeo. Isso pode ocorrer em altas concentrações e altas forças iônicas ou quando pelo menos um dos polímeros é um polieletrólito forte (KRUIF et al., 2004; KASAPIS, 2009).

A co-solubilidade ou miscibilidade ocorre quando a interação entre dois biopolímeros diferentes se comportam de maneira similar à interação entre dois da mesma espécie, ou seja, há uma miscibilidade instantânea (SYRBE et al., 1998).

Por outro lado, a compatibilidade termodinâmica, também chamada de separação associativa de fases ou coacervacão complexa, usualmente ocorre em concentrações relativamente menores (< 3 a 4%) de sólidos totais, baixas concentrações iônicas (< 0,4 mol/L) e quando as duas moléculas estão com cargas líquidas opostas. Tal compatibilidade ocorre em pH entre o ponto isoelétrico (pI) da proteína e o pK_a do polissacarídeo. Sob essas condições, as moléculas atraem espontaneamente umas às outras e ocorre à separação do sistema em duas fases, uma rica em proteína e polissacarídeo e a outra pobre em biopolímeros, mas rica em solvente. Na fase rica em biopolímero, a proteína e o polissacarídeo são mantidos unidos por forças eletrostáticas e podem tomar a forma de coacervado ou precipitado (MOHANTY e BOHIDAR, 2003; KASAPIS, 2009).

2.1 Aspectos termodinâmicos

A energia envolvida na formação do complexo de coacervação, ou complexos solúveis entre polissacarídeos e proteínas tem recebido muita atenção do meio científico e os principais métodos de estudo dos aspectos calorimétricos tem sido a utilização de intrumentos de calormetria de varredura diferencial (DSC) e calorimetria de titulação isotérmica (ITC) (TOLSTOGUZOV, 2002).

A formação de coacervados entre biopolímeros ocorre de forma espontaneamente quando o total de energia livre de Gibbs diminui (ΔG), sendo assim, ΔG< 0. Este valor negativo é alcançado de forma independente à energia acumulada de pelo contato entre os biopolímeros (PEROZZO et al., 2004). Para determinarmos a possibilidade de formação de complexado é preciso elucidar que há um delicado equilíbrio entre a entropia favorável (-*T*Δ*S*) e os altos valores entálpicos (ΔH) para a determinação dos valores de ΔG, pois a entropia favorável é responsável pela liberação das moleculas de água e de contraions já a entalpia diminui a mobilidade dos biopolímeros devido à modificação da interface do mesmo ou do complexado frente à água (JELESAROV e BOSSHARD, 1999; KRUIF et al., 2004; LAUGEL et al., 2006). É possível também relatar que ligações do tipo covalentes também contribuem para o ΔH, entretanto é muito dificil classificar a entalpia de cada ligação não covalente (JELESAROV e BOSSHARD, 1999).

A Calorimetria de titulação isotérmica (ITC) produz dois resultados: ΔH para todos os processos ocorrendo em mais de uma gama estequiométrica (não dependentes do modelo) e a isotérmica de ligação levando em consideração que os valores de entalpia obtidos refletem a progressão das ligações. Quando ΔG e o ΔH são combinados para obtermos o ΔS, as diferentes fontes destes dois termos devem ser considerados se o ΔH e o ΔS forem para permitir comparações qualitativas entre modelos propostos.

GIRARD et al. (2002) utilizaram ITC para determinar a constante de ligação e os valores de entalpia, estequiometria e entropia de complexos formados entre β-lactoglobulina/Pectina de alto e baixo grau de metilação e puderam constatar que os fatores entalpicos foram os responsáveis pela sobilização intrapolimérica enquanto que a formação dos complexos solúveis interpoliméricos foram promovidos pelos fatores entálpicos e entrópicos. HARNSILAWAT et al. (2006a) ao estudar a interação entre β-lactoglobulina e alginato de sódio em pH 3 e 4 encontrou caracteristicas exotérmicas devido a formação das ligações eletrostáticas, em contra partida quando o pH foi alterado para 4 e 5 houve uma transição das fases por diluição dos agregados de β-lactoglobulina. Outros autores também observam caracteristicas endotérmicas (HARNSILAWAT et al., 2006a; NIGEN et al., 2007; ABERKANE et al., 2010). Tal comportamento também pode ser explicado com base no modelo de simulação dinâmica de langevin que descreve dois cenários para a energia de complexação levando em consideração que o polieletrólito pode ser fraco ou forte. Polieletrólitos fracos demonstram que sua força de complexação advem da entalpia de ligação, impulsionada pelas interações eletrostáticas, em detrimento dos contraions liberados pela força de entropia. Por outro lado a liberação de contraions em polieletrólitos fortes contribui para um sinal endotérmico global (BALL et al., 2002; OU e MUTHUKUMAR, 2006).

3 PRINCIPAIS PARÂMETROS QUE INFLUENCIAM A FORMAÇÃO DO COMPLEXO PROTEÍNA/POLISSACARÍDEO

Os fatores que afetam o curso da separação associativa de fases e as características das estruturas formadas podem ser classificados como extrínsecos ou intrínsecos. Entre os extrínsecos estão à mistura macromolecular, pH, a força iônica, o teor de sólidos totais, temperatura, a taxa de acidificação e a taxa de cisalhamento durante a acidificação. Já os parâmetros intrínsecos estão relacionados com a natureza e as características das moléculas poliméricas, como massa molar, concentração total de macromoléculas no meio, densidade de cargas e flexibilidade das cadeias ((DICKINSON, 2003; KRUIF et al., 2004). Iremos apresentar abaixo os fatores extrínsecos e intrínsecos mais relevantes.

3.1 pH

O pH realiza um papel chave na formação de complexos proteína-polissacarídeo por interação eletrostática, pois este promove o aumento ou diminuição do grau de ionização dos grupos laterais funcionais dos biopolímeros (SCHMITT et al., 2005a; GENTÉ et al., 2010). Quando expomos uma proteína abaixo do seu ponto isoelétrico ocorre uma transição da densidade das cargas de uma maior protonação negativa para neutra e finalmente uma maior densidade negativa dos seus grupos laterais. O mesmo efeito também ocorre com os polissacarídeos aniônicos, porém esta transição ocorre mediante ao pK_a do polissacarídeos. Diferentes partículas de biopolímero podem ser formadas através da associação de um único par de polímeros. Este comportamento pode ser melhor elucidado ao observarmos a Figura 2 (DICKINSON, 2003; COOPER et al., 2005; CHANASATTRU et al., 2009).

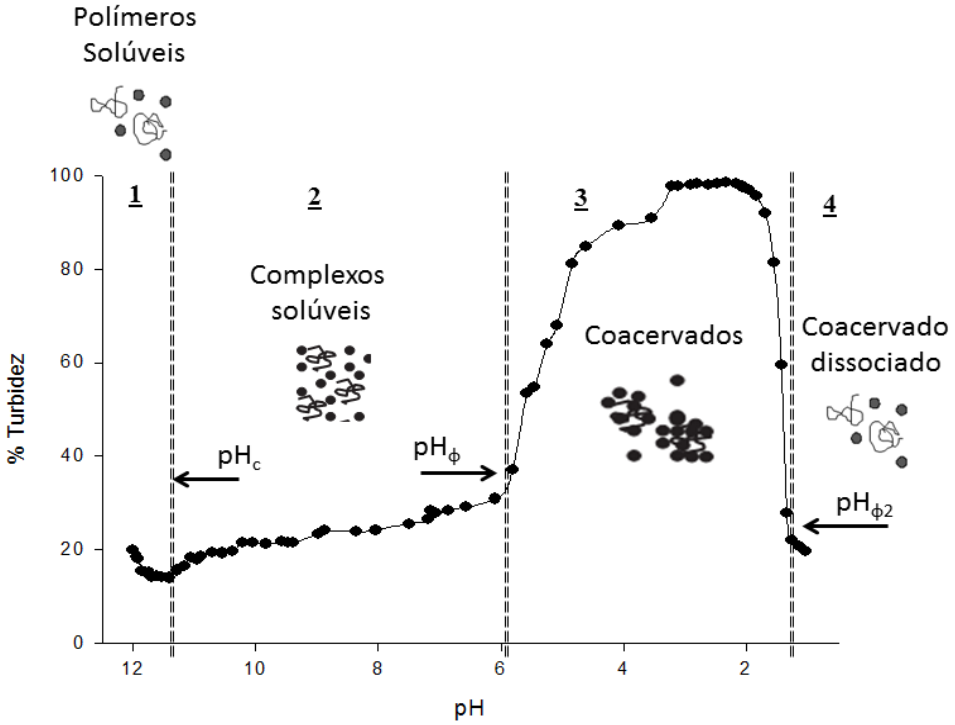

Figura 2: Regiões de estabilidade e instabilidade (1, 2, 3 e 4) do complexado em função do pH. Valores de turbidez de complexos formados entre Lisozima-Goma xanta na proporção de 1:1 de massa total do sistema com 0,1 mol/L NaCl. (Baseado em WEINBRECK, 2004).

1. Não Complexação (pH > pI). Nesta região as moléculas de proteína e polissacarídeo possuem uma grande densidade de carga negativa que promove repulsão eletrostática entre elas. Está repulsão impede que haja uma aproximação suficiente para sua associação (SANCHEZ e RENARD, 2002; ESPINOSA-ANDREWS et al., 2007; PICONE e CUNHA, 2010).
2. Complexos solúveis (pH < pH$_c$ < pH$_\Phi$). Quando o pH é reduzido abaixo de certo valor crítico o qual nos referimos como pH$_c$ podemos perceber um ligeiro aumento nos percentuais de turbidez, que ainda sim são menores que os valores no pH$_\phi$. Isso ocorre devido à fraca associação entre os polímeros que formam, majoritariamente, complexos solúveis, que são relativamente pequenos e por isso eles não impedem a passagem de luz,

levando a uma solução transparente ou ligeiramente turva. Esta associação fraca ocorre com frequência em pHs pouco acima do pI da proteína, pois mesmo ambos tendo densidade de carga negativa em sua superfície, ainda podem ser encontrados pequenos fragmentos de carga positiva na superfície da proteína na qual o polissacarídeo pode se ligar (XIA et al., 1993; TURGEON et al., 2007b). Esta associação fraca pode ser facilmente dissociada quando o pH ou o teor de NaCl é alterado, contudo uma vez formado o complexo solúvel existe a possibilidade de formação de interações covalentes, interações estas de caráter mais permanente que podem ser induzidas por métodos enzimáticos ou agentes físicos e químicos.

3. Coacervado ($pH < pH_{\Phi} < pH_c$). Ao avançar na região seguinte ao pH_c, nos deparamos com um abrupto aumento do percentual de turbidez e denominamos este ponto como pH_{ϕ}. É exatamente nesta região que ocorre a associação entre o polímero e a proteína, denominado como complexo interpolimérico ou coacervado. Estes complexos podem ser relativamente grandes podendo estar na ordem de 100-10000 nm, sendo esta a principal justificativa da não dispersão da luz e consequentemente altos valores de turbidez. Outra importante característica é a alta propensão a formação de coalescência entre os complexados. Isso ocorre devido à baixa densidade negativa em sua superfície. O resultado desta agregação é a precipitação dos complexados e a formação de duas fases, sendo a superior semitransparente, com baixa viscosidade e pobre em polímeros e a inferior rica em polímeros coacervados geralmente viscosos podendo até formar géis.
4. Dissociação do complexado ($pH << pH_{\Phi 2}$). Quando o pH é reduzido a valores abaixo do valor de pK_a do polissacarídeo, os grupos aniônicos da molécula perdem sua densidade de carga positiva, o que enfraquece as interações eletrostáticas entre o polissacarídeo e a proteína, tendo com resultado final a dissociação do complexado.

3.2 Força iônica

Em altas concentrações de NaCl, a densidade de íons transportada pelas proteínas e polissacarídeos é reduzida pela interação com os micro íons do NaCl, resultando numa diminuição na atração eletrostática entre as macromoléculas. Em força iônica elevada, o rastreio das cargas das proteínas e polissacarídeos também pode levar à redução de interações eletrostáticas e, portanto, afetar a formação de complexos (WEINBRECK et al., 2003a; YE e SINGH, 2006). Na baixa força iónica, a concentração micro íon tem somente um pequeno efeito sobre os complexos proteína/polissacarídeos complexos e o número de cargas presentes nas proteínas e polissacarídeos é suficiente para permitir a interação eletrostática

Quando a força iônica do sistema aumenta dois efeitos podem ocorrer. O primeiro efeito é a redução do número de moléculas de proteínas capazes de interagir com as cadeias de polissacarídeos ou polieletrólito, enquanto o segundo é a supressão ou modificação da constante dielétrica para as interações (WEINBRECK et al., 2004a; YE e SINGH, 2006; MATSUNAMI et al., 2007). O efeito da força iônica sobre o complexo coacervado foi descrito por FANG et al. (2006), onde estes puderam demonstrar que a força iônica aumentada foi capaz de suprimir a coacervacão entre gelatina/*κ*-carragena. Eles também relataram que quando os íons bivalentes foram usados à supressão da

coacervacão ocorreu em valores mais baixos de força iônica quando comparado com os monovalentes. Isto pode ser compreendido em termos de ganho de dupla entropia eletrostática, pois dois íons monovalentes são libertados em comparação a um único íon divalente. Uma característica interessante sobre a supressão da coacervacão complexa por adição de sal é que este processo pode ser reduzido com uma diluição do sistema através de um solvente, pois o efeito esperado seria que a sua concentração em massa de contra íons ao redor dos polímeros seja menor, favorecendo novamente assim a formação do complexo (BIESHEUVEL e STUART, 2004).

3.3 Proporção entre proteína e polissacarídeo e concentração total de biopolímero

A proporção de proteína e polissacárido na mistura influencia o equilíbrio de carga dos complexos e, portanto altera o comportamento de formação dos complexos. Para que a complexação máxima seja obtida é necessário conhecer a proporção exata de uma proteína frente a um polissacarídeo a uma dada condição de pH (TAINAKA, 1980; KIZILAY et al., 2011). Vários estudos têm mostrado que a proporção de proteína e polissacarídeo de mistura tem um grande efeito sobre as características do complexo resultante, ou seja, na composição e tamanho do complexo e conseguinte na viscosidade que este apresenta (RU et al., 2012). WANG et al. (2000), relataram que quando relação de polímero e proteína no sistema é baixa, são formados apenas os complexos intrapoliméricos. Entretanto quando esta relação é aumentada, pode ocorrer à formação de coacervado ou complexos interpoliméricos. Outro ponto a ser relatado frente às baixas concentrações é que os coacervados formados tendem a coalescer rapidamente formando coacervados ainda maiores ou tendem a permanecerem carregados ao longo de um amplo intervalo de pH enquanto que em altas concentrações isto não ocorre tão prontamente (SCHMITT et al., 2000; SCHMITT et al., 2001). Quando um complexado é formado com altas concentrações de proteína, este tende a ter grandes dimensões devido ao este excesso. Contudo WANG et al. (2007a) relataram que o aumento da concentração de β-Lactoglobulina em um complexado β-Lg/Pectina foi capaz de proporcionar uma maior elasticidade ao mesmo (SANCHEZ e RENARD, 2002).

Quando comparamos a concentração necessária para a separação das fases por segregação, podemos notar que a complexação pode ocorrer numa ampla faixa de concentração do biopolímero (10^{-2}mg ml^{-1}), quando a força iônica é suficientemente baixa (< 0,2 mol/L), no entanto, em concentrações muito elevadas de biopolímero há uma auto-supressão da interação devido à incompatibilidade termodinâmica, uma vez que, a coacervação complexa é favorecida por fatores entrópicos (TOLSTOGUZOV, 2002). Quando há formação de complexado em altas concentrações de biopolímero ocorre um impedimento do rearranjo do mesmo e o ganho de entropia é completamente perdido, não havendo força para conduzir a separação das fases (LI et al., 1994; TOLSTOGUZOV, 2002).

SCHMITT et al. (2000) puderam notar que a concentração total de biopolímero no sistema também está diretamente ligada à incompatibilidade termodinâmica devido principalmente, pela supressão das ligações, pois em seu estudo de formação de complexado β-lg/Goma acácia esse ponto crítico foi alcançado no pH 4,2, com uma concentração variando em torno de 4,5% até 20% do peso total do sistema. Entretanto em um sistema similar WEINBRECK et al. (2003a) puderam formar complexos entre proteína do soro do leite/goma arábica em pH 3,5, com 15% do peso total do sistema formado por biopolímeros. Acredita-se que isso tenha sido possível devido ao ganho de entropia no pH 3,5, quando comparado ao pH 4,5.

Outro parâmetro relatado é a possibilidade da concentração total de biopolímero modificar os valores de pH_c e do pH_Φ. WEINBRECK et al. (2003a) puderam constatar que altos valores de massa total de biopolímeros foram capazes de aumentar o valor de pH_ϕ, contudo outros trabalhos não encontraram relação alguma entre estes fatores (MATTISON et al., 1999; WEINBRECK et al., 2003b).

3.4 Influência da temperatura

Pode-se notar que a variação na temperatura promove eventualmente uma modificação na conformação estrutural de proteínas e polissacarídeos, além de gerar interações não eletrostáticas entre os mesmos pois, em princípio, baixas temperaturas favorecem as ligações de hidrogênio, enquanto altas temperaturas favorecem as ligações hidrofóbicas (SCHMITT et al., 1999; TOLSTOGUZOV, 2002).

HARDING et al. (1993) estudaram a formação de complexos entre BSA-Alginato variando a temperatura entre 35 e 95 ºC. Eles puderam perceber que entre 35 e 70 ºC os complexos não eram formados em pH 6,8 com concentração de NaCl de 0,1 mol/L. Contudo em temperaturas acima de 70 ºC os complexos foram formados, pois os grupos hidrofóbicos expostos pela desnaturação do BSA favoreceram a complexação. Resultados semelhantes foram encontrados por FANG et al. (2006), que notaram que complexos de gelatina/*κ*-Carragena só eram formados em temperatura acima da sua faixa de transição (25 ºC), enquanto que em temperaturas abaixo desse valor não havia complexação devido à incompatibilidade termodinâmica. Interações hidrofóbicas tem demonstrado grande importância na formação de complexados, haja vista, que em alguns casos a interação eletrostática seria impossível, a menos que as interações hidrofóbicas fossem formadas por aquecimento, primariamente (ZHANG et al., 2007). NIGEN et al. (2007) sugeriram que as interações eletrostáticas entre biopolímeros podem ser na maioria das vezes muito importantes na formação inicial dos complexos, entretanto em uma aplicação de larga escala de coacervacão ou agregação esta seria impulsionada principalmente por ligações de hidrogênio ou de interações hidrofóbicas, em função da temperatura.

Outro ponto importante referente à variação da temperatura foi à constatação de que esta pode afetar a organização estrutural do polissacarídeo (KAYITMAZER et al., 2007b). Ao comparar o efeito da temperatura de 12 e 25 ºC na estrutura de complexos formados entre BSA/Quitosana e BSA/Poli (cloreto de dialildimetilamónio), também conhecido como (PDADMAC), estes puderam constatar que mesmo que os polieletrólitos tivessem a mesma densidade de carga, propriedades reológicas diferentes foram verificadas para quitosana. Também vale ressaltar que alguns estudos tiveram como objetivo avaliar a influência da temperatura no pH_c e pH_ϕ, todavia estes puderam constatar que a temperatura não afetou estes valores (WEINBRECK et al., 2004b; SINGH et al., 2007; LEE e HONG, 2009).

3.5 Cisalhamento e pressão

Forças de cisalhamento podem ter um impacto importante sobre as propriedades dos coacervados e um importante parâmetro para ser observado na escala industrial. Verificou-se que quando as forças de cisalhamento foram aplicadas durante o processo de complexação houve uma reestruturação dos complexos interpoliméricos, causada pela competição entre as forças de interação eletrostática e as forças de cisalhamento além de poder ser observado o impedimento da floculação dos complexados na temperatura adequada devido à alteração da massa molecular dos mesmos causada pelo cisalhamento (SANCHEZ et al., 2001; LANEUVILLE et al., 2005b).

DUBIN et al. (2008) relataram a separação de fases induzida por cisalhamento a uma temperatura próxima da fase quiescente. Este comportamento foi atribuído à temperatura critica associada ou não a velocidade de corte alcançada, pois o líquido apresentou uma característica pseudoplástica acoplada a transição de fase o que conferiu estruturas micelares acopladas de polieletrólitos aos complexos formados (LIBERATORE et al., 2009).

A maioria dos estudos com sistemas polieletrólito/proteina, investigaram o efeito da velocidade de corte ou de cisalhamento sobre o tamanho das partículas de coacervado ou sobre a coalescência de suspensões de coacervado e o que se pode perceber é que o tamanho das partículas de coacervados suspensos diminuiu com a taxa de cisalhamento, enquanto que em uma taxa de cisalhamento constante o tamanho das suspensões de coacervado aumentou (LEE e CABANE, 1997; LANEUVILLE et al., 2005b; LIBERATORE et al., 2009).

GALAZKA et al. (1999) ao estudarem sistemas BSA/carragena relataram que ao aplicarem um tratamento de alta pressão nos complexados, houve um fortalecimento nas interações devido à maior exposição de grupos carregados causada pela parcial desnaturação da proteína. Resultado semelhante foi descrito com complexados formados entre BSA/polissacarídeos aniônicos, pois este estudo demonstrou que o tratamento com pressão promoveu uma maior mobilidade a cadeia da proteína parcialmente desnaturada o que promoveu interações ainda mais fortes e estáveis com polissacarídeo quando comparada com interações com a proteína *in natura* ou desnaturada (SAMANT et al., 1993; GALAZKA et al., 1997)

3.6 Massa molecular

Estudos sobre o efeito do peso molecular nas características dos complexos descobriram que polímeros com maior peso molecular promovem a formação de grandes complexos primários que se agregam mais prontamente em complexos interpoliméricos, uma vez que o polieletrólito atua como uma espinha dorsal para a formação dos complexos e possivelmente devido ao aumento da entropia (WANG et al., 2000; LANEUVILLE et al., 2005b).

Estudos sobre PDADMAC, um polímero catiônico forte, interagindo com micelas carregadas, mostraram que um aumento no peso molecular da micela reduziu a densidade necessária para a carga de coacervacão e aumentou o rendimento de coacervacão, concluindo que era necessária uma massa molar crítica para a formação do coacervado e que essa massa molar estava diretamente ligada ao tamanho final dos complexos (WANG et al., 2000). Cabe também salientar que ao controlar a massa molar dos polieletrólitos é possível manipular a densidade dos complexos e, por conseguinte controlar algumas de suas propriedades funcionais (LANEUVILLE et al., 2005b).

4 REOLOGIA DE COACERVADOS

Coacervados formados entre a associação de proteína-polissacarídeo formam uma fase densa e estruturada rica em biopolímeros e seu comportamento reológico varia de viscoso para viscoelástico, podendo até resultar em uma estrutura gelificada. As propriedades reológicas destes sistemas geralmente são moduladas pelo pH e pelo teor de biopolímeros (TAN e TAM, 2008). As partículas de coacervado podem ser produzidas para fornecer propriedades ou atributos reológicos desejáveis de um produto tal como a espessura ou a cremosidade, ou podem ser concebidos de modo a que eles não venham a

impactar negativamente os atributos esperados de textura de um produto como sua alta ou baixa viscosidade. Alguns alimentos necessariamente devem apresentar uma baixa viscosidade como é o caso de algumas bebidas, no entanto outros alimentos necessitam ser altamente viscosos ou devem apresentar características de um gel, tais como molhos, temperos ou sobremesas (JONES e MCCLEMENTS, 2010).

As propriedades reológicas de coacervados formados entre WPC/goma arábica foram estudados por WEINBRECK et al. (2004c) em função do pH e estes puderam verificar que o pH desempenhou um papel importante na viscosidade do coacervado, pois a viscosidade máxima foi encontrada no pH 4,0 devido a maior interação eletrostática entre os biopolímeros. Abaixo da taxa de cisalhamento de 30 s^{-1} uma viscosidade limitada foi observada, porém acima deste valor houve uma diminuição da viscosidade, pois quanto mais forte for à interação eletrostática entre os polímeros menor será a sua viscosidade devido principalmente à lenta reorganização das moléculas após o cisalhamento. Quando as misturas com a mesma composição em proteína de soro de leite e goma arábica foram expostas a pHs acima do seu ponto isoelétrico houve uma menor interação eletrostática entre os polímeros, o que resultou em um comportamento mais elástico do que viscoso (GUSTAW e MLEKO, 2003). Um comportamento semelhante também foi encontrado SCHMITT et al. (2005b) em complexados formados entre β-lactoglobulina-goma acácia com cargas completamente neutralizadas. Pode-se perceber que o coacervado adotou um comportamento mais elástico após a sua formação devido à reorganização das moléculas de proteína ao longo das cadeias do polissacarídeo. Ao observar esta organização também puderam concluir que o polissacarídeo estava modulando as propriedades reológicas do coacervado, pois à medida que usavam menos polissacarídeo a estrutura reológica do coacervado mais se assemelhava a um gel, característica esta, também relatada por outros autores (BOHIDAR et al., 2005; LANEUVILLE et al., 2006; WANG et al., 2007a; RU et al., 2012).

5 PRINCIPAIS PROPRIEDADES FUNCIONAIS DOS COMPLEXADOS COACERVADOS

5.1 Capacidade gelificante

O processo de formação do gel é resultado da agregação de moléculas desnaturadas com a formação contínua de rede entrelaçada, que exibe certo grau de ordem. A reação inicial do processo de gelificação envolve o enfraquecimento e quebra das ligações de hidrogênio e dissulfídicas, desestabilizando a estrutura conformacional das proteínas. Posteriormente, ocorre a organização das moléculas, produzindo uma estrutura tridimensional capaz de imobilizar fisicamente grande parte do solvente (ZASYPKIN et al., 1997). Na formação da estrutura tridimensional, três ligações podem ser formadas e a integridade física do gel é mantida pelo contrabalanceamento destas forças de atração envolvidas nas ligações e a repulsão entre as moléculas de proteína e destas com o solvente circundante. As ligações covalentes fornecem grande resistência e estabilidade ao gel, enquanto as não covalentes são mais fracas, deixando o gel mais suscetível à quebra. Nas atrações não específicas, podem ocorrer tais atrações entre regiões de uma mesma macromolécula ou entre moléculas (PICONE e CUNHA, 2010). O gama de géis formados entre associação de proteína-polissacarídeo pode ser classificado como interpenetrantes, redes acopladas e rede de fases separadas. A

temperatura, o pH e a força iônica são fatores importantes na capacidade de gelificação das moléculas nos alimentos (DAMODARAN *et al*., 2010; ORDÓÑEZ *et al*., 2005).

Ao analisar um gel formado entre β-lactoglobulina/*κ*-carragena utilizando análise micro reológica com oscilação da temperatura foi possível identificar a contribuição específica de cada biopolímero na formação do gel de fases separadas. Durante o aquecimento foi observado uma contribuição adicional do polissacarídeo no módulo de armazenamento (G`), característica esta não observada nos géis de rede acoplada. Além disso, foi possível observar que houve interação e difusividade entre os biopolímeros após a centrifugação e avaliação dos géis (OULD ELEYA e TURGEON, 2000; KASAPIS, 2008).

FIROOZMAND et al. (2012) avaliaram a influência do pH nos géis formados entre Gelatina/amido oxidado e puderam notar que nas condições normais no pH 5,2 as amostras exibiram separação de fases com uma morfologia característica de tipo spinodal. Porém, ao adicionarem um teor maior de amido (6%) associado a redução do pH (4,2) houve a maior rigidez do gel por inibição da separação das fases. Isso ocorreu devido à transição das fases induzida pela acidificação, pois em pH mais elevado houve incompatibilidade termodinâmica enquanto que em pH mais baixos houve interações associativas (FIROOZMAND et al., 2009). Outro estudo com géis formados com β-lactoglobulina/goma de semente de manjericão pode constatar que após o resfriamento, os géis apresentaram duas fases distintas, sendo que a superior obteve um aumento no modulo de armazenamento devido, a gelificação da proteína enquanto que a fase inferior apresentava um aumento no modulo de armazenamento devido a formação da rede com o polímero principal responsável pela fina microestrutura capaz de reter grande quantidade de água (RAFE et al., 2013).

Efeitos negativos podem ser também obtidos com a adição de polissacarídeos. A presença de pectina em um gel ácido de caseína impediu a agregação da caseína e consequentemente a formação de um gel quando sua concentração alcançou 0,8% de massa do sistema enquanto que a rigidez gel foi reduzida a concentrações mais baixas de pectina (MATIA-MERINO et al., 2004). Da mesma forma, se a proteína do soro do leite formar complexos com polissacarídeos o tamanho destas partículas devem ser monitorados, pois grandes complexos podem perturbar a formação da rede e resultar em géis mais fracos com uma textura arenosa (ROCHA et al., 2009).

5.2 Capacidade espumante

O estudo das propriedades interfaciais em interfaces ar-água constitui uma abordagem fundamental para compreender como polímeros podem absorver e interagir como espumas. Várias proteínas de alimentos tais como clara de ovo e gelatina, são amplamente utilizadas na produção de espuma, contudo com o objetivo de ampliar as funcionalidades destas proteínas alguns polissacarídeos têm sido adicionados (WIERENGA e GRUPPEN, 2010). Dois modelos de adsorção foram propostos dependendo da proteína inicial ou da proporção de mistura de polissacarídeo (GANZEVLES et al., 2006a): i) termodinamicamente limitado à adsorção de proteínas (barreira eletrostática) na interface, em que os complexos são carregadas por causa de um excesso de polissacarídeo, ii) uma difusão limitada a adsorção dos complexos quando a proporção de proteína e de polissacárido está perto de neutralização de cargas.

A formação e a estabilidade de espumas são as características funcionais mais importantes da albumina proveniente da clara do ovo e de proteínas do soro do leite, com muitas aplicações na indústria de alimentos. Produtos como *mousses* e sistemas proteína-açúcar, como o *mashmallow* e merengue, contém em sua composição a ovoalbumina, que

auxiliam de forma significativa na aeração (PELEGRINE e GASPARETTO, 2003). Vale ressaltar que as espumas formadas por proteínas junto à outros agentes de estabilização, como a gelatina e e outros polissacarídeos, apresentam maior estabilidade em relação à maioria das proteínas puras (DAMODARAN *et al*., 2010). Os polissacarídeos tem um impacto importante sobre a estrutura da interface e mediante a esta informação foi demonstrado que as proteínas não se organizam da mesma forma na interface quando complexadas com um polissacarídeo, pois a complexação de proteínas permite que haja uma preservação da sua conformação inicial, até mesmo na interface através da manutenção de um microambiente aquoso favorável (KUDRYASHOVA et al., 2007). Usando um modelo de β-lactoglobulina-pectina em pH 4,5, GANZEVLES et al. (2006b) demonstraram que as redes interfaciais com maior módulo de cisalhamento foram formadas quando a proteína foi adsorvida em primeiro lugar na interface em comparação ao complexos de adsorção, contudo, o módulo de cisalhamento das redes interfaciais de β-lactoglobulina-pectina formados a partir do complexo de adsorção foram maiores que a proteína sozinha alcançando seu valor máximo quando suas cargas foram neutras. SCHMITT et al. (2001) relataram comportamento muito semelhante com complexos β-lactoglobulina/goma acácia na proporção de neutralização de cargas. A elasticidade da superfície de dilatação (E') e a viscosidade (η_d) foram significativamente maiores quando comparada a β-lactoglobulina adsorvida sozinha. O efeito da razão de mistura de β-lactoglobulina-pectina sobre a estrutura da camada adsorvida foi relatado por GANZEVLES et al. (2008), onde se pode verificar que os complexos neutros foram capazes de formar uma estrutura muito mais densa e menos e espessa que os complexos carregados.

Ainda há necessidade de um estudo mais detalhado sobre a aplicação desta tecnologia nos alimentos propriamente ditos, no entanto, os resultados apresentados até o presente momento oferecerem um comportamento promissor em vários produtos, tais como sorvetes e as espumas obtidas a partir produtos lácteos (WIERENGA e GRUPPEN, 2010).

5.3 Capacidade emulsificante

As propriedades comumente hidrofóbicas dos complexos proteína-polissacarídeo possuem uma funcionalidade muito valorizada na interface óleo/água, que é a capacidade emulsificante. Para que uma emulsão seja formada por meio desta interação, primeiramente esta deve passar por um processo de homogeneização, que pode ser feito por alta pressão ou cisalhamento a altas velocidades (DICKINSON, 2001). Estas emulsões são caracterizadas geralmente por gotículas de óleo envoltas por uma fina camada de proteína complementar. Quando as condições de emulsificação são suficientes, à razão proteína/óleo e alcançada e uma emulsão não floculada pode ser formada, mas se gotas agregarem-se ou houver floculação isso pode ser um sinal de baixa concentração da razão proteína/óleo ou pode ser um excesso de proteína não adsorvida no sistema (DICKINSON, 2010). A fim de resolver este problema de estabilidade, é adicionada ao sistema, um surfactante ou um polissacarídeo irá gelificar o sistema impedindo a depleção ou floculação do emulsionado (DICKINSON, 2003; MOSCHAKIS et al., 2006; DICKINSON, 2009)

Dois tipos de métodos são comumente usados para gerar emulsões estabilizadas por complexos proteína-polissacarídeo: i) a emulsificação de uma fase de óleo em um complexo aquoso disperso, ii) a emulsificação de uma fase de óleo com uma solução aquosa dispersa por um biopolímero, seguido por um passo de enxague e posterior adsorção por um segundo biopolímero (emulsão camada por camada). Obviamente, as

duas técnicas levam a diferentes composições e estruturas interfaciais, e também a diferentes características de estabilidade frente à cremeação ou floculação, dependendo do pH, força iónica ou temperatura (GUZEY e MCCLEMENTS, 2006). Para que uma emulsão seja mantida constante com um teor baixo ou moderada de óleo, se faz-se necessário uma suave transformação do meio líquido para sólido. Os métodos mais populares de gelificação de proteínas são o tratamento térmico, acidificação ou tratamento enzimático (VAN VLIET et al., 2004c).

Em um estudo realizado sobre a formação de emulsões formadas entre β-lactoglobulina-pectina, verificou-se que a membrana multicamadas foi capaz de conferir estabilidade com a concentração de NaCl de 50mM em uma ampla de pHs ácidos (GUZEY et al., 2004). A provável explicação para este comportamento foi a repulsão eletrostática entre as gotículas formadas pelo complexo, promovida pelo NaCl, em detrimento das forças de van der Waals. (GUZEY e MCCLEMENTS, 2007). Características semelhantes foram encontradas em emulsões formadas entre β-lactoglobulina-ι-carragena ou β-lactoglobulina-goma acácia estabilizadas utilizando NaCl ou calor (90 °C), entretanto, quando as emulsões formadas entre os complexos β-lactoglobulina-goma houve floculação logo após a adição de 50mM de NaCl. Isso provavelmente deve ter ocorrido devido à baixa interação eletrostática entre os biopolímeros (GU et al., 2005; HARNSILAWAT et al., 2006b).

Foi demonstrado que complexo eletrostático formado entre WPI-goma xantana podem ser utilizados em sistemas água/óleo/água ou óleo/água/óleo podem ser utilizados para liberação de fármacos lipofílicos (BENICHOU et al., 2007b, a). O sistema de goma arábica- gelatina foi o primeiro a ser relatado para fins de encapsulamento e certamente é o mais estudado (THIES, 2007). Este sistema possui várias características chave para numerosas aplicações, pois forma coacervados viscosos durante a formação da microcápsula geralmente preparadas entre 50 e 60 º C. No resfriamento, a rigidez do gel aumenta resultando numa concha gelificada estável em torno do composto a ser protegido. Se a entrega do bioativo for no corpo humano, a gelatina é facilmente derretido na boca e as aplicações das emulsões coacervadas podem variar de liberação de sabor (YEO et al., 2005; PRATA et al., 2008) a proteção de nutrientes para mascarar compostos como gosto desagradáveis ou odor (PIERUCCI et al., 2007; LECLERCQ et al., 2010).

6 APLICAÇÕES INDUSTRIAIS DOS COMPLEXOS COACERVADOS

6.1 Controle e liberação de proteínas e/ou drogas a base de proteínas

Através da formação dos hidrogéis iônicos ligeiramente reticulados há possibilidade de controle e liberação de proteínas e/ou drogas a base de proteínas. Quando este hidrogéis sofrem estímulos ambientais como alteração da temperatura, pH, força iônica, íons específicos e metabólitos, há uma alteração em sua rede tridimensional, que pode promover a liberação ou retenção destas proteínas (BROMBERG e RON, 1998; PEPPAS e LEOBANDUNG, 2004; MALMSTEN et al., 2010).

Um aumento na concentração de grupos ionizáveis no polímero pode causar um aumento, ou uma diminuição, da liberação de proteína, dependendo do sinal de carga do polímero. Por exemplo, a quantidade de mioglobina liberada a partir de um hidrogel catiônico foi aumentada quando um homopolímero de metacrilato de 2-hidroxietilo (HEMA) foi substituído por um copolímero catiônico de metacrilato de dietilaminoetilo HEMA (ENDE et al., 1995). Outro ponto a ser citado é que proteínas com baixos pesos

moleculares e as proteínas globulares são facilmente liberadas quando comparadas com as proteínas de estrutura fibrosa.

Proteínas transportadas por hidrogéis têm sido utilizadas como materiais biocompatíveis em cronobiologia, ou seja, no estudo dos fenômenos biológicos temporais para o tratamento de doenças cronobiológicas (PEPPAS e LEOBANDUNG, 2004). A utilização de Alginato hidrofóbicamente modificado foi capaz de controlar a liberação de BSA e hemoglobina humana.

6.2 Encapsulamento

A microencapsulação é uma tecnologia utilizada para a proteção, estabilização e liberação lenta de ingredientes alimentares (SCHMITT et al., 1998). Os materiais de encapsulação ou de revestimento utilizados são geralmente constituídos de amido, derivados de amido, proteínas, gomas, lipídeos, ou qualquer combinação dos mesmos (BURGESS, 1994; JONES e MCCLEMENTS, 2010; KATONA et al., 2010). Os métodos de encapsulação de ingredientes alimentares incluem spray drying, liofilização, revestimento em leito fluidizado, extrusão, alta pressão, co-cristalização, inclusão molecular e coacervacão (SHAHIDI e HAN, 1993; RAO e MCCLEMENTS, 2012). A formação de complexados entre polissacarídeo-proteína é um método físico comum utilizado em microencapsulação de óleos e sabores para alimentos e bebidas, todavia este também tem sido utilizado para encapsular medicamentos, aditivos cosméticos, aromas, pesticidas, células vivas e vacinas (SCHMITT et al., 1998; CHANG et al., 2006; DONG et al., 2007; LECLERCQ et al., 2009; VOETS et al., 2009; HAMMAN, 2010).

BÉDIÉ et al. (2008) utilizaram complexos de WPI + pectina de baixo grau de metoxilação para avaliar a sua funcionalidade como uma matriz de retenção de tiamina. Foram avaliados dois processos de acidificação, sendo um durante a mistura e outro por titulação após a mistura. Através destes dois métodos foram avaliadas as características morfológicas do complexo, além da eficiência de encapsulamento do mesmo frente ao pH e a relação proteína-polissacarídeo. Pode-se notar que o maior rendimento ocorreu pelo método de acidificação durante a mistura. E isso ocorreu devido à busca acidificação das soluções durante o seu preparo, porém, a acidificação após a mistura proporcionou complexos menores e mais homogêneos, o que confere uma boa alternativa para aplicação em alimentos líquidos onde a preocupação com é sedimentação é recorrente.

Uma emulsão alimentícia instantânea foi formulada contendo azeite e sumo de limão utilizando complexos de polímeros, tais como alginato, goma arábica, maltodextrina e carboximetilcelulose tendo em vista o desenvolvimento de um novo produto microencapsulado. As misturas de maltodextrina-goma arábica apresentaram os menores valores médios de partícula, porém estas apresentaram formas arredondadas com pequenas depressões nas análises de microscopia electrónica de varredura, além de apresentarem características amorfas nas análises de difração de raios-X. Este estudo demonstrou uma possível forma de encapsulamento por secagem a frio que, segundo os autores, podem ser usados para produção de molhos de salada instantâneos (SILVA et al., 2013). ZIMET e LIVNEY (2009), demonstraram que nano complexos de β-Lg/pectina de baixo grau de metoxilação foram capazes de nano encapsular ácido docosaexaenoico (DHA), que é um ácido graxo essencialmente do tipo ω-3. Ao avaliar a formação da complexação no pH 4,5, pode-se perceber que o excesso de pectina foi capaz que reter cerca de 166 vezes mais DHA quando comparado com os valores encontrados no sistema e isso permitiu a formação de partículas diluíveis e transparentes com 0,05% de b-Lg e DHA, na proporção de 1:2 (b-Lg: DHA). Estas características conferiram ao encapsulado

uma eficiência de proteção de cerca de 80% mediante um teste de stress de 100h a 40 ºC, além de sugerir uma nova forma de enriquecimento de bebidas ácidas e claras.

Uma promissora aplicação do encapsulamento formado entre coacervados proteína + polissacarídeo é a utilização desta cápsula como forma de proteção de bioativos no trato digestivo superior (em condições de pH ácido), para em seguida, ser liberada no intestino delgado, onde o pH prevalece básico, o que favorece a biodisponibilidade e a bioestabilidade de algumas moléculas (LAMPRECHT e KAWASHIMA, 2006; BALAMURALIDHARA et al., 2011). A adição de surfactantes foi sugerida para melhorar a eficiência de encapsulamento (MAYYA et al., 2003), diminuir o diâmetro das gotículas e acelerar coacervacão de um sistema de goma arábica + gelatina (TAN e TAM, 2008). ZIANI et al. (2012) estudaram a influência do tipo de tensoativo (Tween 20, 60 e 80) e do tipo de óleo (vitamina E, D_3 e óleo de limão) sobre o encapsulamento dos componentes lipofílicos na micela do surfactante. Estes puderam constatar que os diferentes óleos e surfactantes tinham uma influência direta na natureza das dispersões formadas, pois microemulsões não podiam ser formados usando vitamina D ou E em soluções com 1% de Tween, devido ao tamanho de relativamente grande de moléculas lipofílicas em relação ao interior hidrofóbico das micelas tensoativas. Por outro lado, as microemulsões podiam ser formadas a partir de óleo de limão em concentrações relativamente elevadas de óleo e surfactante. Outro ponto importante relatado foi à constatação que o tipo de tensoativo não influenciou de modo tão significativo na formação das micelas, contudo Tween 20 parece ser menos capaz de solubilizar óleo de limão, quando comparado a Tween 60 ou 80, fato este que presumivelmente ocorre devido às suas pequenas dimensões.

Mediante as aplicações anteriores dos encapsulados, possivelmente a mais bem sucedida na indústria alimentícia tem sido de libertação de aroma por encapsulamento. As cápsulas de liberação de aroma consistem geralmente de uma gota de óleo vegetal rodeado por uma fina camada de coacervado, que dependendo das condições da matriz do alimento (teor de água), estas moléculas podem passar por permeabilidade por micro poros nesta fina barreira hidratada devido ao seu baixo tamanho molecular (menor que 200 Da) (GIVEN JR, 2009). No entanto, um dos principais desafios para a aplicação de encapsulamento de aromas por coacervacão é que diferentes óleos possuem diferentes graus de hidrofilicidade o que determina diferentes taxas de liberação da molécula. Contudo estudos mais detalhados destas taxas podem possibilitar alimentos industrializados ainda mais atraentes ao consumidor (TAYLOR, 2002; GIVEN JR, 2009).

6.3 Purificação de proteínas

Os coacervados formados podem ter suas interações dissociadas, o que proporciona um bom método de fracionamento de proteínas. A diferença entre o ponto isoelétrico da proteína e a densidade de carga do polímero a um dado pH são os principais fatores que permitem a precipitação seletiva de uma proteína numa mistura com polissacarídeos aniônicos (HANSEN e CHANG, 1968; CASAL et al., 2006). Este método oferece uma vantagem interessante que é a não utilização de solventes orgânicos, além de permitir que haja uma reutilização dos polissacarídeos em uma larga escala de purificação.

A recuperação de proteína de soro de leite pode ser realizada em condições de pH básico quando a quitosana foi utilizado para a formação do complexo, pois até 90% de β-Lg pode ser removida a partir do soro do queijo complexado com quitosana. A faixa de pH para recuperação variou entre 8 a 10 e forças iónicas foi variada entre 0,08 a 0,3 mol/L (MONTILLA et al., 2007). Curiosamente, uma variedade de outras proteínas como as

proteínas de soja e de batata podem ser fracionadas usando o mesmo princípio (SMITH et al., 1962; VIKELOUDA e KIOSSEOGLOU, 2004). Pois esta técnica mostrou eficiente até para o isolamento de imunoglobulinas Y (IgY) a partir de complexos formados entre proteínas da gema do ovo e polissacarídeos aniônicos (CHANG et al., 2000). As condições de isolamento de IgY de ovo foram optimizadas através da adição de várias concentrações de Alginato de sódio, κ-carragena, CMC, e pectina em um sistema contendo gema de ovo diluída. Pode-se verificar que a variação do pH afetou tanto a quantidade de complexados formados quanto o grau de recuperação da imunoatividade da IgY quando avaliada pelo método de imunodifusão de radial simples. Outro ponto a ser citado foi que o grau de recuperação variou entre 33 e 74% e que dentre os polissacarídeos testados, o uso de 0.15% de pectina no pH 5,0 exibiu o melhor grau de recuperação da imunoatividade da IgY.

6.4 Estruturação de biofilmes alimentícios

Ao longo dos últimos anos, várias pesquisas têm sido realizadas para desenvolver e aplicar biopolímeros a partir de uma variedade de commodities agrícolas e / ou resíduos da industrialização de produtos alimentícios (KUORWEL et al., 2011). Estes materiais apresentam a possibilidade de obtenção de filmes finos para revestimentos ou cobertura de alimentos frescos ou processados com um objetivo principal de estender a vida de prateleira. Os principais biopolímeros utilizados na formulação dos biofilmes são os amidos, os derivados de celulose, quitosana / quitina, gomas, proteínas (animal ou à base de plantas) e lipídios (CUTTER, 2006). Os filmes comestíveis e/ou revestimentos formados a partir destes biopolímeros oferecem algumas vantagens como uma segura comestibilidade, biocompatibilidade, uma boa aparência estética, propriedades de barreira, sendo não tóxico e não poluente e com baixo custo, além de possuir também capacidade de transporte de aditivos alimentares tais como agentes antimicrobianos ou antioxidantes (APPENDINI e HOTCHKISS, 2002; BOURTOOM, 2008; DUTTA et al., 2009).

DUAN et al. (2011) estudaram a capacidade encapsulante de óleo de peixe biofilmes formados a partir da interação quitosana/WPI. Estes filmes foram incorporados com 1,5 ou 2% m/v de óleo de peixe, 2% m/v de glicerol, Tween 80 na proporção 3 vezes maior que a massa de óleo de peixe ou 0,5% de óleo essencial de alecrim ou orégano. Ao avaliar esta capacidade encapsulante, estes puderam que perceber que após o armazenamento de 30 dias a 2 ºC destes filmes, houve um aumento no teor total de óleo nos filmes previamente formados com óleo de peixe, pois o percentual variou de 28 % com somente óleo de peixe, 32% para óleo de peixe e glicerol, 33,4% para óleo de peixe e Tween 80 e 37% para óleo de peixe e óleo de alecrim ou orégano. O teor de umidade e atividade de água não apresentou diferença significativa entre as diferentes formulações, contudo o aumento de óleo de peixe de 1,5% a 2% diminuiu a resistência à tração dos filmes, mas não o alongamento do mesmo. A adição de óleo de orégano diminuiu a oxidação do óleo de peixe encapsulado o que corroborou juntamente com as outras características para um meio simples e econômico de encapsulamento e estabilização de óleo de peixe em biofilmes, podendo ser aplicados em diferentes alimentos.

6.5 Demais propriedades

Proteínas complexadas eletrostaticamente com polissacarídeos aniônicos podem ter sua solubilidade aumentada devido à inibição da precipitação que esta sofre em pHs próximos ao seu ponto isoelétrico (JONES e MCCLEMENTS, 2010). A formação destes

complexos também pode proporcionar um meio prático de controlar a agregação térmica de proteínas globulares, pois a força desta interação pode inibir a agregação térmica das proteínas. Este comportamento ocorre provavelmente devido a limitação do número de acesso aos sítios reativos da proteína, consequência esta do baixo coeficiente de difusão do biopolímero. (TURGEON et al., 2007b; JONES e MCCLEMENTS, 2010).

7 BIOPOLÍMEROS

7.1 Ovo de Galinha

O ovo de galinha é conhecido como um dos alimentos mais completos, ocupando posição de destaque na área da nutrição por ser uma fonte de proteína de baixo custo, capaz de fornecer elementos indispensáveis à saúde. Além de ser rico em proteínas, é excelente fonte de minerais e vitaminas, principalmente as do complexo B, além das vitaminas A, D e E, que por serem lipossolúveis, depositam-se apenas na gema (FONSECA, 1985; SIM e NAKAI, 1994; LEANDRO et al., 2006).

Do ponto de vista legal, a simples designação de ovos indica os ovos oriundos de galinha, sendo necessário, quando se trata de ovos de outras aves, mencionar a espécie da qual procedem (BRASIL, 1990). De acordo com FONSECA (1985) o ovo é constituído de gema, clara (ou albúmen), casca e membranas da casca e, de uma maneira geral, sua composição depende de fatores como idade, tamanho, alimentação e estado sanitário das aves, sendo constituído basicamente de 74% de água, 12,5% de proteínas, 12% de lipídeos, 1% de sais minerais e 0,5% de carboidratos (FONSECA, 1985; SARCINELLI et al., 2007a).

A casca representa de 8 a 11% da composição proporcional do ovo e é formada por uma matriz de fibras entrelaçadas de natureza protéica e carbonato cálcico intersticiais, sendo composta por 94% de carbonato de cálcio, 1% de carbonato de magnésio, 1% de fosfato de cálcio. A matéria orgânica, bastante reduzida, apresenta-se principalmente na forma de proteínas (STADELMAN e COTTERILL, 1995a; ORDONEZ, 2005).

A gema representa de 27 a 32% da composição proporcional do ovo e contém grande fração de nutrientes como vitaminas, proteínas de alto valor biológico, fosfolipídios, ácidos graxos essenciais e minerais. É constituída de aproximadamente 47% de água, 35% de lipídeos, 16% de proteína, 1% de carboidratos e 1% de minerais (SARCINELLI et al., 2007b). De acordo com YAMAMOTO (1997) a maioria dos lipídeos da gema está associada a proteínas na forma de lipoproteínas. A fração lipídica da gema é composta por cerca de 70 % de triglicerídeos, 25% de fosfolipídios e 5% de colesterol com os ácidos graxos tendo papel relevante por serem os principais elementos dos triglicerídeos e fosfolipídios, possuindo diferentes comprimentos de cadeia e graus de saturação. Os ácidos graxos insaturados estão presentes em maior percentual nos ovos convencionais disponíveis no mercado, representando cerca de 64 % do total (KOVACS-NOLAN et al., 2005; MINE, 2007).

Já a clara, também chamada de albúmen, participa com 56 a 61% da composição total do ovo e armazena a maior parte da água presente no ovo, correspondendo a 88% de seu conteúdo (ORDONEZ, 2005). Além disso, contém grande quantidade de proteínas de alto valor nutricional e funcional (que varia de 9,7 a 10,6% de acordo com a idade da ave), cerca de 0,03 % de lipídeos, 0,4 a 0,9% de carboidratos e 0,5 a 0,6% de cinzas, correspondentes ao conteúdo mineral. O sistema proteico é constituído por fibras de

ovomucina incluídas em solução aquosa de numerosas proteínas globulares (STADELMAN e COTTERILL, 1995a; KOVACS-NOLAN et al., 2005) .

7.1.1 Importância econômica

Em 2001, o Brasil ocupou a sétima posição na produção mundial de ovos, com pouco mais de 1 milhão de toneladas produzidas de ovo com casca. Em 2013 sua colocação mundial mudou como pode ser visto na Figura 3, pois este passou a ocupar a sexta colocação mundial com uma produção anual total de pouco mais de 1.6 milhões de toneladas (FAO-DATABASE, 2011).

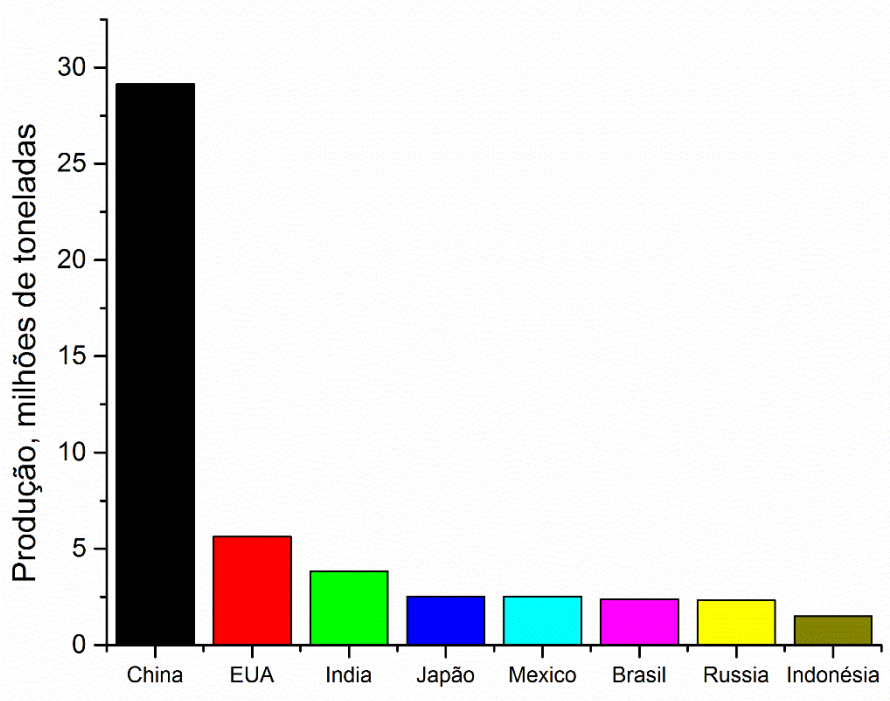

Figura 3: Produção de ovos de galinha com casca dos principais países produtores em 2013. Fonte: FAOSTAT.® FAO Divisão de estatística, 2015

A produção global de ovos tem crescido rapidamente nos últimos anos. Ela duplicou desde 1990, quando a produção mundial foi de cerca de 37 milhões de toneladas, em comparação com quase 75 milhões de toneladas em 2013, como pode ser visto na Figura 4 (FAO-DATABASE, 2011). Especialistas da Comissão Internacional do Ovo (IEC) esperam que se as taxas de crescimento de produção permaneçam constantes, pois dentro de alguns anos o volume de produção de ovos pode ser tão grande quanto à

produção de carne de bovina o que elevaria ainda mais a importância econômica deste produto no cenário agroindustrial (IEC, 2012).

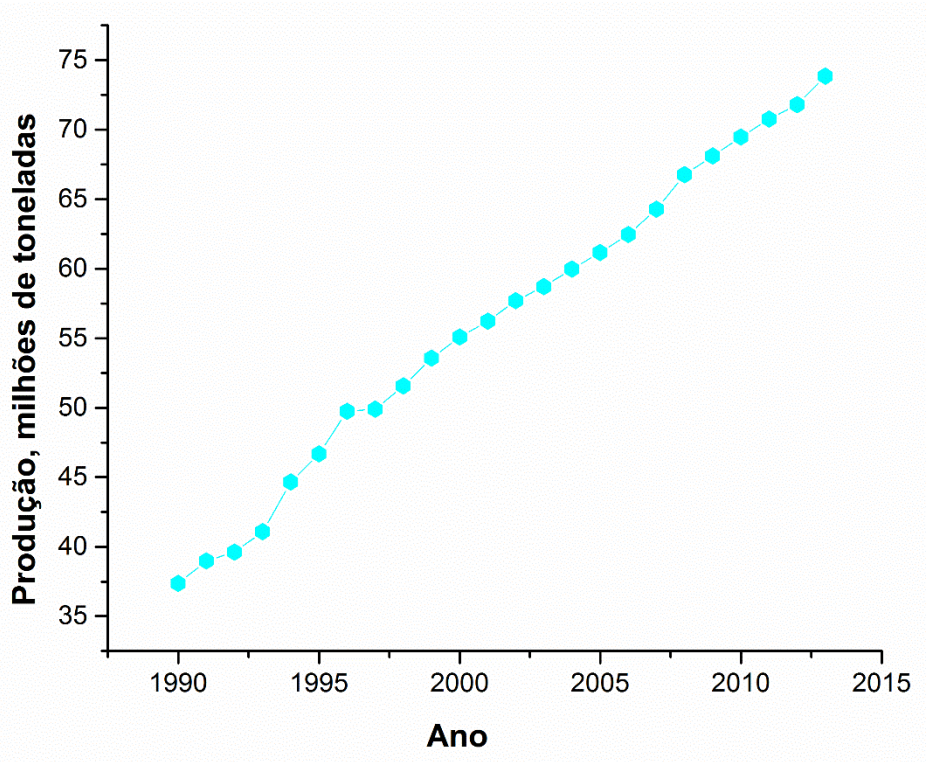

Figura 4: Dinâmica de produção de ovos de galinha com casca no mundo. Fonte: FAOSTAT.® FAO Divisão de estatística, 2015.

Atualmente, o país continua com a sexta colocação mundial em uma crescente progressão na sua produção anual (FAO-DATABASE, 2011). No primeiro trimestre de 2009 a produção de ovos de galinha no país foi de 580.832 milhões de dúzias, considerando-se apenas estabelecimentos com 10.000 ou mais galinhas de postura. Isto reflete uma variação positiva de 2,2% em comparação ao mesmo período de 2008 totalizando ao final de 2009 uma produção de 1.798,257 milhões de dúzias (IBGE, 2012) o que já em 2007 colocou o ovo como sendo o 14º produto agropecuário brasileiro com rendimento de 1.198 U$ Bilhões (FAO-DATABASE, 2011).

A principal região produtora de ovos de galinha é a região sudeste com 50,4% do total nacional. São Paulo é o maior estado produtor, representando 31,3% da produção nacional (IBGE, 2012). (SIM e NAKAI, 1994), destacou em seu trabalho que em média 20 % do ovo "in natura" de uma granja segue diretamente para o mercado consumidor. O restante destina−se ao uso industrial, geralmente na forma de clara, gema ou ovo integral desidratado. O produto desidratado é normalmente utilizado como ingrediente alimentar no preparo de biscoitos, bolos, doces, maioneses e massas. Os principais constituintes da gema, lipídios e proteínas, têm importância particular e essencial em vários segmentos da indústria e aqui podemos citar alguns exemplos como o processamento da indústria de alimentos onde está é utilizada para elaboração de novos emulsificantes, na formulação de cosméticos como shampoos e condicionadores e como componente bioativo da indústria farmacêutica onde ela é utilizada como excelente meio de crescimento de vírus para elaboração de vacinas (GARCIA ROJAS et al., 2007; AEB, 2012).

7.2 Proteínas da Clara do ovo

Segundo MINE (2007) a clara do ovo é constituída de quatro camadas distintas, sendo elas: (1) Albúmen fluido externo, sendo 23% do total da clara. Está em contato com a membrana da casca. Quando o ovo se rompe sobre uma superfície plana, este albúmen é, precisamente, o que se estende com rapidez; (2) Albúmen denso, sendo 57% do total da clara. Encontra-se unido aos dois extremos do ovo. Apresenta um aspecto de gel; (3) Albúmen fluido interno, sendo 17% do total da clara. Encontra-se localizado entre o albúmen denso e a gema; (4) Chalaza, sendo 3% do total do albúmen. São filamentos dispostos em espiral, que vão desde a gema até os dois pólos do ovo e atravessam o albúmen denso. Colaboram para manter a gema no centro do ovo.

A proporção com que estas zonas aparecem varia em função da raça da ave, peso do ovo e do tempo em que foi posto (STADELMAN e COTTERILL, 1995a). Quando o peso do ovo aumenta com a idade da ave, também aumenta a presença de albúmen denso, enquanto o albúmen fluido interno diminui.

A clara de ovo apresenta alto teor de umidade (cerca de 88 % de água) e conteúdo de sólidos variando de 11 a 13 %, sendo as proteínas as maiores constituintes e a quantidade de lipídeos insignificante. Assim como a gema, a clara apresenta baixo teor de carboidratos, sendo, entretanto, relativamente abundante em *Mg* e *Na* (STADELMAN e COTTERILL, 1995a).

Segundo STADELMAN e COTTERILL (1995a) a clara pode ser considerada um sistema protéico constituído por fibras de ovomucina em uma solução aquosa de numerosas proteínas globulares, sendo a ovoalbumina, conalbumina (ovotransferrina), ovomucoide, lisosima, globulinas e a ovomucina as principais proteínas consideradas no sistema (Tabela 1). A composição protéica das camada fluida e densa diferencia-se apenas no conteúdo em ovomucina, que é cerca de quatro vezes maior na camada densa.

Análises calorimétricas mostram três principais endotermas que ocorrem durante o aquecimento da clara de ovo, que correspondem à desnaturação da ovotransferrina, lisozima e ovoalbumina. As proteínas da clara são termo lábeis e formam géis fortes sob aquecimento. Durante a desnaturação pelo calor, são formadas estruturas intermoleculares estáveis entre a ovoalbumina, ovotransferrina e lisozima. Sob uma temperatura de cerca de 60 ºC, as proteínas da clara sofrem o desdobramento, expondo seus grupos sulfidrila internos (que podem afetar a formação de ligações disulfidicas) e a hidrofobicidade superficial aumenta. A desnaturação das proteínas da clara pode ser afetada pelo pH, concentração de sal, açúcar ou tratamentos prévios de aquecimento (EMBUSCADO e HUBER, 2009).

Tabela 1: Proteínas da clara do ovo e suas principais propriedades funcionais

Proteína	% na Clara	Massa Molar (KDa)	Ponto Isoelétrico	Temperatura de Desnaturação (°C)	Características
Ovoalbumina	54	45	4,6	84,0	Fosfoglicoproteína, Capacidade de formar géis
Conalbumina (ovotranferrina)	12	76	6,1	61,0	Combina-se com íons metálicos, inibidor bacteriano
Ovomucóide	11	28	4,1	79,0	Inibidor proteinase
Lisozima	3,4	14,3	10,7	75,0	Lise bacteriana
Ovoglobulina G_2	4	45	5,6	92,5	Espumante
Ovoglobulina G_3	4		5,8	-	Espumante
Ovomucina	3,5	550 a 830	4,5 - 5,0	-	Viscosidade
Ovoflavoproteína	0,8	32	4,0	-	Fixa riboflavina
Ovoinibidor	1,5	49	5,1	-	Inibidor proteinase
Ovoglicoproteína	1,0	24,4	3,9	-	Sialoproteina, viscosidade
Ovomacroglobulina	0,5	830 x 1000	4,5	-	Fortemente antigênica
Avidina	0,05	67	10	85,0	Complexação com Biotina

Fonte: Adaptada de Ordóñez *et al*.(2005) e Stadelman e Cotterill (1995)

7.2.1 Ovoalbumina

A ovoalbumina é a proteína majoritária da clara, representando em torno de 54% do total. Trata-se de uma fosfoglicoproteína, constituída por três componentes (A_1, A_2 e A_3) que se diferenciam pelo número de grupos fosfato (dois, um ou nenhum, respectivamente), com o grupo carboidrato sendo constituído por duas moléculas de N-acetilglicosamina e quatro unidades de manose. A cadeia polipeptídica é composta por 385 resíduos de aminoácidos, incluindo 4 grupamentos sulfidrila e uma ligação dissulfídica simples (ORDONEZ, 2005).

A ovoalbumina pode ser desnaturada e coagulada com relativa facilidade nas interfaces após a agitação ou batedura em solução aquosa (espumas e emulsões), porém é relativamente estável ao tratamento térmico. O aquecimento da clara a 62°C por 3,5 minutos sob pH 9,0 é capaz de alterar apenas 3 a 5% da ovoalbumina, enquanto em pH 7,0 praticamente não há alteração (STADELMAN e COTTERILL, 1995a).

Quando os ovos são armazenados por longos períodos, ocorre a conversão da ovoalbumina em S-ovoalbumina, proteína mais termoestável devido a um intercâmbio sulfidrila-dissulfeto. A S-ovoalbumina é encontrada em pequena quantidade na clara quando o ovo está fresco, porém, após seis meses de armazenamento sob refrigeração,

pode chegar a representar 81% da ovoalbumina da clara (LINDEN et al., 1996; FENNEMA, 2000).

7.2.2 Conalbumina ou Ovotransferrina

A conalbumina, também conhecida por ovotransferrina, representa em torno de 12% do total de proteínas da clara. É uma glicoproteína constituída por uma cadeia polipeptídica, podendo existir em equilíbrio sob três formas distintas conforme o conteúdo de ferro (dois, um ou nenhum átomo por molécula) formando complexos metálicos que são mais termoestáveis que a proteína nativa. É a proteína da clara mais sensível ao calor, desnaturando-se a 61ºC, sendo, quando comparada à ovoalbumina, menos suscetível à desnaturação nas interfaces (ORDONEZ, 2005).

A sua habilidade em se associar ao ferro relaciona-se com sua atividade antimicrobiana a partir do momento que é capaz de inativar microrganismos que necessitam deste íon para sobrevivência. Quando não ligada ao ferro, a conalbumina é mais sensível ao tratamento térmico do que a ovoalbumina, porém menos suscetível à desnaturação de superfície (LINDEN et al., 1996).

7.2.3 Ovomucoide

A ovomucóide, presente em um percentual de 11% da clara, é uma glicoproteína com nove pontes dissulfeto, o que faz com que seja mais estável à coagulação pelo calor, precipitando-se apenas em presença de lisozima e em meio alcalino. Possui uma única cadeia polipeptídica com segmentos helicoidais (aproximadamente 22%) e segmentos desorganizados (estrutura primária) e há ocorrência de um resíduo de cistina, em média, a cada 11 resíduos de aminoácidos. Contém de 20 a 25% de carboidratos constituídos por D-manose, D-galactose, glicosamina e ácido siálico. A ovomucóide se diferencia bioquimicamente da ovoalbumina e da conalbumina pornão se coagular na presença de calor. Outra característica é a ação de anti-enzima para a tripsina, diminuindo a atividade da protease (STADELMAN e COTTERILL, 1995a).

7.2.4 Ovomucina

A ovomucina é uma glicoproteína sulfatada que representa 3,5% do total das proteínas da clara e se difere das demais proteínas por conter galactosamina, ésteres sulfúricos, grande quantidade de cistina (que interliga as subunidades por meio de pontes intermoleculares) e ácido siálico (contém cerca de 50% de todo o presente na clara) (SGARBIERI, 1996).

A estrutura estirada da molécula é resultado das repulsões eletrostáticas devido às cargas negativas dos resíduos de ácido siálico responsáveis pela viscosidade da capa gelificada do albúmen. Cabe ressaltar que esta proteína é insolúvel em água (LINDEN et al., 1996).

Quando em solução com a lisozima, pode interagir por ligações eletrostáticas formando complexos insolúveis em água. Na faixa de pH 7,2 a 10,4, a interação dessas proteínas decresce com o aumento de pH. O complexo vai se desfazendo conforme o pH se aproxima do ponto isoelétrico da lisozima (10,7). O complexo ovomucina–lisozima provavelmente tem papel importante na diminuição da viscosidade da clara de ovo durante o armazenamento (STADELMAN e COTTERILL, 1995a).

7.2.5 Liozima (Ovoglobulina G_1

A lisozima (cerca de 3,5% do total de proteínas) possui massa molar mais baixa (14,307) e o pI mais alto (10,7) dentre todas as proteínas da clara. É formada de um único polipeptídio com 129 resíduos de aminoácidos e quatro pontes dissulfeto, não possuindo grupos sulfidrila livres. Representa uma proteína básica com alta porcentagem de histidina, lisina e arginina que apresenta estabilidade ao calor, frio e a muitos agentes de desnaturação, porém não é estável em álcalis. Algumas proteases, como a tripsina e a papaína, não a atacam. Sua grande estabilidade pode ser atribuída à estrutura compacta da molécula, com quatro pontes dissulfeto intermolecular e a presença de apenas três moléculas de água por molécula de lisozima (YAMAMOTO, 1997).

Sua ação enzimática inclui a clivagem de polissacarídeos, ligação glicosídica β-1,4 entre N-acetilglicosamina e ácido murâmico, em parede celular de bactérias. Além da atividade glicosídica, possui também atividade de transglicosidade de esterase, exercendo ação antimicrobiana. A lisozima da clara de ovo é homóloga à lisozima humana e à α-lactalbumina (YAMAMOTO, 1997; MINE, 2007).

7.2.6 Outras Proteínas

A avidina é uma glicoproteína básica composta de quatro subunidades idênticas, podendo se fixar a cada uma delas uma molécula de biotina. Por ser encontrada na clara do ovo quase sempre na forma livre de biotina, apresenta também atividade antimicrobiana. Já as ovoglobulinas G_2 e G_3 são bons agentes espumantes e, diferentemente de outras globulinas, não apresenta atividade inibitória de proteinases. O Ovoinibidor, outra proteína presente na clara, é capaz de inibir tripsina, quimotripsina e enzimas microbianas enquanto a ovomacroglobulina é uma glicoproteína de elevada massa molar que representa 0,5% das proteínas da clara e apresenta ponto isoelétrico em pH 4,5. É fortemente antigênica e mostra muita reatividade cruzada contra ovomacroglobulina de outras espécies de aves (ORDONEZ, 2005; MINE, 2007).

Os polissacarídeos são polímeros naturais de alta massa molar, formados por mais de 20 unidades de monossacarídeos dispostos de forma linear ou ramificada, podendo ser solúveis ou insolúveis em água (DAMODARAN et al., 2010). Segundo LEWIS et al. (1993) são hidrocolóides que conferem maior viscosidade e consistência gelatinosa aos alimentos aos quais são adicionados, pois contêm grupos hidrofílicos e hidrofóbicos que favorecem suas propriedades emulsificantes. São exemplos de polissacarídeos o amido, o glicogênio, a celulose, a pectina, a carragena e a goma xantana. A seguir serão descritas algumas características dos polissacarídeos utilizados neste trabalho.

7.3 Goma Xantana

A goma xantana é um polímero aniônico, um exopolissacarídeo microbiano produzido por *Xanthomonas campestris* em grandes fermentadores. É atóxica e foi aprovada no Brasil como aditivo em alimentos desde 1965 pela Legislação Brasileira de Alimentos. Sua estrutura primária é composta de repetidas unidades pentassacarídicas, formada por duas unidades de glicose, três unidades de manose (sendo o primeiro resíduo de manose normalmente acetilado no C-6) e duas unidades de ácido glicurônico. A cadeia

principal consiste de unidades de glicose ligadas na posição 1 e 4 (DAMODARAN et al., 2010) (Figura 5).

Quando em solução e com o aumento da temperatura, a goma xantana passa por

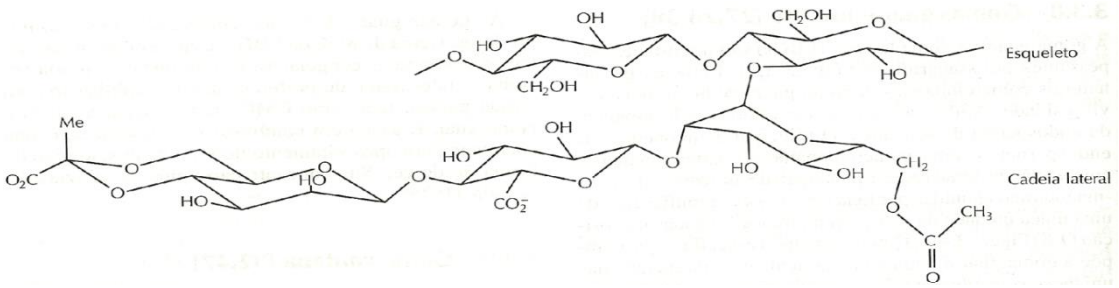

Figura 5: Estrutura da unidade repetitiva do pentassacarídeo da goma xantana (DAMODARAN et al., 2010)

uma transição conformacional irreversível de um estado ordenado e rígido para um estado mais desordenado e flexível (desnaturado). A temperatura de transição é, ainda, função de alguns fatores como a concentração da goma e a força iônica do meio, mas geralmente ocorre até 50ºC (URLACHER e NOBLE, 1997).

Na indústria alimentícia seu uso é bastante valorizado devido a algumas características como a alta solubilidade, tanto em água quente como fria, por produzir alta viscosidade em soluções mesmo em baixas concentrações, por não provocar mudanças perceptíveis na viscosidade das soluções, em uma ampla faixa de temperatura, de 0 a 100ºC (o que a torna única entre as gomas alimentícias), por ser solúvel e estável em soluções ácidas, por ser bastante compatível com sal, por ser estabilizante de suspensões e emulsões e por conferir estabilidade a produtos submetidos a congelamento e descongelamento. Além disso, tem a propriedade de interagir com a goma guar de forma sinérgica para produzir aumento da viscosidade da solução e gelifica somente em presença de outras gomas, especialmente a goma locusta (DAMODARAN et al., 2010).

Pelo fato de possuir propriedades reológicas únicas e muito úteis para as indústrias, a goma xantana vem sendo amplamente utilizada para espessar e estabilizar suspensões e emulsões. Como a viscosidade das soluções é pouco alterada pela temperatura, não se tornando mais espessas quando resfriadas, a xantana é insubstituível em produtos como molhos para salada e calda de chocolate aos quais necessitam fluir de forma fácil quando retirados do refrigerador ou em temperatura ambiente ou outros molhos que não devem ser muito espessos quando frios nem muito fluidos quando aquecidos (HUI et al., 2005).

7.4 Pectina

A pectina é um polissacarídeo natural presente em quase todas as plantas terrestres e responsável por propriedades estruturais de frutas e vegetais (CLEGG, 2010). As pectinas comerciais possuem como componente majoritário o ácido galacturônico parcialmente esterificado com um grupo metoxila (Figura 6) e são obtidas geralmente pela hidrólise de substâncias pécticas sob aquecimento em meio ácido (60 a 100ºC em

pH 1,5 a 3,0), estando entre as principais fontes à casca de frutas cítricas (20 a 30% de pectina) e a polpa de maçã (10 a 15% de pectina).

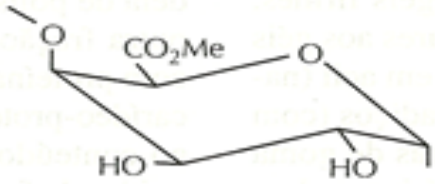

Figura 6: Unidade monomérica predominante de uma pectina de alta metoxilação (DAMODARAN et al., 2010)

As pectinas podem ser classificadas de acordo com o grau de esterificação (GE) ou metoxilação (GM). As que possuem GE superior a 50% (mais da metade dos grupos carboxila sob a forma de éster metílico) são denominadas pectinas de alto teor de metoxilação (ATM) e as com GE inferior a 50%, são de baixo teor de metoxilação (BTM). Em ambos os casos, os grupos carboxilas remanescentes estão presentes como uma mistura na forma de ácidos livres (-COOH) e sais ($-COO^-Na^+$) (ORDONEZ, 2005; DAMODARAN et al., 2010).

Possuem uma capacidade única de formar géis espalháveis na presença de açúcar ou ácido ou na presença de íons cálcio, sendo usados principalmente como ingrediente na indústria de alimentos na produção de geleias, sucos de frutas, artigos de confeitaria e na fabricação de filmes biodegradáveis comestíveis As pectinas têm pouco uso como espessantes devido à sua baixa capacidade de formar soluções viscosas, quando comparadas com outros biopolímeros (SPERBER et al., 2009).

A gelificação das soluções de pectina ATM ocorre quando há ácido (com pH ideal na faixa de 2,8 a 3,5) e açúcar (cerca de 65%) em quantidade suficiente, de maneira que à medida que o pH da solução diminui, os grupos carboxila, altamente hidratados e carregados, são convertidos em grupos não carregados e apenas levemente hidratados. Como resultado da perda de algumas de suas cargas e de sua hidratação, as moléculas poliméricas podem então associar-se em porções ao longo de seu comprimento formando junções e uma rede de cadeias poliméricas que aprisionam a solução aquosa de moléculas de soluto, formando zonas de junção que são favorecidas em alta concentração de açúcar. Os géis formados por pectinas ATM possuem grande quantidade de grupos éster metílicos ($COOCH_3$) cujas interações hidrofóbicas sustentam a estrutura do gel (NEIRYNCK et al., 2004).

Já as pectinas BTM gelificam apenas na presença de 0,01 a 0,05g/L de cátions divalentes (que proporcionam pontes cruzadas), sem a necessidade de altos teores de açúcar, sendo portanto, muito úteis na confecção de geleias e marmeladas com baixo teor deste componente (ORDONEZ, 2005; DAMODARAN et al., 2010).

7.5 Carragena

Carragena é o termo utilizado para identificar um grupo ou família de galactanas sulfatadas extraídas alcalinamente de algas vermelhas das espécies *Gigartina, Hypnea, Eucheuma, Chondrus* e *Iridaea*. São biopolimeros de cadeias lineares de unidades D-galactopiranosil unidas por ligações D-glicosídicas alternadas α-1,3 e β-1,4 com a maioria das unidades de açúcar apresentando um ou dois grupos semi estér sulfato esterificados no grupo hidroxila dos átomos de carbono C-2 e ou C-6. As principais estruturas são denominadas carragena kappa (κ), iota (ι) e lambda (λ). As unidades dissacarídicas demonstradas na Figura 7 representam os blocos constituintes predominantes de cada

tipo, mas não são, necessariamente, unidades estruturais repetidas (SHUMILINA e SHCHIPUNOV, 2002; CAMPO et al., 2009).

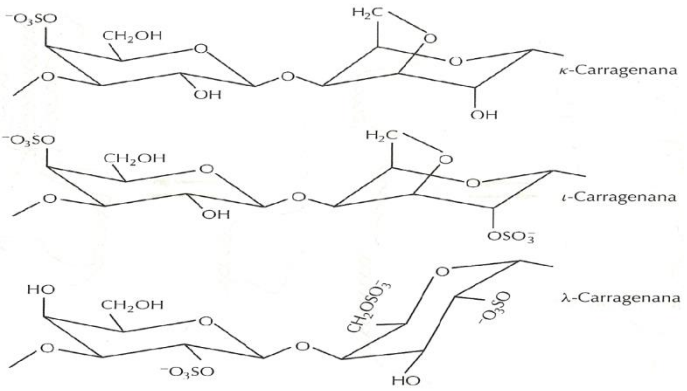

Figura 7: Estruturas unitárias idealizadas das carragenas tipos κ, ι e λ (DAMODARAN et al., 2010).

As carragenas comerciais são misturas de polissacarídeos não homogêneos, contendo diferentes proporções dos três principais tipos estruturais (κ, ι e λ), produzidos com misturas de espécies de algas vermelhas. Possuem geralmente 60% da fração κ (gelificante) e 40% da fração λ (não gelificante), sendo estáveis em valores de pH acima de 7,0, com diminuição na estabilidade na faixa de pH de 5,0 a 7,0 e degradando-se rapidamente em pH abaixo de 5,0 (TOLSTOGUZOV, 2007).

As carragenas apresentam moléculas muito flexíveis, sendo que em altas concentrações podem formar uma estrutura mais ordenada na forma de duplas hélices, a qual pode levar à formação de géis. Quanto maior for o grau de sulfatação das moléculas, maior é a solubilidade a frio. A λ-carragena apresenta três radicais sulfatos para cada dois resíduos de galactose, com fortes repulsões eletrostáticas. Com isso, há maior mobilidade das moléculas, que, separadas umas das outras, não tendem a se associar, diminuindo a capacidade de gelificação, porém apresentando a propriedade de espessante tanto a frio quanto a quente. Já a κ-carragena possui um grupo sulfato para duas unidades de galactose, sendo capaz de apresentar alto poder de gelificação (TOLSTOGUZOV, 2007). A κ -carragena forma géis termo reversíveis que ocorrem a partir do ordenamento molecular com o resfriamento. A presença dos cátions K^+ e Ca^{2+} no sistema contribuem tanto para induzir a gelificação a temperaturas mais elevadas, quanto para a formação de géis mais fortes. As carragenas do tipo iota e kappa além de serem amplamente utilizadas como agentes espessantes em produtos que se preparam a altas temperaturas, também resultam géis estáveis em água à temperatura ambiente sem necessidade de refrigeração. Esses géis são transparentes e termo reversíveis, conseguindo uma ampla variedade de texturas desde muito elásticas e coesas, até géis firmes e quebradiços, dependendo da combinação das frações que se utiliza (CAMPO et al., 2009).

Uma das propriedades mais importantes que diferencia a carragena de outros hidrocolóides é sua capacidade única de formar complexos e interagir com proteínas do leite, sob diversas condições. Suas propriedades funcionais são amplamente utilizadas pela indústria alimentícia, com diversas aplicações como espessante, gelificante, emulsão e estabilização de gorduras; estabilidade de congelamento-degelo; controle de textura; propriedades de fusão controladas; controle de viscosidade no processamento, sendo útil em produtos lácteos, carnes enlatadas, gelatinas, geléias, produtos *light*, macarrão, entre outros (TOLSTOGUZOV, 2007; DAMODARAN et al., 2010).

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CAPÍTULO II

EFFECTS OF SALT AND PROTEIN CONCENTRATIONS ON THE ASSOCIATION AND DISSOCIATION OF OVALBUMIN-PECTIN COMPLEXES

Published at journal Food **Hydrocolloids,** v. 47, n. 5, p. 124-129, 5. 2015

ABSTRACT

Formation of ovalbumin-pectin coacervate complexes was analyzed in various NaCl concentrations and with various protein:polysaccharide ratios by measuring zeta (ζ)-potentials, and by X-ray diffraction. The titration curve of a 1:1 ovalbumin:pectin coacervate complex formed in 0.01 M NaCl displayed a region containing insoluble complexes, a region of considerable complex formation, and a region of complex dissociation. Changes in protein concentrations led to shifts in the region of insoluble complex formation (at the isoelectric point). At an ovalbumin:pectin ratio of 8:1, complex formation was suppressed. At NaCl concentrations of 0.05 and 0.1 M, ovalbumin self-aggregation increased. When NaCl concentrations increased from 0.1 to 0.4 M, complex dissociation was suppressed. X-ray diffraction of the ovalbumin-pectin coacervate complex showed a partially defined crystalline region from 27-20 ° suggesting that the structure of the complex is more organized than the individual amorphous polymers. Finally, this study addressed the effect of ovalbumin self-aggregates on ovalbumin-pectin complex formation.

1 INTRODUCTION

Technical applications involving complexes formed with polyelectrolytes and oppositely charged colloids have received considerable attention since the early 20th century (HARTLEY, 1948). The term "complex" includes soluble complexes that involve liquid-liquid phase separations and coacervate complexes that involve solid-liquid separations and precipitations (WEINBRECK et al., 2003b). Polymer and protein complexes have been used in the development of controlled-release proteins and protein-based drugs (MALMSTEN et al., 2010), to encapsulate bioactive compounds, cosmetic additives, flavorings, and living cells (KATONA et al., 2010; LECLERCQ et al., 2010), to separate proteins (HANSEN e CHANG, 1968; MONTILLA et al., 2007), and to define biofilm structure (DUAN et al., 2011; KUORWEL et al., 2011).

Factors affecting complex formation and associative phase separations can be classified as intrinsic or extrinsic. Extrinsic factors include the macromolecules in the mixture, pH, ionic strength, amount of total solids, the rate of acidification, and the shear rate during acidification. When the pH is increased and is close to the p*I* (also known as the critical pH, pH_c), turbidity values of soluble complexes formed by non-covalent bonds increase slightly. When the pH is lowered, soluble complexes grow in size and number until a critical point, $pH_{\varphi 1}$. At $pH_{\varphi 1}$, insoluble complexes form and macroscopic separation occurs (JONES e MCCLEMENTS, 2010). When complexes have an overall neutral charge (zeta (ζ)- potential equals 0), the pH is ideal, pH_{ot} and complex formation is optimal. Reducing pH ($pH_{\varphi 2}$) leads to dissociation of protein and polysaccharide molecules due to positive protonation (WEINBRECK et al., 2003a).

Intrinsic factors include the characteristics of the polymer molecules, such as molecular weight, charge density, chain flexibility, and concentrations of the polymers (KRUIF et al., 2004; SCHMITT e TURGEON, 2011). The ratio of protein and

polysaccharide in a mixture affects charge balance, thereby altering complex formation. The protein:polysaccharide ratio for maximum complex formation at a given pH must be determined (TAINAKA, 1980; TURGEON et al., 2007a). When the ionic strength is high, the charges on the proteins and polysaccharides are screened, the electrostatic interactions are reduced, and complex formation is inhibited (WEINBRECK et al., 2003a; YE e SINGH, 2006). At low ionic strength, the effects on the protein and polysaccharide charges are small and do not impede electrostatic interactions and complex formation .

Although many biopolymer coacervation studies have focused on interactions between milk proteins (e.g. bovine serum albumin, milk protein isolate and β-lactoglobulin) (WEINBRECK et al., 2003a; SCHMITT et al., 2005c; LI et al., 2012) and vegetable proteins (e.g. soy protein and pea protein) (LIU et al., 2009a; HUANG et al., 2012) with anionic polysaccharides (e.g. acacia, alginate, pectin) (HARNSILAWAT et al., 2006a; ESPINOSA-ANDREWS et al., 2007; SPERBER et al., 2009), some have focused on egg proteins (KUDRYASHOVA e DE JONGH, 2008; AL-HAKKAK e AL-HAKKAK, 2010; MLEKO et al., 2010; SOUZA et al., 2013; NIU et al., 2014). Approximately 65% of egg white protein is ovalbumin. Ovalbumin has been used extensively in food technology because of its emulsifying and stabilizing properties (SIM e NAKAI, 1994).

In addition, ovalbumin has been found to have antimutagenic and anticarcinogenic (VIS et al., 1998), immunomodulatory (GOLDBERG et al., 2003) and antioxidant (protection of linoleic acid and docosahexaenoic acid) properties (NARA et al., 1995).

Pectin is a natural polysaccharide present in nearly all land plants and is responsible for the structural properties of fruits and vegetables (LI et al., 2012; RU et al., 2012). Pectins are characterized according to their degree of esterification (DE) or their degree of methoxylation (DM). Those with DMs greater than 50% (more than half of the carboxyl groups are methyl esters) are called pectin high methoxyl (HM), and those with less than 50% DM are called pectin low methoxyl (LM). In both cases, the remaining carboxyl groups are free acids (-COOH) and salts ($-COO^-Na^+$) (SPERBER et al., 2009; LOPES DA SILVA e RAO, 2010).

The purpose of this study was to evaluate the effects of varying of pH, NaCl concentrations and protein: polysaccharide ratios on the formation of ovalbumin-pectin coacervate complexes.

2 MATERIALS AND METHODS

2.1 MATERIALS

Ovalbumin (Ova; purity > 90%; 4.4 x 10^4 Da) and pectin high methoxyl (Pec; GM > 69%; 2.2 x 10^5 Da) were obtained from Sigma Chemicals (St. Louis, USA). Sodium chloride (NaCl, purity > 99%), hydrochloric acid (HCl, 0.5 M), and sodium hydroxide (NaOH, 0.5 M) were purchased from VETEC® Ltda, (Rio de Janeiro, Brazil). The water used was ultrapure with a conductivity of 0.05 µS/cm ± 0.01 (Gehaka-Master P&D – Brazil). Stock solutions of pectin (0.1% w/w) and ovalbumin (0.1-1% w/w) were prepared by gently stirring the powders in deionized water for 6 hours at room temperature (25 °C).

2.2 Formation of complexes

2.2.1 Preparation of complexes

The concentration of pectin used was 0.1% w/w and the concentration of ovalbumin used varied from 0.1-1% w/w. Five ratios of Ova:Pec (1:1, 2:1, 3:1, 5:1, 10:1) were evaluated. To determine the effect of NaCl on complex formation, Ova:Pec complexes were formed in five concentrations of NaCl (0.01 M, 0.05 M, 0.1 M, 0.2 M, 0.4 M). The Ova:Pec mixtures were stirred and adjusted to pH 9.0.

2.2.2 Turbidimetric measurements

pH-dependent turbidity was measured at a wavelength of 400 nm using a spectrophotometer (Biochrom mod. LIBRA S12, England) calibrated with ultrapure water to 100% transmittance (*T*). Turbidity was defined as 100 - %*T*. With the aid of a magnetic stirrer (Nova Técnica, NT 101, Brazil) and a pH meter (Tecnopon, mPA-210, Brazil) the pH of the solutions were adjusted (9.0 to 1.0) with HCl, 0.5 M. Measurements of complexes and the solutions with biopolymers isolates were made at room temperature (25 ºC ± 1), and each sample was measured four times at one minute intervals.

2.3 Zeta - Potential

A Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK) was used to determine zeta (ζ)-potentials. Pectin and ovalbumin stock solutions and Ova:Pec complexes formed in 0.01 M NaCl were diluted to 0.01% w/w and transferred to an MPT-2 autotitrator (Malvern Instruments, Worcestershire, UK) that adjusted pH using 0.5 M NaOH, 0.25 M HCl and 0.025 M HCl solutions. pH was varied from 9.0 to 1.0 by 0.5 unit increments with a confidence interval of ± 0.1 unit. ζ-potentials were calculated using the Smoluschwsky mathematical model. The Stokes-Einstein equation was used to calculate the average size of the particles. Complexes with different Ova:Pec ratios were diluted as described above and the pH was adjusted to 3.55. Each experiment was performed three times and sample readings were done in triplicate at 25 °C.

2.3.1 X-ray Diffraction

X-ray diffraction was performed to determine whether polymers alone and in complex were crystalline or amorphous. Pectin, Ovalbumin and Ova:Pec coacervates were analyzed in triplicate using an X-ray diffractometer (Zhimadzu XRD-6100, Japan) set at a range of 15 to 60 ° (2Θ), a scan rate of 1.2 Å/min, and 40 kV.

3 RESULTS AND DISCUSSION

3.1 Effect of pH

When Ova:Pec coacervate complexes were formed at varying pHs, we found three distinct regions of turbidity (A, B, C; Figure 1) the transition regions ($pH_{\varphi 1}$, pH_{ot} and $pH_{\varphi 2}$) were determined by the intersections of the two tangents. In region A, the turbidimetric titration analysis (WEINBRECK et al., 2003a; KLASSEN et al., 2011) and dynamic light scattering analysis (SPERBER et al., 2009) have showing that polysaccharide-protein complexes form soluble complexes through non-covalent attractions between polymers when the pH is near the isoelectric point of the protein (the p*I* of ovoalbumin is ~ 4.9)), and this complex formation (referred to as pH_c) increases turbidity slightly however, we did not find significant differences in turbidity when titrating between pH 9.0 and 5.18. The absence of pH_c behavior observed in region A may be due to higher deprotonation because the pH of the system was above the p*K*a of pectin (p*K*a 3.5), and this deprotonation may have reduced the non-covalent attractions between the biopolymers. The absence of pH_c was also described by RU et al. (2012) in a system containing 5:1 BSA (p*I* 4.7) and pectin (p*K*a 3.5) in 0.1 M NaCl; when titrating these complexes from pH 7-1, they observed that the polymers remained soluble between pH 7.0 and pH 4.7 ($pH_{\varphi 1}$).

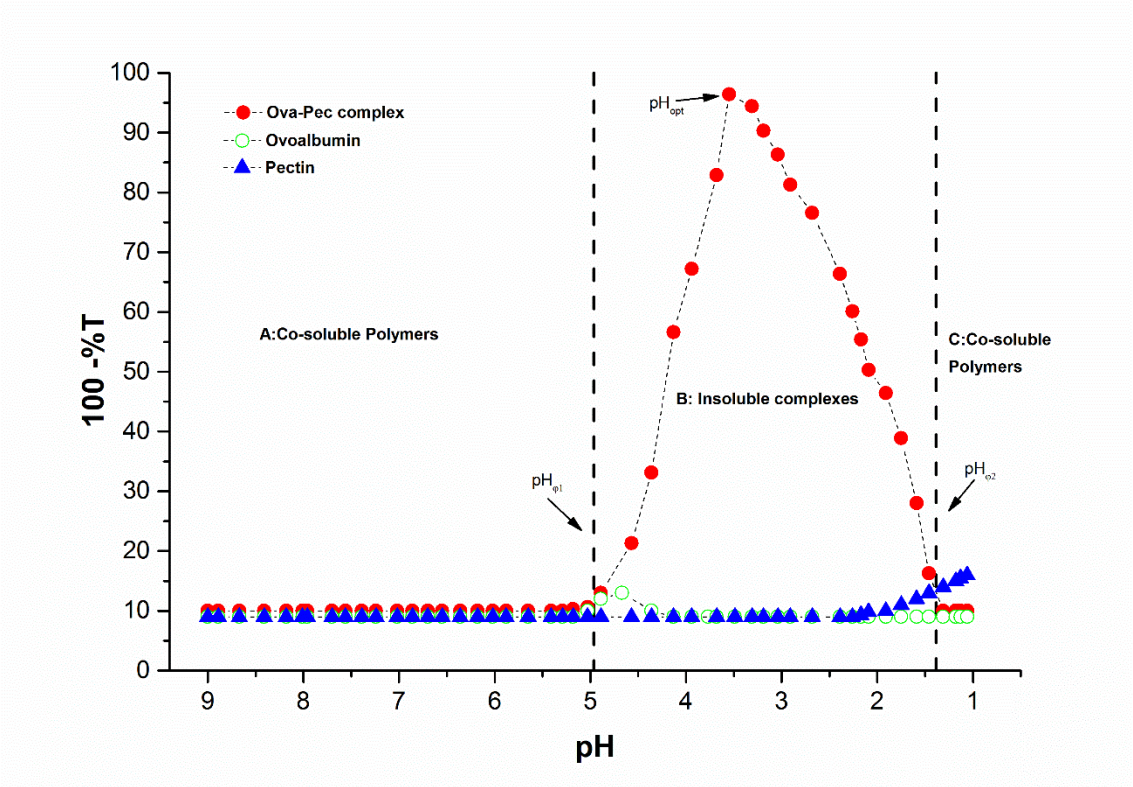

Figure 1: Turbidity (100- %T) of a system containing Ova/Pec (1:1), a function of pH using 0.01 M NaCl and determination of stability regions of instability (A, B and C) of complex coacervates.

At pH 4.89, near the p*I* of ovalbumin, the turbidity increased indicating the formation and aggregation of complexes and the onset of macroscopic phase separation ($pH_{\varphi 1}$). In region B, as pH< $pH_{\varphi 1}$, ovalbumin was protonated until the electrical charges

between ovalbumin and pectin were equivalent and turbidity reached 96.4% at pH 3.55 (pH_{ot}). At pH 3.55, the system acidified and pectin was no longer deprotonated, thus pectin was able to interact with ovalbumin. In region C, at pH 1.31 ($pH_{\varphi 2}$), low turbidity values indicated the dissociation of the biopolymers. Similar pH-dependent transitions in the formation of ovalbumin;gum arabic, β- lactoglobulin:pectin HM and BSA:pectin complexes have been described by NIU et al. (2014), SPERBER et al. (2009), and LI et al. (2012), respectively.

It should be noted that ovalbumin itself aggregated as demonstrated by a small increase in turbidity near its p*I*. Although these small aggregates did not appear to prevent interactions between ovalbumin and pectin the aggregation of protein can interfere on charge patches or on charged residues of protein during electrostatic with polysaccharide (LI et al., 2014b). Pectin aggregated at pH < 1.7 but not at pH < $pH_{\varphi 2}$ (TURGEON et al., 2007a).

3.2 Effect of Ratio Ova/Pec

The stoichiometries of the biopolymers are important variables in the formation of coacervate complexes (KIZILAY et al., 2011; SCHMITT e TURGEON, 2011). Different Ova:Pec ratios resulted in pHφ1 shifts (Figure. 2AB). When the Ova:Pec ratio was increased from 2:1 to 5:1, the maximum turbidity was followed by a plateau (Figure. 2A). Dependence of pHφ1 on different protein:polymer ratios has been previously reported by LIU et al. (2009a) with >4:1 pea protein: gum arabic ratios, by KLASSEN et al. (2011) with >20:1 canola protein:ι-carrageenan/alginate ratios, by SINGH et al. (2007) with >2:1 gelatin type A and B:gum agar ratios, and by YE et al. (2006) with > 1:10 sodium caseinate:gum arabic ratios. The authors attributed this dependency on binding of protein molecules to polysaccharide chains, which promotes aggregation of protein molecules until a plateau is reached, however is possible to say too that when protein is in excess, aggregates can be through the packing of proteins (LI et al., 2014b). This aggregation was also responsible for the gradual suppression of disassociation between biopolymers and a gradual increase in the size of the complexes (Figure. 2AB). At pH 1, the $pH_{\varphi 2}$ was low and this was likely due to the formation of small 443 nm diameter ovalbumin aggregates around the polysaccharide chain, which suppressed the dissociation of the complexes. As the protein:polysaccharide ratio was increased to 10:1, the size of the aggregates increased to a diameter of 2,357 nm and the dissociation of the complexes was suppressed. KLASSEN et al. (2011), RU et al. (2012), and YE et al. (2006) reported similar observations.

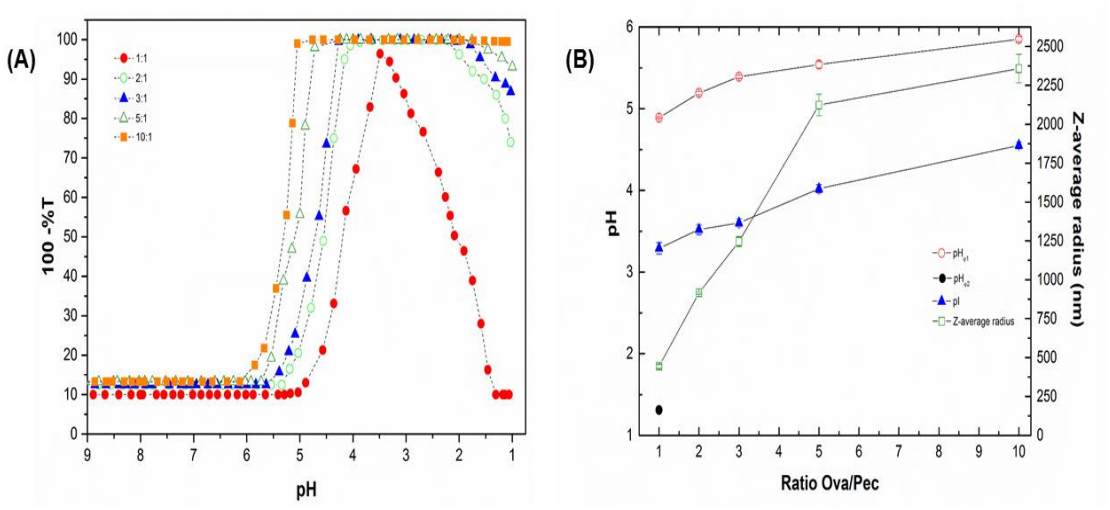

Figure 2: (A) Turbidity (100-T%) versus pH of the systems containing Ova/Pec at different ratios with concentration 0.1 M

The pH-dependence of the ζ-potential for ovalbumin, pectin and complexes with varying Ova:Pec ratios is shown in Figure 3. Ovalbumin exhibited a positive ζ-potential of 25.3 ± 0.4 mV at pH 1. As the pH increased, the ζ-potential decreased to neutral at p*I* 4.93, then further decreased to a ζ-potential of -22.3 ± 0.6 mV at pH 9. NIU et al. (2014) also observed this when they evaluated the ζ-potentials of ovalbumin from pH 1-7. Pectin exhibited positive ζ-potentials at pH < 3.50 (pK_a) with a maximum ζ-potential of + 9.37 ± 0.3 mV at pH 1.62. At pH > 3.50, ζ-potentials became negative and reached -29.5 ± 0.5 mV at pH 9.0. This can be explained by protonation of pectin carboxyl groups when pH < pK_a or by deprotonation of pectin carboxyl groups when pH > pK_a; the carboxyl groups of pectin have pK_a's varying between 2.9 and 3.5 that are similar to the pK_a of monomeric galacturonic acid reported by LOPES DA SILVA e RAO (2010). Complexes containing varying Ova:Pec rations had intermediate ζ-potentials and p*I*'s ranging from 3.55-4.55 (Figure. 2B) indicating that ovalbumin was strongly linked with pectin, probably through electrostatic interactions between the anionic carboxyl groups on pectin and the cationic amino groups on ovalbumin (JONES e MCCLEMENTS, 2010). As the protein:polysaccharide ratio was increased, the p*I*'s of the complexes shifted in a positive direction, the diameters of the complexes increased, and instability increased, for example, the ζ-potentials of the 10:1 complexes were ~0 at pH 1-4.5. LI et al. (2012) reported significant positive shifts in p*I*'s of 1:1 BSA:sugar beet pectin complexes due to saturation of electrostatic bonds between the carboxylic groups of pectin and BSA. In contrast, ELMER et al. (2011) found that as the protein ratio was increased, the p*I* shifted in the negative direction; they attributed this result to electrostatic binding between pea protein and chitosan.

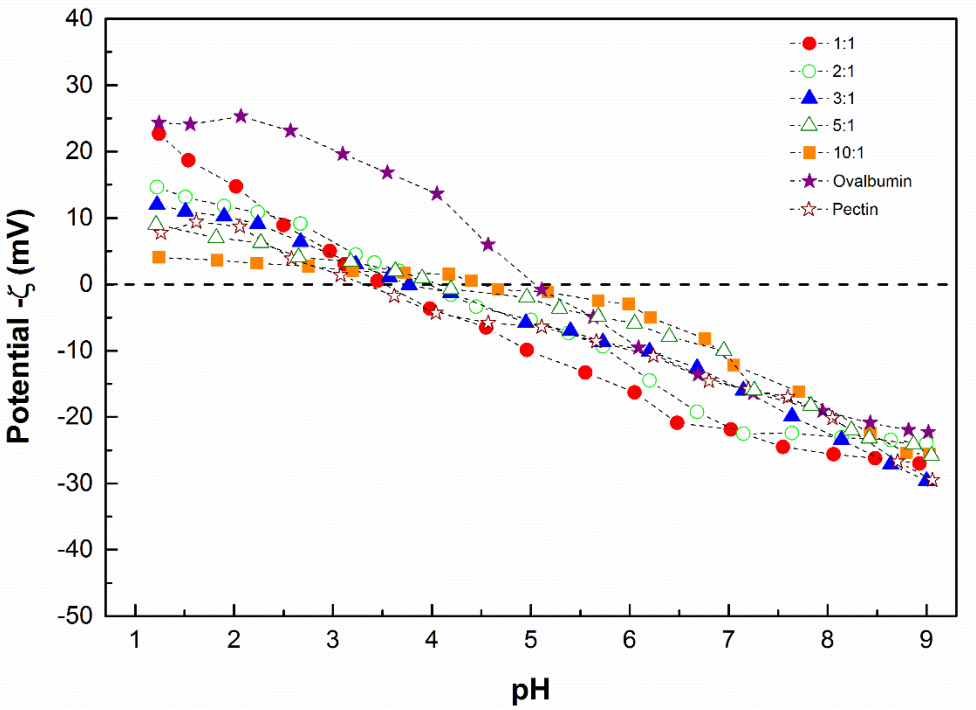

Figure 3: Potential-ζ depending on the pH of the systems containing pectin or Ovalbumin with 0.1% (w/w) or complex Ova/Pec at different ratios with a fixed concentration (0.1 M NaCl).

Recently SADAHIRA et al. (2014) could find that the electrical neutrality and the size of the formed electrostatic complexes depend on the egg white:pectin ratio showing that complexes obtained with protein:pectin ratio 15:1 were close to electroneutrality with large size (95.91 ± 8.19 µm) than those obtained with ratio 55:1 (45.92 ± 3.47 µm) not electrically neutral assigning this result of higher concentration of non complexed protein

3.3 Effect of ionic strength

When titrating complexes formed in various concentrations of NaCl, we observed a region of constant turbidity from pH 9.0–5.5 followed by an abrupt increase in turbidity values ($pH_{\varphi 1}$) (Figure. 4). When the NaCl concentration ranged from 0.01 to 0.1 M, plateaus of maximum turbidity were reached and were followed by suppressions of pH_{ot} and slight negative displacements of $pH_{\varphi 1}$ (Figure 4B). At 0.01 M NaCl, we observed Ova:Pec coacervate complex formation, but at 0.05-0.1 M NaCl, we observed ovalbumin aggregation. We attribute this NaCl effect to the fact that protein molecules are amphoteric polyelectrolytes containing both positive and negative charges, thus there are both attractive and repulsive electrostatic forces between the charges on the proteins and polysaccharides (HATTORI et al., 2000). The electrostatic interaction model for a protein-polyelectrolyte complex by SEYREK et al. (2003) states that the attractive and repulsive electrostatic forces between a protein and a polysaccharide may be related to the average distances between the positive sites on the protein and the negative sites on the polysaccharide (R_+), the average distances between negative sites on the protein and the polysaccharide (R_-), and the Debye length (R_d), which is the distance at which significant charge separations persist. When Ova:Pec coacervation occurred at a low concentration of NaCl (0.01M), we speculated that $R_+ < R_d < R_-$, allowing the salt to screen the electrostatic repulsion between ovalbumin and pectin without disturbing the

electrostatic attraction between them, thus allowing the formation of complexes at higher pH's. However, when the salt concentration was increased to 0.05-0.4 M, that $R_d < R_+ < R_-$ resulting in screening of both repulsive and attractive electrostatic interactions between the polymers, in promoting ovalbumin self-aggregation, and in a decreased $pH_{\varphi 1}$. WANG et al. (2007b) reported self-aggregation of β-lactoglobulin in a β-lactoglobulin:pectin coacervate complex and a reduction in $pH_{\varphi 1}$ values when the salt concentration was increased from 0.01 to 0.1 M. In addition, they found a further reduction in $pH_{\varphi 1}$ when the salt concentration was > 0.2 M. We found the same further reduction in $pH_{\varphi 1}$ when the salt concentration was > 0.1 M. We also noticed a slight displacement in $pH_{\varphi 2}$ when salt concentrations were between 0.01-0.1 M; however, when salt concentrations were > 0.1 M, $pH_{\varphi 2}$ and dissociation of the complexes were suppressed (Fig. 4B).. This has also been observed by KLASSEN et al. (2011) with 20:1 canola protein:ι-carrageenan or alginate complexes in 100 mM NaCl and by RU et al. (2012) with 5:1 BSA:Pectin complexes in 0.4 M NaCl. This work provides further evidence of suppression of dissociation due to pectin self-aggregation. The effect of salt depends on the natures of the polymers, for example, formation of 2:1 ovalbumin:gum arabic complexes was suppressed in 1-60 mM NaCl (NIU et al., 2014).

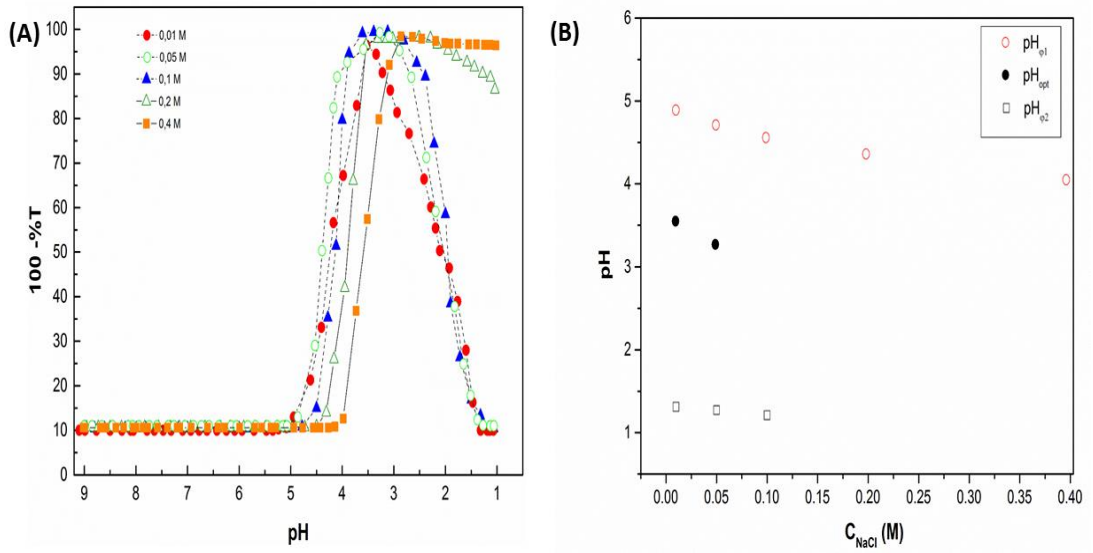

Figure 4: (A) Turbidity (100-T%) depending of pH of the systems containing Ova / Pec (1:1) at different NaCl concentration. (B) Variation pHφ1 pHop and pHφ2 of complexes formed between Ova / Pec in different concentrations of NaCl.

3.4 X-Ray Diffraction

X-ray diffraction analysis provides atomic coordinates, direct structural information, and permits determination of the amorphous or crystalline characteristics of molecules (CHANDRASEKARAN, 1998). We found pectin to be primarily amorphous with a small partially crystalline band between 23-27° (Fig. 5) as did MISHRA et al. (2009). Ovalbumin was also found to be amorphous, and this amorphous characteristic is directly linked to polymer hydrophilicity and hygroscopicity (PICKUP et al., 2014). The Ova:Pec complex was amorphous and showed a partially crystalline band between 23-27° that was more pronounced than the band seen with pectin alone suggesting that the interactions between ovalbumin and pectin promoted a more defined structure.

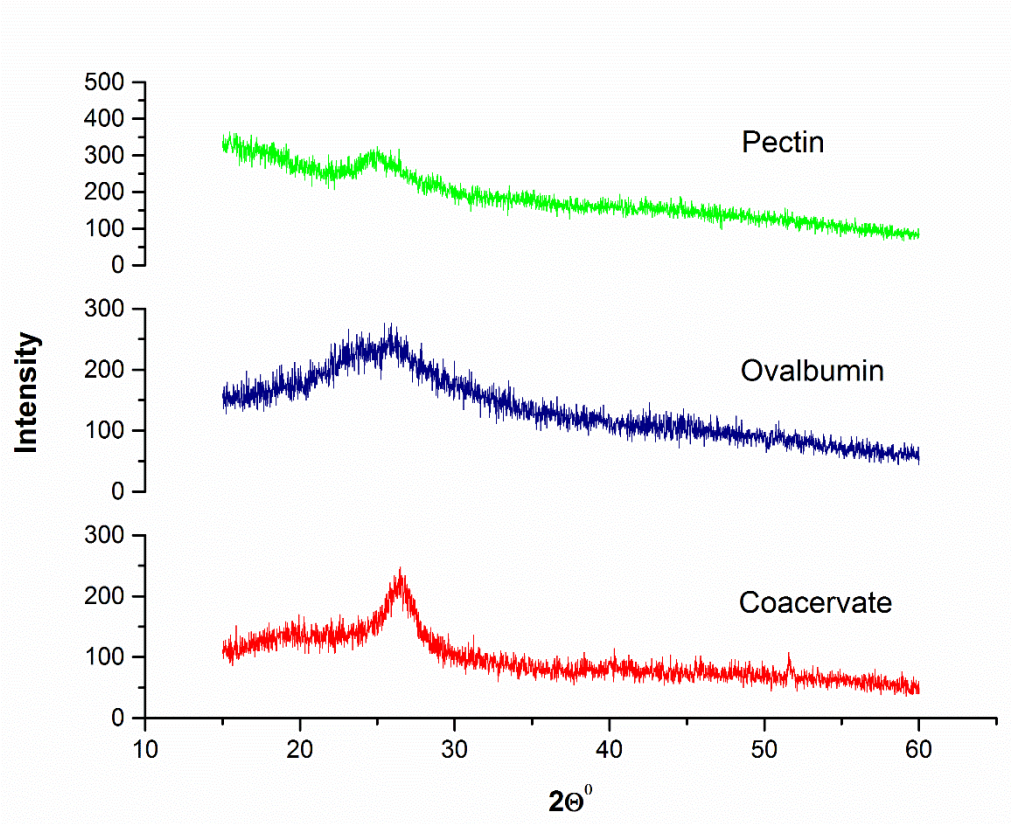

Figure 5: X-ray diffraction of powdered pectin, ovalbumin and complex Ova/Pec (1:1) prepared at pH 3.5 with 0.01 M NaCl.

4 CONCLUSIONS

The formation of ovalbumin-pectin complexes was found to be dependent on protein and NaCl concentrations. A 1:1 Ova:Pec ratio displayed three distinct regions of complex formation, and when the protein:polysaccharide ratio ranged from 1:1 to 1:5, ovalbumin aggregation led to a shift in pH_c followed by a suppression of complex dissociation. At a 10:1 protein:polysaccharide ratio, complex dissociation was abolished. Similarly, increasing NaCl concentrations screened electrostatic interactions between polymers, promoted ovalbumin self-aggregation, and decreased $pH_{\varphi 1}$ and $pH_{\varphi 2}$. This study addressed the affect of ovalbumin self-aggregates on ovalbumin-pectin complex formation.

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CAPÍTULO III

EFFECT OF THE RATIO PROTEIN-POLYSACCHARIDE AND IONIC FORCE ON FORMATION OF COACERVATES BETWEEN LYSOZYME AND PECTIN

ABSTRACT

Proteins and polysaccharides are the most frequently used hydrocolloids in the food industry, and their interaction can provide products such as complexes coacervates, which can be used as ingredients and biomaterials or in microencapsulation systems. The complexation between lysozyme (Lyso) and citrus pectin (Pec) was studied in situ by acidification (12.0-1.0) using zeta potential and turbidity measurements. The complexes were analyzed in different NaCl concentrations with different protein:polysaccharide ratios. In the ratio 1:1 with 0.01 mol/L of NaCl the region of the formation of insoluble complex corresponded a pH range 7.0 until 2.0 which represent a great range to apply this complex on different matrix food. However, when ratio was increased from 1:1 to 3:1, the pH range between $pH_{\varphi 1}$ to $pH_{\varphi 2}$ increased meantime, the critical pH value decreased disappearing in the ratios 5:1 and 10:1. When NaCl concentration was increased from 0.01 mol/L to 0.2 mol/L was possible to see a progressive reduction of turbidity and the pH range of complex formation achieving in a total suppression of complex in 0.4 mol/L. The microscopy images of samples revealed that complexes present spheroid like appearance with the heterogeneous structure containing a single core polymeric phase. The Lyso/Pectin complex particles described here may represents a great potential in various commercial applications in the biotechnological, pharmaceutical, and food industries, for example, as delivery systems to encapsulate, protect, and release bioactive components or as functional ingredients to modify texture of products.

1 INTRODUTION

New challenges and emerging opportunities in the area of food product development are to effectively control ingredient functionality in common food types using appropriate delivery vehicles such as emulsions and gels (SCHOLTEN et al., 2013). A great number of industries are interested in incorporating bioactive functional components into their products so as to maintain or promote human health, for example, the food, cosmetics, and pharmaceutical industries (FATHI et al., 2014; MCCLEMENTS, 2015b; MCCLEMENTS, 2015a). In the food industry, these components include bioactives (e.g., coenzyme Q10, and lipophilic compounds) (STRATULAT et al., 2013; STRATULAT et al., 2014), proteins (e.g., immunoglobulin, and Lactoferrin) (LI et al., 2009; RAEI et al., 2015), carbohydrates (e.g., prebiotics and dietary fibers) (LIAO et al., 2005; IRAVANI et al., 2014), and minerals (e.g., iron and calcium) (BRAITHWAITE et al., 2014) that have been demonstrated to have potential health benefits.

The key to the develops a successful new ingredient depends of physicochemical characteristics of the bioactive component itself and of the surrounding food matrix (such as pH and ionic strength), and also is worth mentioning factors such as low water-solubility, poor bioavailability, adverse ingredient interactions, and detrimental effects on the physicochemical and sensory attributes of foods (such as taste, texture, appearance, and stability) (AUGUSTIN e SANGUANSRI, 2015). A promising approach to overcome many of these challenges is the use of biopolymer delivery systems (coacervate) that

convert the bioactive components into a form where they can be conveniently incorporated into foods and other products.

The formation of complex coacervates between biopolymers (proteins and polysaccharides) in solutions has been under extensive investigation and review (BURGESS, 1990; YE, 2008; SCHMITT e TURGEON, 2011; KAYITMAZER et al., 2013; SCHOLTEN et al., 2013). In general, mixtures of biopolymers lead to either phase separation through thermodynamic incompatibility (biopolymers segregate into separate phases) or complex coacervation (biopolymers associated) (TURGEON et al., 2007a). Segregative phase separation occurs at high concentrations and high ionic strengths when both biopolymers carry similar charges, thereby resulting in electrostatic repulsion between the molecules. On the other hand, associative phase separation or complex coacervation usually occurs at relatively dilute concentrations, low ionic strengths and when both biopolymers carry opposite charges (BURGESS, 1990; KRUIF et al., 2004). The general picture for coacervation between protein and anionic polysaccharide is that the charge "patches" on the protein molecules cause the protein to interact with anionic polysaccharides to form soluble protein/polysaccharide complexes when pH > pI, initiated at the first critical pH (pH_c). At the second critical pH ($pH_{\varphi 1}$), abrupt increases in turbidity illustrate the continued aggregation of soluble complexes into insoluble protein/polysaccharide complexes due to charge neutralization (WEINBRECK et al., 2003a; KRUIF et al., 2004). For carboxylic acid-based polysaccharides, such as pectin, when pH decreases to the third critical pH ($pH_{\varphi 2}$), insoluble complexes dissociate into soluble complexes, or into uninteracted protein molecules and polysaccharide chains (JONES e MCCLEMENTS, 2010).

Coacervates or polysaccharide-protein complexes are attractive to the industry because they are natural products obtained by commonly used ingredients are therefore inexpensive and easy to regulatory approval food, but also there is another very significant advantage that is incrementing the functional properties. When the formed coacervate is then arises biopolymer with new functional properties usually superior functional properties of the individual polymers (DICKINSON, 2003). Pectin is a polysaccharide well-known for its long and safe use in the food industry as a gelling and thickening agent and as a stabilizer (LOPES DA SILVA e RAO, 2010). Because of its gelling characteristics, bioadhesivity, biocompatibility and non-toxicity properties, pectin is also a promising polymer for use in the delivery of pharmaceuticals. Pectin has, e.g., been used in mucoadhesive drug delivery systems to increase the retention time of the dosage form in the gastrointestinal tract, and thus enhance drug absorption after oral administration (LIU et al., 2005). Lysozyme has shown a wide range of application in the food and pharmaceutical industry (ABEYRATHNE et al., 2013). In the pharmaceutical industry, lysozyme has been shown effective in preventing and controlling several viral skin infections, including herpes simplex and chicken pox, as well as acting as exerting anti-inflammatory action (SAVA, 1995). In the food industry, lysozyme is one of the major bacteriolytic proteins having the capability of controlling foodborne pathogens such as *Listeria monocytogens* and *Clostridium botulinum* (RADZIEJEWSKA et al., 2008; LEŚNIEROWSKI e CEGIELSKA-RADZIEJEWSKA, 2012), which are considered 2 main pathogens that cause problems in the food industry.

The present study has aimed to elucidate the influence of pH, ionic strength and ratio of lysozyme/pectin in the kinetics of the formation of complex coacervates.

2 MATERIALS AND METHODS

2.1 Materials

Lysozyme (Lyso purity > 90%) and pectin high methoxyl (Pec; HM) were obtained from Sigma Chemicals (St. Louis, USA). Sodium chloride (NaCl, purity > 99%), hydrochloric acid (HCl, 0.5 M), and sodium hydroxide (NaOH, 0.5 M) were purchased from VETEC® Ltda, (Rio de Janeiro, Brazil). The water used was ultrapure with a conductivity of 0.05 µS/cm ± 0.01 (Gehaka-Master P&D – Brazil). Stock solutions of pectin (0.1% w/w) and lysozyme (0.1-1% w/w) were prepared by gently stirring the powders in deionized water for 6 hours at room temperature (25 °C).

2.2 Formation of complexes

2.2.1 Preparation of complexes

The concentration of pectin used was 0.1% w/w and the concentration of lysozyme used varied from 0.1-1% w/w. Five ratios of Lyso:Pec (1:1, 2:1, 3:1, 5:1, 10:1) were evaluated. To determine the effect of NaCl on complex formation, Lyso:Pec complexes were formed in five different concentrations of NaCl (0.01 M, 0.05 M, 0.1M, 0.2 M, 0.4 M). The Lyso:Pec mixtures were previously stirred and adjusted to pH 12 for turbidimetric measurements.

2.2.2 Turbidimetric measurements

pH-dependent turbidity was measured at a wavelength of 600 nm using a spectrophotometer (Biochrom mod. LIBRA S12, England) calibrated with ultrapure water to 100% transmittance (T). Turbidity was defined as 100 e %T. With the aid of a magnetic stirrer (Nova Tecnica, NT 101, Brazil) and a pH meter (Tecnopon, mPA-210, Brazil) the pH of the solutions were adjusted (12.0-1.0) with HCl, 0.5 M. Measurements of complexes and the solutions with biopolymers isolates were made at room temperature (25 C ± 1), and each sample was measured four times at 1 min intervals.

2.3 Zeta - Potential

A Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK) was used to determine zeta (ζ)-potentials. Lysozyme and pectin stock solutions and Lyso:Pec complexes formed in 0.01 M NaCl were diluted to 0.01% w/w and transferred to an MPT-2 autotitrator (Malvern Instruments, Worcestershire, UK) that adjusted pH using 0.5 M NaOH, 0.25 M HCl and 0.025 M HCl solutions. pH was varied from 9.0 to 1.0 by 0.5 unit increments with a confidence interval of ± 0.1 unit. ζ-potentials were calculated using the Smoluschwsky mathematical model. The Stokes-Einstein equation was used to calculate the average size of the particles. Complexes with different Lyso:Pec ratios were diluted as described above and the pH was adjusted to 3.55. Each experiment was performed three times and sample readings were done in triplicate at 25 °C.

2.4 Optical microscopy and atomic force microscope

An optical microscope (Nikon, model LV 150,USA) amplified 20x with coupled camera (High-definition DSFi1) and Software (NSI-Elements D 3.0) was used to obtain images of complex. The preparation of samples was adapted from methodology proposed by SOUZA et al. (2013) in which centrifugation of samples containing precipitated (coacervate) was kept under refrigeration for 24 h assuring formation of complexation. Superior liquid phase was removed with pipette, and precipitated was resuspended in 3.0 mL buffer citrate pH 4.1 and afterward an aliquot of 1 mL was taken to be observed by means of an optic microscope.

The complex samples were imaged using an atomic force microscopy in a FlexAFM (Nanosurf, Basel, Switzerland). As described for optical microscopy the complex samples precipitated (1 mL) was resuspended in 10 ml buffer citrate pH 4.1 and afterward an aliquot of 2 µl were deposited in freshly cleaved mica and allowed to stand in air before imaging. The experiments were carried out in air, operating in contact mode (constant force). Oxidized sharpened silicon tips and a spring constant of 0.2 N/m were used for imaging. data were analyzed with the Easyscan 2 software®.

3 RESULTS AND DISCUSSION

3.1 pH-Induced phase separation for the Lyso-Pec complexes

The effect of pH on complex formation was investigated at the ratio 1:1 Lyso-Pec mixing ratio, NaCl concentration (0.01M). The complex coacervation phenomenon of Lyso-Pec mixture was investigated the pH range (12-1) via the dropwise addition of HCL. In the control group, homogenous solutions of Lyso and Pec were studied under the same conditions.

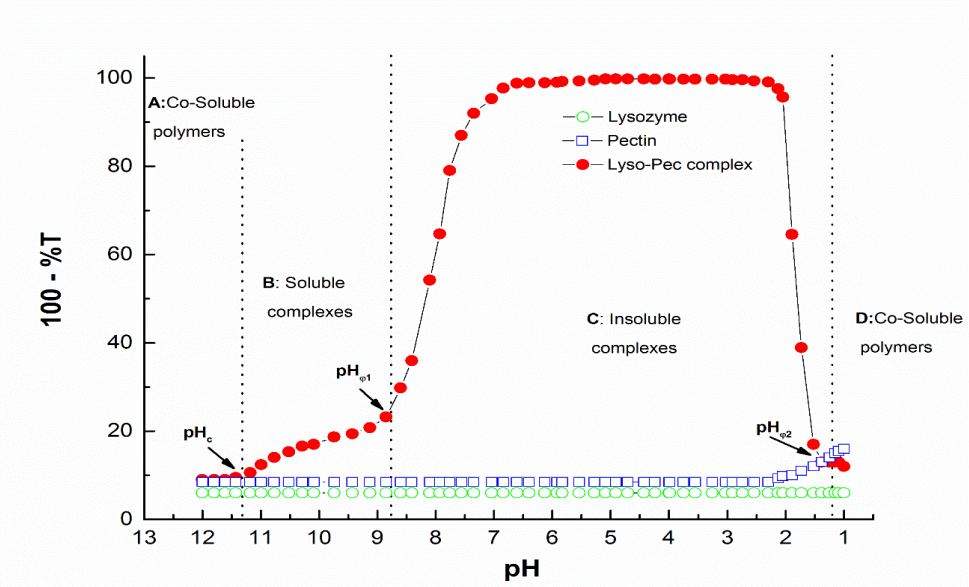

Figure 1: Turbidity of systems containing individual biopolymers and Lyso-Pec complex at ratio 1:1 as a function of pH using 0.01 M NaCl and determination of stability and instability regions (A, B, C and D) of complex coacervate

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In the figure 1 is possible see that four phases (A, B, C and D) were obtained with changing of turbidity of the Lyso-Pec mixture. The critical pH transitions points (pH_c, $pH_{\varphi 1}$ and $pH_{\varphi 2}$) were determined by intersection point of two curve tangents. The turbidity arises mainly from the change in mass and size of aggregates in the solution, and as we can see, the mixture remained almost a constant value above pH_c (11.37) in the region A. However, the turbidity curve had a slight rise as the pH value decreased from pH_c to $pH_{\varphi 1}$ (pH 8.74) (region B). I occurred because there was the formation of soluble complexes between Lyso and Pec because, although both biopolymers were deprotonated the Lyso became more protonated as soon as pH approached the p*I* (10.7) which promoted weak electrostatic interaction with each other forming soluble complexes (KRUIF et al., 2004; SCHMITT e TURGEON, 2011). In so far as the drops of HCL were added the electrostatic interaction became stronger influenced, mainly by protonation of protein, and mixture turbidity increased sharply forming insoluble complex (region C). At this region, the electrostatic attraction between the protein and polysaccharide molecules is sufficiently strong to promote the formation biopolymers packed densely which decrease the presence of solvent internally and consequently the precipitation occur (JONES e MCCLEMENTS, 2010). It is worth mentioning that this region corresponds a range from pH 7.0 until 2.0, which represent a great range to apply this complex on different matrix food (UBBINK et al., 2008). In the region D, the pectin became highly protonated (pH < pK_a 3.5), which furthered the gradually disassociation between biopolymers and the mixture system became transparent showing that interaction Lyso-Pec ceased at a lower pH ($pH_{\varphi 2}$ = 1.52).

The self-aggregation of protein can interfere directly on charge patches or on charged residues of protein during electrostatic interaction with polysaccharide (LI e HUANG, 2013) meanwhile, as we is possible to see the lysozyme did not aggregate even in pH near of p*I*; However the pectin aggregated at pH < 1.7 but was possible observe that this aggregation was not noticeable at $pH_{\varphi 2}$. Similar behavior was mentioned by SOUZA e GARCIA-ROJAS (2015).

In order to identify the density of charge of individual biopolymers and Lyso-Pec complex formed at ratio 1:1 the ζ-potentials as function of pH was conducted and results are present at figure 2. As it is possible to notice the Lyso has a change in the electrical charge on the individual protein solution, ranging from a negative charge at pH 11.9 to a positive charge at pH 1.6 with a zero load point (p*I*) near 10.7 as reported in the literature (STADELMAN e COTTERILL, 1995b). Same behavior was observed with pectin ranging from negative charge at pH 11.8 to a positive charge at pH 1.2 with a zero load point (pK_a 3.5) as reported in the literature (LOPES DA SILVA e RAO, 2010). It can be observed that the values found for the complex is the values of the isolated polymer intermediates. This behavior indicates the interaction between the carboxyl groups of the polysaccharide and protein amine groups, featuring electrostatic binding. In the pH 11.9 is possible to see that ζ-potentials of complexes was closer then pectin meanwhile, when pH was decreased about pH 11.5 is possible see that complex became less negative charged which can explain the weak electrostatic interaction and formation of soluble complexes (COOPER et al., 2005; KIZILAY et al., 2011). About the pH 6.7 the region of electrical charge equivalence pH (EEP) (LE e TURGEON, 2013) was found indicating that biopolymers were strongly bonded and this pH correspond the beginning of the region of plateau in the figure 1. Finally, in pH 2.0 is possible to note that pectin present a decrease of protonation from pH 2.0 -1.2 and we suggest that is may corresponds the self-aggregation discussed above.

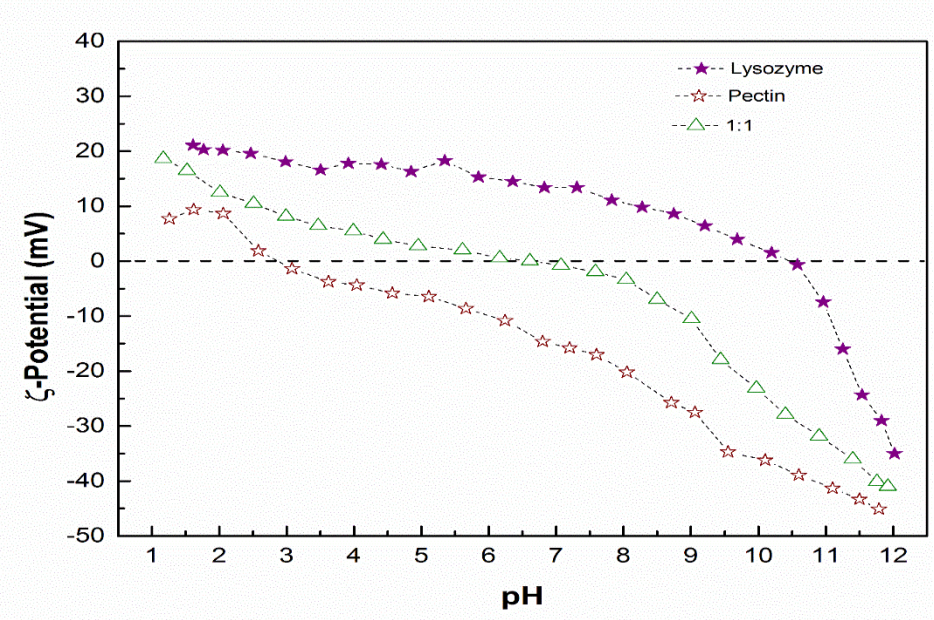

Figure 1: Potential-ζ of Lysozyme or Pectin systems with 0.1% (w/w) or Lyso-Pec at ratio 1:1 as a function of pH with a fixed concentration of 0.01 M NaCl.

3.2 Effect of ratio Lyso-Pec

The effect of the biopolymer mixing ratio is a critical point in controlling the charge balance in the mixed systems (ZEEB et al., 2014). In figure 3, the titration curves of turbidity (100-T%) versus pH for the Lyso-Pec mixtures as a function of the ratio showed that increased of protein affected directly the kinetic of formation of complexes. When the ratio was enlarged from 1:1 to 3:1 is possible to note in the figure 3B that pH range between $pH_{\varphi 1}$ to $pH_{\varphi 2}$ increased meantime, the pHc decreased disappearing in the ratios 5:1 and 10:1. As the ratio of protein was increased, a large number of positively charged lyso molecules were available to neutralize the negative pectin carboxyl groups even in the pH > p*I* of protein (GIRARD et al., 2003b) which, gradually increased the strength of interaction between protein and polymer resulting in shifting of pHφ1 and pHc from pH 8.74 to 10.71 and pH 11.32 to 11.65 respectively. In the ratio 5:1 and 10:1, the strength of interaction between protein and polysaccharide was large enough to suppress the formation of soluble complexes (pH_c) resulting in a rapid increase of turbidity (Figure 3A) and formation of complexes ($pH_{\varphi 1}$) in pH ≥ 11.50 (Figure 3B). The strength of interaction also affected directly the average size of complexes and $pH_{\varphi 2}$ of those systems. In the ratios 1:1 and 2:1 are possible notice a slight increase of the average size of complexes; however, when the ratio was enlarged from 3:1 to 10:1, the average size of complexes increased drastically caused mainly by the large number of protein bound with pectin. This finding reflects the abundance of protein molecules available per polysaccharide chain. The values of $pH_{\varphi 2}$ tend to have smaller pH values when the ratio increases from 1:1 to 10:1. The smaller pHφ2 indicates that the Lyso/pectin coacervates are more difficult to dissociate at the higher ratio, which is in accordance with the foregoing discussion about ratio effects previously reported by RU et al. (2012), KLASSEN et al. (2011), LIU et al. (2010), and WEINBRECK et al. (2003a).

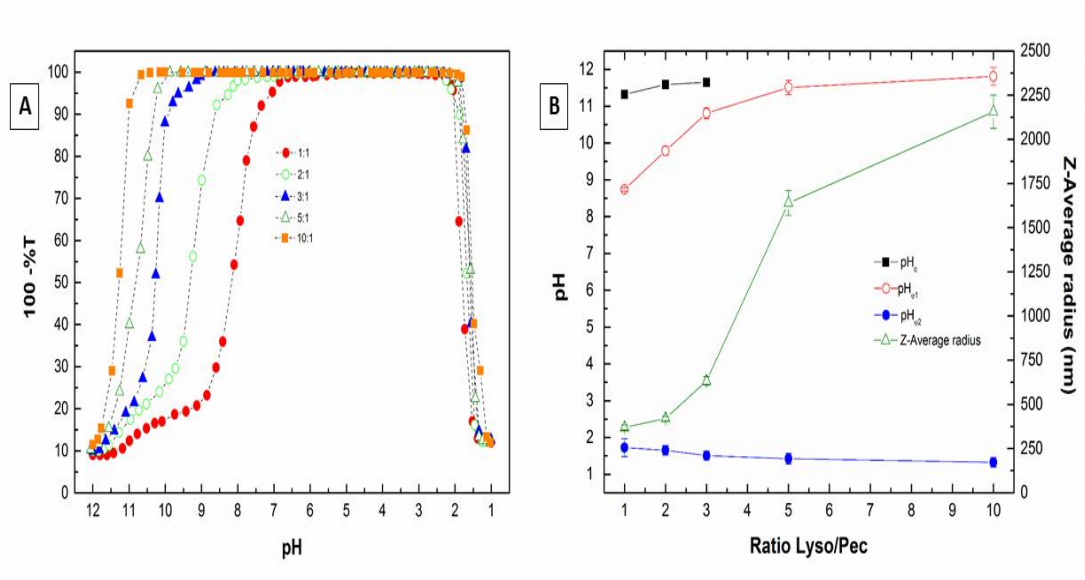

Figure 2: (A) Turbidity (100-T%) as a function of pH of the systems containing Lyso-Pec at different Ratios (B) Variation of pH_c, $pH_{\varphi 1}$ and $pH_{\varphi 2}$ and average particle diameter as a function Lyso/Pec ratio with 0.01M NaCL

3.3 Effect of ionic strength

The net charge carried by the proteins and polysaccharides may be reduced by interaction with the microions, resulting in a decrease in the electrostatic attraction between the macromolecules. At the high ionic strength, screening the charges of the proteins and polysaccharides also leads to reduce electrostatic interactions and hence affect the formation of complexes (YE, 2008; SCHMITT e TURGEON, 2011). At the low ionic strength, the microions concentration can favor complexation by promoting greater solubility of the polymers, leading to the winding of the molecule and the exposing of their loads, thus favoring electrostatic binding (BURGESS, 1990). In figure 4, the titration curves of turbidity (100-T%) versus pH for the Lyso-Pec mixtures (ratio 1:1) as a function of the NaCl concentration showed that increased of NaCl affected directly the kinetic of formation of complexes.

In the concentration of 0.01 mol/L (figure 4A) is possible note that the ability of competitive adsorption between Na^+ and Cl^- was not able to compete with the positive and negative charge biding site of lysozyme and pectin respectively, showing that the effect of NaCl concentration on complex formation could be favoring the maximum electrostatic interaction (turbidity ~ 100%) (SCHMITT et al., 1998; TURGEON et al., 2007a). However, when NaCl concentration was increased from 0.05 until 0.2 M, the kinect and turbidity of systems were directly affected by the amount of ions resulting in a progressive reduction of turbidity and the pH range of complex formation. At 0.4 mol/L the ability of competitive adsorption between Na^+ and Cl^- becomes strong enough to suppress the electrostatic interaction between biopolymers, which explain the lower turbidity during all pH range studied. WEINBRECK et al. (2003a) investigated the effect of NaCl concentration on the coacervation of whey proteins–gum arabic as a function of pH at a 2:1 mixing ratio and found that the turbidities for all the turbidimetric titration curves decreased with increasing NaCl concentration. RU et al.

(2012) and NIU et al. (2014) also observed similar trends in bovine serum albumin–pectin and ovalbumin-gum arabic complexes respectively.

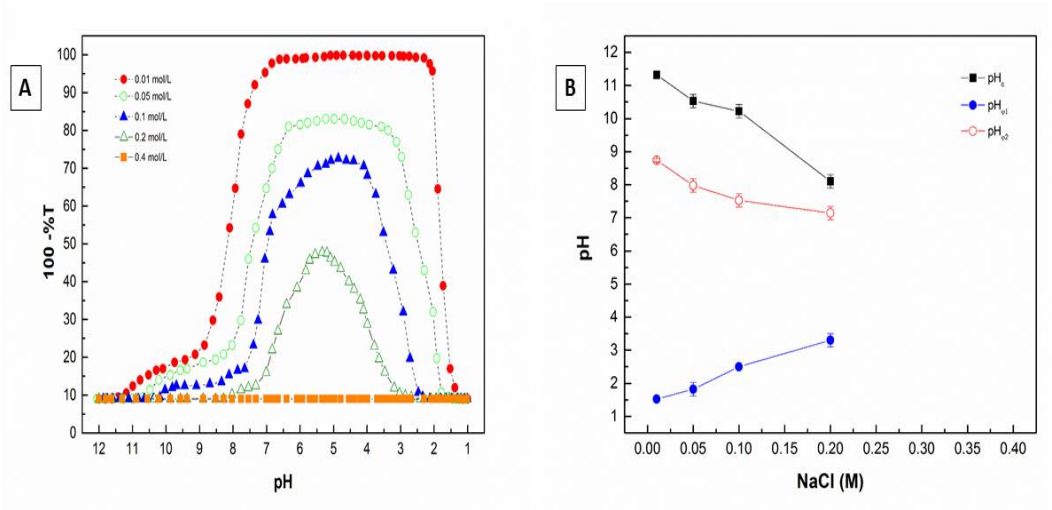

Figure 3: (A) Turbidity (100-T%) as a function of pH of the systems containing Lyso-Pec (ratio 1:1) at different NaCl concentrations (B) Variation of pHc, pHφ1 and pHφ2 as a function of different concentrations of NaCL

In the figure 4B, the critical pH values (pH_c and $pH_{\varphi 1}$) shifted toward lower pH when NaCl was added to the mixtures, but the $pH_{\varphi 2}$ shifted to higher values; the range of biopolymer interactions became narrower. It was supposed that NaCl from 0.01 to 0.2 mol/L screened part of the charge groups on the Lyso and Pec surfaces, the formation of insoluble complex occurred only after Lyso took on more positive net charges as pH decreased. However, the reactive groups on the Lyso polysaccharide became protonated, so the $pH_{\varphi 2}$ shifted to higher pH values. GULÃO et al. (2014) also observed similar trends in lactoferrin–gum arabic mixtures in the ratios 2:1 and 3:1. LIU et al. (2009b) studied the effect of salt on the formation of pea protein isolate–gum arabic complexes and the results revealed that all critical pH values were independent of NaCl levels at concentrations of $\leq$ 7.5 mM. Nevertheless, YE et al. (2006) observed a slight increase in the absorbance values in the sodium caseinate–gum arabic mixtures at low levels of NaCl (5 and 10 mM) between pH 5.0 and 3.0, however, at high NaCl concentrations (50 and 100 mM), the absorbance values enlarged with increasing NaCl concentration.

3.4 Morphological characteristics of coacervate complexes

The morphological characteristic of the complex particles is presented on figure 5. Observing morphology of dehydrated complexes in figure 5 A and B is possible to verify that during electrostatic interaction, the particles formed were slightly irregular in shape but had a spheroid like appearance. PEINADO et al. (2010) described the similar appearance for Lactoferrin/Pectin complexes. As discussed above there is the possibility to form complex Lyso/Pec (capsules) in a pH ranges from pH 7.0 until 2.0, which represent a great range to apply this complex as a nutraceutical ingredient in pharmaceutical or food industry. (MCCLEMENTS, 2015a; YAO et al., 2015).

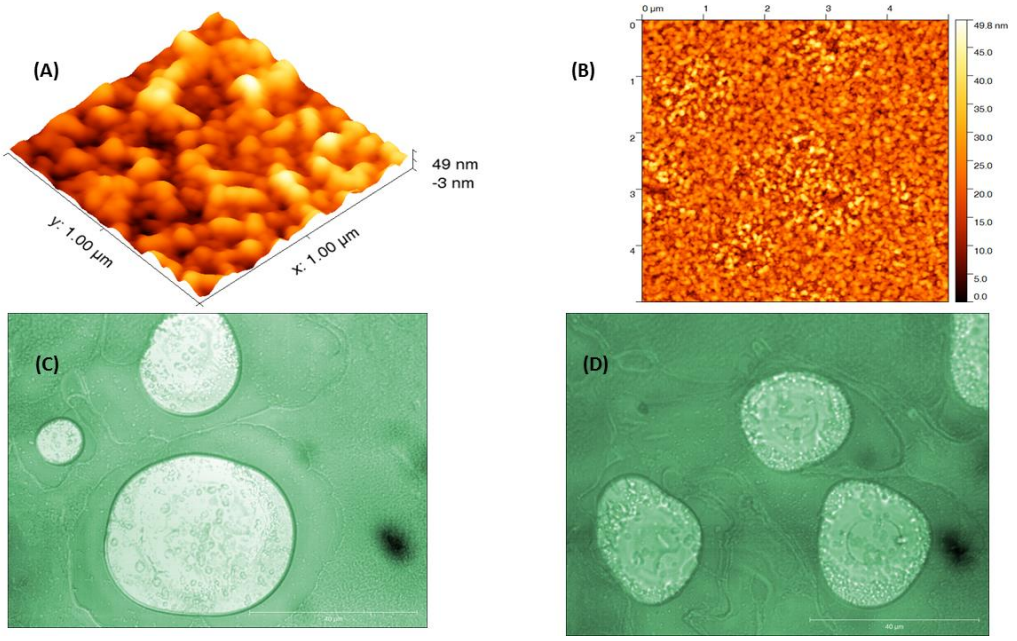

Figure 4: Morphological images of Lyso/Pec complexes at ratio 1:1 with 0.01 mol/L NaCl. (A) and (B). Atomic force microscopic images of samples. (C) and (D) optical microscopy images (50 x) of samples at pH 4.0.

For better understanding the new biopolymer formed, precipitated obtained in pH 4.0 were separated and kept hydrated with the objective of demonstrating new structures formed in the natural state and the results can be seen at figure 5 C and D. The optical microscopy of samples revealed capsules with heterogeneous structure containing a single core polymeric phase (NESTERENKO et al., 2013). Although the mechanisms for the identification of biopolymers in coacervation processes are not well elucidated, it is possible suggest that pectin forms a layer around the lysozyme. Similar structure was described by NGUYEN et al. (2011) for the liposomes coated with HM-pectin.

4 CONCLUSIONS

Structural transitions of protein/polysaccharide during complexation were studied using Lyso/Pectin mixtures and was found four distinct regions of complex formation; however, the region of formation of complex coacervates corresponded large pH range from 7.0 until 2.0. The increase in the ratio promotes Lyso/Pectin coacervation and increase in the ratio raised the strength of interaction between biopolymers as well as the

average-size particle of complex. Was possible to note that when the ratio was enlarged from 1:1 to 3:1, the pH range between $pH_{\varphi 1}$ to $pH_{\varphi 2}$ increased meantime, the pHc decreased disappearing in the ratios 5:1 and 10:1. The ability of competitive adsorption between Na+ and Cl- affected directly the structural transitions and strength of interaction of the complexes. In the concentration 0.01 mol/L the NaCl was not able to compete with the positive and negative charge biding site of lysozyme and pectin respectively. However, when concentration of NaCl was increased from 0.01 mol/L to 0.2 mol/L was possible to see a progressive reduction of turbidity and the pH range of complex formation achieving in a total suppression of complex in 0.4 mol/L. The microscopy images of samples revealed that complexes present spheroid like appearance with the heterogeneous structure containing a single core polymeric phase. The Lyso/Pectin complex particles described here may represents a potential application in various commercial applications in the biotechnological, pharmaceutical, and food industries, for example, as delivery systems to encapsulate, protect, and release bioactive components or as functional ingredients to modify texture of products.

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CAPÍTULO IV

COMPLEXATION OF EGG WHITE PROTEINS AND K-CARRAGEENAN: EFFECT OF IONIC STRENGTH AND PROTEIN-POLYSACCHARIDE RATIO

ABSTRACT

In foods, proteins and polysaccharides are the most important structure-forming ingredients. The complexation between lysozyme/κ-carrageenan and ovalbumin/κ-carrageenan was studied *in situ* by acidification (12.0-1.0) using zeta potential, turbidity and rheological measurements. The complexes were analyzed in different NaCl concentrations with different protein/polysaccharide ratios. As the protein/polisacharide ratio increased from 1:1 to 10:1, critical structure forming events (i.e., those associated with soluble, insoluble and large insoluble complexes) shifted to higher pHs with ovalbumin/κ-carrageenan followed by decrease of G' values of the ratios 5:1 and 10:1. The increased of the ratio with lysozyme/κ-carrageenan complexes suppressed the critical pH transitions points forming large insoluble complexes from pH 12.0 until 1.0 and the values of G' increased simultaneously reaching the higher value of the ratio 10:1. Addition of salt to the ovalbumin/κ-carrageenan and lysozyme/κ-carrageenan mixtures suppressed the electrostatic interaction between proteins and κ-carrageenan shifting to lowering pHs the critical pH transitions points, meanwhile at the ratio 3:1 with 0.01 mol/L the complex coacervates yield reached 79.6% ± 0.6 and 93.7% ± 4.8 for the ovalbumin and lysozyme complex. The rheological data associated with microscopy images showed that intrapolymer complexes with heterogeneous structure were formed for both complexes, and we suggest that complexes represent a great potential to improve or extent texture, mechanical stability, consistency, and taste of food products.

1 INTRODUTION

Protein/polysaccharide coacervation is of continuing interest due to great flexibility in the engineering of mechanical and structural properties of foods, cosmetics, and pharmaceuticals (TURGEON et al., 2007a). In foods, proteins and polysaccharides are the most important structure-forming ingredients and their use in mixed systems can improve or extent their texture, mechanical stability, consistency and, ultimately, appearance and taste (SAMANT et al., 1993; DICKINSON, 2003; VAN VLIET et al., 2004b).

Mixing a protein with a polysaccharide into an aqueous solution may drive to one of several situations depending on the polymer–polymer and solvent–polymer attractive or repulsive interactions and a greater understanding of the factors affecting biopolymers interactions is required, particularly those of electrostatic origin (ZEEB et al., 2014). Attractive interactions between two biopolymers can become evident in various ways: (i) formation of small soluble complex, manifesting itself in murky solutions, (ii) formation of a homogeneous weak gel, if interactions are weak, and (iii) precipitation of both biopolymers, if interactions are strong (SEMENOVA e DICKINSON, 2013).

The environmental parameters of a system, such as pH, ionic strength, protein to polysaccharide ratio, amount of total solids, the rate of acidification, and the shear rate during acidification play critical roles in protein/polysaccharide coacervation (BURGESS, 1990; TURGEON et al., 2007a; SCHOLTEN et al., 2013). Alteration of the surface charge of both the protein and the polysaccharide by a change in pH initiates and prolongs the ongoing of coacervation. Proteins and polysaccharides associate together at the critical pH (pH_c) where the protein and polysaccharide are oppositely charged, which forms soluble protein/ polysaccharide coacervates (WEINBRECK et al., 2003a). Coacervation at the pH of visual phase separation (pH_φ) beyond pH_c produces insoluble

protein/polysaccharide coacervates, resulting in an abrupt increase in turbidity (GULÃO et al., 2014). Further coacervation increases the size, but decreases the number of protein/polysaccharide coacervates near the pH value of maximum turbidity (pH_{max}) (KAIBARA et al., 2000). The addition of salt to the system suppresses coacervation due to a screening effect that prevents electrostatic interaction and reduces the pH_c, pH_φ, and pH_{max} values of protein/polysaccharide coacervation (ANTONOV et al., 2009; SOUZA e GARCIA-ROJAS, 2015). The higher the protein to polysaccharide ratio, the higher is the pH_φ value of protein/polysaccharide coacervation (LIU et al., 2009b; KIZILAY et al., 2011).

Coacervates or polysaccharide-protein complexes are attractive to the industry because they are natural products obtained by commonly used ingredients are therefore inexpensive and easy to regulatory approval food (JONES e MCCLEMENTS, 2010). Ovalbumin is the major egg white protein synthesized in the hen's oviduct and accounts for 54% of the total egg white proteins (STADELMAN e COTTERILL, 1995a). The molecular weight of ovalbumin is 45 kDa with 386 amino acids. Ovalbumin does not have a classical N-terminal ladder sequence (HUNTINGTON e STEIN, 2001), but has 3 sites of postsynthetic modification in addition to the N-terminal acetyl group. The amino acid composition of ovalbumin is unique compared with other proteins and for this reason ovalbumin present many functional properties such as antimutagenic, anticarcinogenic (VIS et al., 1998), immunomodulatory (GOLDBERG et al., 2003) and antioxidant (NARA et al., 1995). Ovalbumin has been used extensively in food technology because of its emulsifying and stabilizing properties. Lysozyme is another important protein found in egg white. The molecular weight of lysozyme is 14,400 Da and consists of a single polypeptide chain with 129 amino acids. In nature, this protein is found as a monomer but is occasionally present as a dimer with more thermal stability. It is considered as a strong basic protein present in egg white (HUOPALAHTI et al., 2007). Lysozyme has 4 disulfide bridges leading to high thermal stability, and its isoelectric point is 10.7. In the food industry, lysozyme is one of the major bacteriolytic proteins having the capability of controlling foodborne pathogens such as *Listeria monocytogens* and *Clostridium botulinum* (RADZIEJEWSKA et al., 2008; LEŚNIEROWSKI e CEGIELSKA-RADZIEJEWSKA, 2012), which are considered 2 main pathogens that cause problems in the food industry. Carrageenan is a polymer obtained from several genera and species of marine algae class Rodophyta (CAMPO et al., 2009). Carrageenan is a sulfated polygalactan with 15 to 40% of ester-sulfate content and an average relative molecular mass well above 100 kDa. It is formed by alternate units of D-galactose and 3,6-anhydro-galactose (3,6-AG) joined by α-1,3 and β-1,4-glycosidic linkage. Carrageenan is classified into various types such as λ, κ, ι, ε, μ, all containing 22 to 35% sulphate groups (NECAS e BARTOSIKOVA, 2013). Carrageenan has no nutritional value, however is used in food preparation for its gelling, thickening, and emulsifying properties (VAN DE VELDE et al., 2002).

The present study has aimed to elucidate the influence of protein, pH, ionic strength and ratio of protein/polysaccharide in the kinetics of the formation of complex coacervates as well the rheology properties of the coacervates.

2 MATERIALS AND METHODS

2.1 Materials

Lysozyme (Lyso purity > 90%), Ovalbumin (Ova; purity > 90%) and κ-carrageenan (Ca) were obtained from Sigma Chemicals (St. Louis, USA). Sodium chloride (NaCl, purity > 99%), hydrochloric acid (HCl, 0.5 M), and sodium hydroxide (NaOH, 0.5 M) were purchased from VETEC® Ltda, (Rio de Janeiro, Brazil). The water used was ultrapure with a conductivity of 0.05 μS/cm ± 0.01 (Gehaka-Master P&D – Brazil). Stock solutions of κ-carrageenan (0.1% w/w), ovalbumin (0.1-1% w/w) and lysozyme (0.1-1% w/w) were prepared by gently stirring the powders in deionized water for 6 hours at room temperature (25 °C).

2.2 Formation of complexes

2.2.1 Preparation of complexes

The concentration of κ-carrageenan used was 0.1% w/w and the concentration of lysozyme or ovalbumin used varied from 0.1-1% w/w. Five ratios of protein:Ca (1:1, 2:1, 3:1, 5:1, 10:1) were evaluated. To determine the effect of NaCl on complex formation, Lyso:Ca or Ova:Ca complexes were formed in five concentrations of NaCl (0.01 M, 0.05M, 0.1M, 0.2M, 0.4 M). The protein:Ca mixtures were previously stirred and adjusted to pH 12 for turbidimetric measurements.

2.2.2 Turbidimetric measurements

pH-dependent turbidity was measured at a wavelength of 400 nm using a spectrophotometer (Biochrom mod. LIBRA S12, England) calibrated with ultrapure water to 100% transmittance (T). Turbidity was defined as 100 e %T. With the aid of a magnetic stirrer (Nova Tecnica, NT 101, Brazil) and a pH meter (Tecnopon, mPA-210, Brazil) the pH of the solutions were adjusted (12.0-1.0) with HCl, 0.5 M. Measurements of complexes and the solutions with biopolymers isolates were made at room temperature (25 ºC ± 1), and each sample was measured four times at 1 min intervals.

2.2.3 Complexo insolúvel

From the data of turbidimetric titration was chosen the region (pH) with the highest value of turbidity for different ratios of protein/ κ-carrageenan and their respective concentrations of NaCl. These complexes were formed, and 72 hours after the samples were centrifuged under the temperature of 5 ºC (CIENTEC, TC-6000, Brazil) at 6000 rpm for 30 minutes. The supernatant was removed, and the precipitate was frozen and lyophilized (Terroni mod. Enter 1B, Brazil). Complex coacervate yield (CCY) was determined using the following equation (BÉDIÉ et al., 2008).

$$CCY = \frac{Weight\ of\ CC}{total\ weight\ of\ protein + polissacharide + NaCl}\ x\ 100$$

All analyzes were performed with three independent samples and a simple analysis of variance was performed using the Tukey test in order to determine the difference between the means ($p < 0.05$).

2.3 Zeta - Potential

A Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK) was used to determine zeta (ζ)-potentials. Lysozyme, ovalbumin and κ-carrageenan stock solutions and Lyso:Ca or Ova:Ca complexes formed in 0.01 M NaCl were diluted to 0.01% w/w and transferred to an MPT-2 autotitrator (Malvern Instruments, Worcestershire, UK) that adjusted pH using 0.5 M NaOH, 0.25 M HCl and 0.025 M HCl solutions. pH was varied from 12.0 to 1.0 by 0.5 unit increments with a confidence interval of ± 0.1 unit. ζ-potentials were calculated using the Smoluschwsky mathematical model. Each experiment was performed three times and sample readings were done in triplicate at 25 °C.

2.4 Characterization of the complexes

2.4.1 Rheological measurements

The rheological properties of complex coacervates were determined using a rotational rheometer Haake™ Mars II Rotational Rheometer (Thermo Scientifi Inc., Alemanha) with cone and plate attachments (20 mm, angle: 1°) and a gap of 0.025 mm between the elements. The ratios of Lys:Ca and Ova:Ca coacervates with different concentrations of NaCl were formed, and after 24 hours the samples were centrifuged under the temperature of 5 ºC (CIENTEC, TC-6000, Brazil) at 6000 rpm for 30 minutes. Before analysis, all coacervates were accommodated in the rheometer plate (10 minutes) along with the protective dome in order to stabilize the temperature and prevent evaporation of water during analysis. Strain sweep tests were carried out to determine the linear viscoelastic range (0,1–100%). The storage modulus (G'), the dissipation module (G'') and apparent viscosity (η^*) were measured while the frequency varied from 0.1 to 100 rad / s. All samples were analyzed at 25 °C with four independent repetitions.

2.4.2 Microscope images

An optical microscope (Nikon, model LV 150,USA) amplified 20x with coupled camera (High-definition DSFi1) and Software (NSI-Elements D 3.0) was used to obtain images of complex. The preparation of samples was adapted from methodology proposed by SOUZA et al. (2013) in which centrifugation of samples containing precipitated (coacervate) was kept under refrigeration for 24 h assuring formation of complexation.

The lyophilized samples were analyzed with the aid of a scanning electron microscope (Evo Ma 10, Zeiss, Germany), which was operated in the secondary electron mode with an accelerating voltage of 20 kV.

The complex samples were imaged using an atomic force microscopy in a FlexAFM (Nanosurf, Basel, Switzerland). The complex samples precipitated (1 mL) was resuspended in 10 ml of the buffer and afterward an aliquot of 2 µl were deposited in freshly cleaved mica and allowed to stand in air before imaging. The experiments were carried out in air, operating in contact mode (constant force). Oxidized sharpened silicon tips and a spring constant of 0.2 N/m were used for imaging. Imaging data were analyzed with the Easyscan 2 software®.

3 RESULTS AND DISCUSSIONS

3.1 Effect of pH and ratio on protein/Ca coacervation

The protein surface charge depends upon the charge state of the amino acid side chain on the own surface. The amino acid side chain has its own pK_a, and the charge state is modulated by the environmental pH (BELITZ e GROSCH, 1999). The lysozyme and ovalbumin are both proteins from egg white, however, the isoelectric point (p*I*) of Lyso (p*I* 10.7) and Ova (4.5) (STADELMAN e COTTERILL, 1995a; ABEYRATHNE et al., 2013) show that amino acid charge state of those proteins can present a particular pH-induce phase interaction for the complexes formed with κ-carrageenan and result of this interaction are presented in the figure 1.

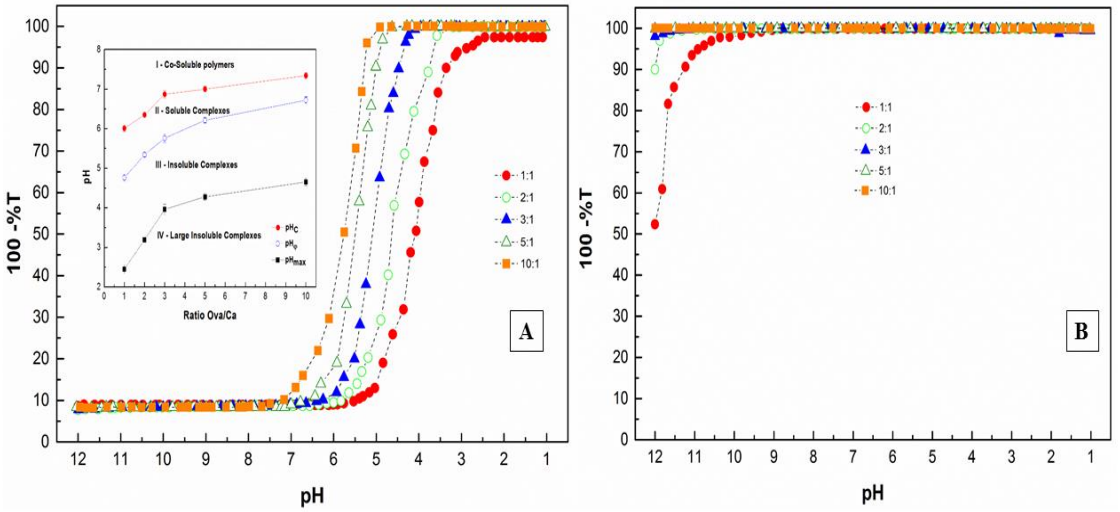

Figure 1: Turbidity (100 – %T) of Ova/Ca (A) and Lyso/Ca (B) complexes as a function of pH in different ratios in a fixed concentration of 0.01 mol/L NaCL.

In the figure 1A is possible see that four phases (I, II, III and IV) were obtained with changing of turbidity of the Ova-Ca mixtures in the different ratios. The critical pH transitions points (pH_c, pH_φ and pH_{max}) were determined by intersection point of two curve tangents. The turbidity arises mainly from the change in mass and size of aggregates in the solution, and as we can see, the mixture remained almost a constant value above pH_c of complexes showing that biopolymers were co-soluble in the system (phase I). The association between ovalbumin and κ-carrageenan at pH_c enhanced by reduction of the deprotonation and the domination of the cations on the Ova surface, which produces a weak electrostatic interaction with each other forming soluble complexes (phase II) (SCHMITT e TURGEON, 2011). The turbidity of the Ova/Ca coacervates abruptly increased at pH_φ when the number and the size of coacervates enlarged. As far as the drops of HCL were added in the systems the electrostatic interaction became stronger influenced, mainly by protonation of protein, and mixture turbidity increased sharply reaching the maximum of interactions (pH_{max}), thus forming insoluble and large insoluble complexes (phase III and IV) (JONES e MCCLEMENTS, 2010). In the figure 1A, was also possible to see that the critical pH transition points, and turbidities of complexes were directly influenced by the ratio of Ova/Ca complexes. The coacervation (pH_c, pH_φ and pH_{max}) in Ova/Ca mixtures with the high ratio of Ova to Ca (10:1) occurred at higher pH values, compared to a mixture with the low ratio (1:1 and 2:1). As the ratio of protein

was increased, a large number of positively charged ovalbumin molecules were available to neutralize the negative κ-carrageenan sulfate group ($-OSO_3^-$) even in the pH > p*I* of protein (GIRARD et al., 2003b) which, gradually increased the strength of interaction between protein and polymer resulting in shifting of pHφ, pHc and $pH_{max.}$. Similar behavior was described by CHAI et al. (2014) in bovine serum albumin/ κ-carrageenan coacervates. In the ratio 1:1, is also possible to note that maximum turbidity achieved was 97.4 ± 0.6 %, meanwhile when the ratio was increased (2:1 until 10:1) the maximum turbidity was obtained. We suggest that it occurred due to the increased of strength of interaction.

Different of Ova/Ca complexes the Lyso/Ca complexes showed in the figure 1B a behavior totally different against the influence of pH and ratio of complexes. In the ratios 1:1, 2:1 and 3:1 the pH_{max} found was 8.82 ± 0.25, 10.49 ± 0.2 and 10.86 ± 0.24 respectively, however, were not possible to determine the pH_c and pH_φ. We suggest that it occurred due to protonation of lysozyme, even in the pH 12.0, influenced mainly by amino acids arginine (pK_a 12.0) and lysine (pK_a10.5) associated with higher deprotonated κ-carrageenan (pK_a 2.0) sulfate groups (BELITZ e GROSCH, 1999; CAMPO et al., 2009). At the lower ratio (1:1) the electrostatic interaction was stronger enough to form the insoluble complex in the pH 12.0. Nonetheless, when the ratio was increased more positively charged lyso molecules were available to bind with carrageenan sulfate groups resulting in shifting of pH_{max} to pH 12.0 at the ratios 5:1 and 10:1. It is worth mentioning that pH range of Lyso/Ca complex region corresponds a range from pH 12.0 until 1.0, which, until this moment, was not reported in the literature and it may represent a great range to apply this complex on the food, cosmetics, and pharmaceutical industries (FATHI et al., 2014; MCCLEMENTS, 2015b; MCCLEMENTS, 2015a).

In order to identify the density of charge of individual biopolymers and protein-Ca complexes formed at ratio 3:1 the ζ-potentials as function of pH was conducted and results are present at figure 2. As it is possible to notice the Lyso and Ova have a change in the electrical charge on the individual protein solution, ranging from a negative charge at pH 11.95 to a positive charge at pH 1.0 with a zero load point (p*I*) near 10.7 to Lyso and 4.7 to Ova as reported in the literature (ABEYRATHNE et al., 2013). Different behavior was observed with κ-carrageenan as the pH of the system was acidified. The κ-carrageenan is a highly anionic polysaccharide with many sulfate groups and for this reason, the sulfate groups remain considerably negative charge during acidification. It can be observed that the values found for the mixture containing both polymers are the values of the isolated polymer intermediates. This behavior indicates the interaction between the sulfate groups of the polysaccharide and protein amine groups, featuring electrostatic binding. In the pH 11.9 is possible to see that Ovo/Ca complex was not formed, however as soon as the pH became more acid the protonation of ovalbumin molecules promoted the formation of soluble complex about the pH 7.0 which agree with the results discussed above. When we observe the ζ-potentials of Lyso/Ca complex is possible conclude that coacervation occurred during all pH studied, however the region of electrical charge equivalence pH (EEP) (LE e TURGEON, 2013) was reached about the pH 5.5 - 4.5 indicating that biopolymers were strongly bonded.

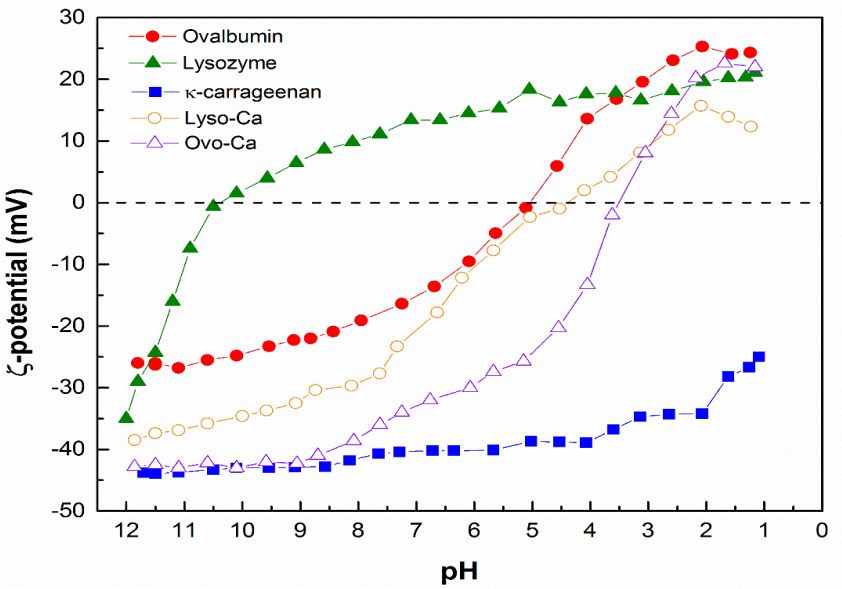

Figure 2: **:** ζ-potential of Lysozyme, ovalbumin and κ-carrageenan systems with 0.1% (w/w) or Lyso/Ca and Ova/Ca complexes at ratio 3:1 as a function of pH with a fixed concentration of 0.01 M NaCl.

3.2 Effect of ionic strength on protein/Ca coacervation

The net charge carried by the proteins and polysaccharides is reduced by interaction with the micro-ions, resulting in a decrease in the electrostatic attraction between the macromolecules at high salt concentration. At the high ionic strength, screening the charges of the proteins and polysaccharides leads to reduce electrostatic interactions and hence affect the formation of complexes (WEINBRECK et al., 2003a; YE, 2008). At the low ionic strength, the micro-ion concentration has only a small effect on protein–polysaccharide complexes. The number of charges present in the proteins, and polysaccharides is sufficient to allow electrostatic interaction. In figure 3, the titration curves of turbidity (100-T%) versus pH for the Protein/Ca mixtures (ratio 3:1) as a function of the NaCl concentration showed that increased of NaCl affected directly the kinetic of formation of complexes.

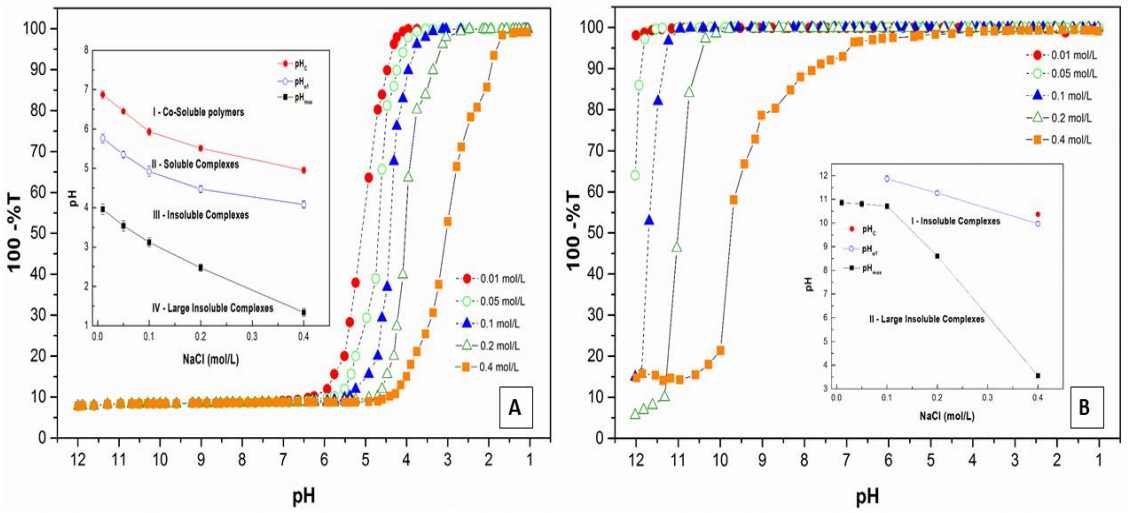

Figure 5: Turbidity (100 – %T) of Ova/Ca (A) and Lyso/Ca (B) complexes as a function of pH in different NaCl concentration at the ratio 3:1.

Addition of salt to the Ova/Ca (Figure 3A) and Lyso/Ca (Figure 3B) mixtures suppressed the electrostatic interaction between proteins and κ-carrageenan. The pH_c, pH_φ and pH_{max} values of Ova/Ca coacervation reduced by the increase of ionic strength, meanwhile Lyso/Ca complex showed a different behavior of coacervation when the ionic strength was increased. In the figure 3B, is possible to note that in 0.01 mol/L of NaCl, the complexation occurred instantly in the pH 12.0 with pH_{max} = 10.86 ± 0.24, nevertheless, when the NaCl concentration was enhanced from 0.01 to 0.1, the pH_{max} shifted slightly to 10.71 ± 0.11 and for the first time, was possible to determine the pH_φ. We suggest that it occurred due to the ability of competitive adsorption of NaCl (Na^+ and Cl^-) which suppressed the protonation of the amino acids arginine and lysine resulting pH_{max} close of p*I* of lysozyme (MINE, 2007; SCHMITT e TURGEON, 2011). When the NaCl concentration was enlarged to 0.2 and 0.4 mol/L the competitive adsorption of the NaCl was able to shift the pH_{max} to the lower pH = 3.55 showing that high concentrations of sodium chloride becomes strong enough to suppress the higher affinity to bind between Lysozyme and κ-carrageenan and additional acidification of the system is required to overcome the screening effect of salts (SEYREK et al., 2003; ANTONOV et al., 2009). Similar dependence of additional acidification of system is observed in Ova/Ca complex (Figure 3A). In the NaCl concentration of 0.01 mol/L the pH_{max} occurred at pH 3.96 ± 0.13 (pH < p*I* of Ova); However, when NaCl was enlarged, the pH_{max} decreased, shifting linearly to pH 1.34 ± 0.1. The decreases of the pH_c, pH_φ also showed that pH range of the co-soluble polymers increased with the increased of the ionic strength of NaCl.

Grounded on the data discussed above, the turbidity and ζ-potential of Protein/Ca complexes were collected in the pH < pH_{max} (Lyso/Ca complexes = pH 5.0 and Ova/Ca complexes = pH 3.0) with fixed NaCl concentration (0.01mol/L) with the purpose to determine, the complex coacervates yield (CCY). The complex coacervates formation was ensured for all the ratios after three days, and analyses are presented in the table 1.

Table 1: Interrelationship between complex coacervates yield, turbidity and ζ-potential of large insoluble complexes at different ratios

Ovalbumin			
Ratio	**Turbidity (100 –T%)**	**ζ-potential (mv)**	**CCY (%)**
1:1	97.4 ± 0.6[a]	-27.4 ± 2.3[a]	44.8 ± 8.4[a]
2:1	99.9 ± 0.8[b]	-15.7 ± 1.6[b]	73.3 ± 2.2[b]
3:1	100 ± 0.9[b]	+11.6 ± 0.8[c]	79.6 ± 0.6[c]
5:1	100 ± 0.7[b]	+16.2 ± 1.8[d]	71.8 ± 1.6[b]
10:1	100 ± 0.5[b]	+18.9 ± 1.5[d]	66.8 ± 3.2[b]
Lysozyme			
Ratio	**Turbidity (100 –T%)**	**ζ-potential (mv)**	**CCY (%)**
1:1	99.9 ± 1.3[a]	-35.7 ± 0.9[a]	64.2 ± 2.2[a]
2:1	99.9 ± 0.9[a]	-25.3 ± 2.7[b]	83.0 ± 1.4[b]
3:1	100 ± 1.5[a]	-19.8 ± 1.1[c]	93.7 ± 4.8[c]
5:1	100 ± 0.5[a]	-11.6 ± 0.6[d]	92.4 ± 3.6[c]
10:1	100 ± 0.7[a]	+1.3 ± 0.4[e]	95.5 ± 5.4[c]

Ratio = protein:polysaccharide; CCY = complex coacervate yield of biopolymers complex values are mean of three replicates ± standard error. [a,b, c, d and d] Means in the same column with different lower case letters are significantly different (P< 0.05)

In the Ova/Ca complex, is possible to note that at the ratio 1:1 the turbidity and the yield was lower than other ratios studied. It occurred because in the ratio 1:1 the amount of protonated protein molecules were not enough to bind in all deprotonated κ-carrageenan sulfate groups, which explain the ζ-potential of -27.4 ± 2.3 (ESPINOSA-ANDREWS et al., 2013). When the ovalbumin concentration was increased in the ratios 2:1 and 3:1 is possible to see that turbidity became almost totally turbid with yield of 73.3 ± 2.2 and 79.6 ± 0.6 respectively, however the ζ-potential showed that the region of electrical charge equivalence pH may correspond a ratio between 2:1 and 3:1 (ZEEB et al., 2014). In the ratios 5:1 and 10:1 is possible to note that turbidity remained almost totally turbid; Nevertheless the ζ-potential became more positive charged followed by the decreasing of yield. It occurred due to the excess of protonated protein molecules, which affected the stoichiometry of the systems reducing the yield of complex (WANG et al., 2013; ZEEB et al., 2014).

In the Lyso/Ca complex is possible to see similar behavior described to Ova/Ca complex, meanwhile the turbidity did not present significant difference for all the ratios studied. In the ratio 1:1, the yield was the lower for all the ratios studied followed by the high negative ζ-potential, meanwhile when the ratio was increased from 1:1 to 10:1 the yield increased reaching 95.5 ± 5.4 with ζ-potential (+1.3 ± 0.4) about the region of electrical charge equivalence pH indicating that all deprotonated κ-carrageenan sulfate groups were bonded with protonated protein molecules (KIZILAY et al., 2011; KAYITMAZER et al., 2013).

3.3 Rheology properties of Protein/Ca coacervates

Although no chemical reaction occurs between salt ions and polysaccharide, changes in the viscosity, elasticity, fluidity, and stability of the food system may occur by the addition of salt changes but are worth mentioning that the pH, temperature, and protein/polysaccharide ratio also influence the rheological properties of such systems (OULD ELEYA e TURGEON, 2000; VAN VLIET et al., 2004b). The figure 4 shows the results of small deformation oscillatory measurements of Lyso/Ca and Ova/ca complexes in different ratios with different NaCl concentrations. In the Figure 4C, a gel-like behavior is observed in the Lyso/Ca and Ova/Ca coacervates (FOSTER e WOLF, 2011). In contrast, the κ-carrageenan solution exhibits a viscoelastic behavior as a polymer solution with the G’ and G’’ smaller when compared with coacervates formed at the same concentration of κ-carrageenan (data not showed). In the figure 4C, is worth mentioning that G’ and G’’ are frequency-dependent, and G’ is significantly higher than G’’, the coacervates have highly interconnected gel-like network structure with mainly elastic behavior. In addition, the complexes viscosity decreases nearly linearly with frequency, showing the general shear-thinning phenomenon (WANG et al., 2007a). Therefore, the elastic behavior of the Lyso/Ca and Ova/Ca complexes may originate mainly from the electrostatic interaction between the protein and polysaccharide chains (ROCHA et al., 2014). Similar dynamic rheological results were mentioned for the coacervates formed between BSA/pectin (RU et al., 2012) and β-lactoglobulin/pectin (WANG et al., 2007a).

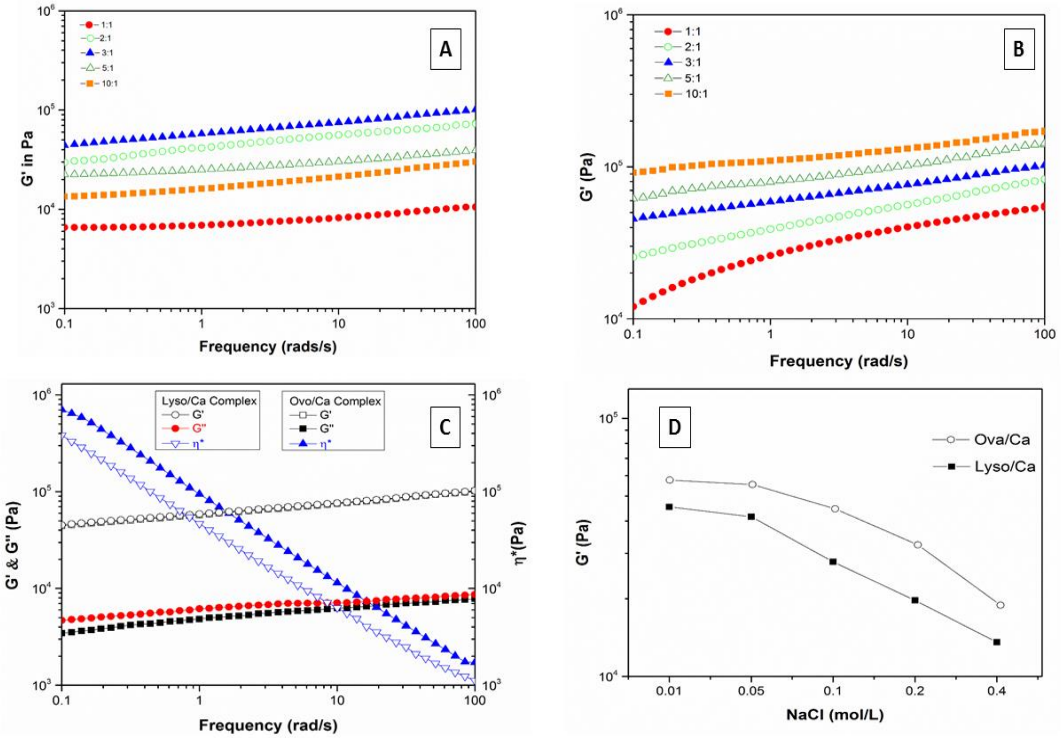

Figure 4: The storage modulus G' curves for the Ova/Ca (**A**) and Lyso/Ca (**B**) complexes prepared at different ratios at fixed NaCl concentration (0.01 mol/L). (**C**) Complex viscosity η*, storage modulus G', and loss modulus G'' versus angular frequency for the Ova/Ca and Lyso complexes prepared the ratio 3:1 (NaCl 0.01 mol/L). (**D**) Variation of the storage modulus at 0.1 rad/s frequency as a function of NaCl concentration for the Ova/Ca and Lyso/Ca (B) complexes.

In order to identify the influence of the ratio on the rheological properties of coacervates formed the dynamic rheological measurements were performed, and the results are presented in the figure 4A and B. When the ratio of ovalbumin (Figure 4A) reaches 3:1, the maximum yield of the coacervates is reached, and the maximum shear modulus is observed. However, further increases the ratio to 10:1, the excess amounts of Ova molecules decreased the G' value of the coacervates. These results corroborate with the effect of Ova/Ca ratio discussed above. The higher G' values at the ratio 3:1 indicate a stronger intermolecular Ova/Ca network, possible due to interactions between protein-protein and protein-polysaccharide molecules (LANEUVILLE et al., 2006; LE e TURGEON, 2013), nevertheless, is also possible indicate that the positive charges in the Ova, and the negative charges in the pectin chains were nearly balanced at ratio 3:1. This phenomenon was also observed in coacervation of ovalbumin and gum Arabic due to the occurrence of charge neutralization between the biopolymers (NIU et al., 2014). The Lyso/Ca coacervates (Figure 4B) showed a different rheological behavior when the ratio was increased. When the ratio was enlarged from 1:1 to 10:1 is possible to note that G' reached the highest value showing that even though the yield did not show significant difference in the ratio 10:1 (table 1) the high amount of lysozyme allowed a great interaction between protein-protein and protein-polysaccharide molecules achieving the highest shear modulus. Similar behavior at the ratio 10:1 was described on rheological properties by CHAI et al. (2014) and by RU et al. (2012) with BSA/ κ-carrageenan and BSA/pectin coacervates respectively.

Dynamic rheological measurements were also completed to investigate the impact of ionic strength on the rheological properties of the Lyso/Ca and Ova/Ca coacervate (Figure 4D). In general, the presence of salt in the solution weakens the interaction, and stifles the formation of protein/polysaccharide coacervates (BURGESS, 1990; KRUIF et al., 2004; WEINBRECK et al., 2004b), and as we can see in the figure 4D the values of G' are inversely proportional to NaCl concentration, mainly because the formation of the weaker binding between protein and polysaccharide by the added salt. BIESHEUVEL e STUART (2004) suggest that complexation is driven by the increase of entropy due to the expulsion of small ions from the double layer around the individual polyelectrolytes chains, which increase the free energy to the system. The large amount of salt hinders the release of small ions thus reducing the available site for the interaction suppressing the driving force for complexation. The complex coacervation is mainly driven by electrostatic interaction, meantime during the process of coacervation process of rearrangement of the biopolymers may involve non-electrostatic interactions, such as hydrogen bonding, hydrophobic interactions, or even van der Waals forces (DE VRIES e COHEN STUART, 2006; JONES e MCCLEMENTS, 2010; SCHMITT e TURGEON, 2011). We suggest that the slight decrease in G' with the increasing salt concentration from 0.01 to 0.1 mol/L for the Lyso/Ca and Ovo/Ca coacervate may correspond the coexist of electrostatic and non-electrostatic interactions.

3.4 Morphological characteristic of coacervate complexes

The morphological characteristic of the complex particles is presented on figure 5. The new biopolymers were separated and kept hydrated with the objective of demonstrating new structures formed in the natural state, and the results can be seen at figure 5 A and B. The optical microscopy of samples revealed that intrapolymer complexes with heterogeneous structure were formed for both complex (KAYITMAZER et al., 2007a).

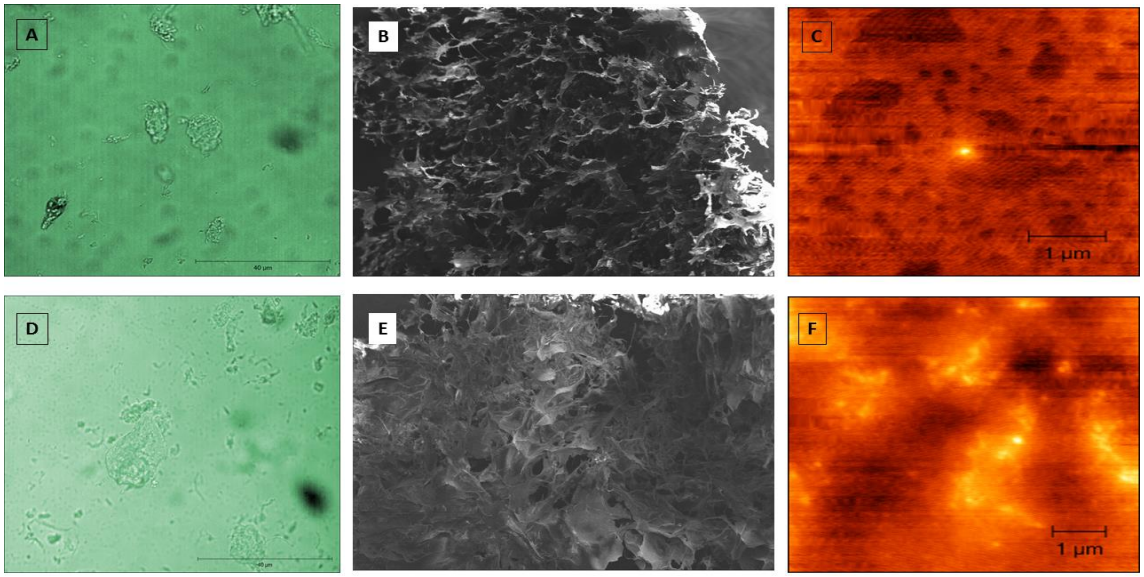

Figure 5: Morphological images of Ova/Ca and Lyso/Ca complexes at ratio 3:1 with 0.01 mol/L NaCl. (A) (B) and (C) correspond optical, scanning electron (scale 100 µm) and atomic microscopic images of Ova/Ca complexes. (D), (E) and (F) correspond optical, scanning electron (scale 100 µm) and atomic microscopic images of Lyso/Ca complexes.

In the figure 5 B, C E and F is possible to note that three-dimensional network was formed during complexation and is also possible to mention that Ova/Ca complex showed a network more homogeneous when compared with Lyso/Ca complex. LE e TURGEON (2013) studied the formation of interpolymer complexes between β-Lactogolublin and xanthan gum and authors suggested that protein aggregated along the polysaccharide chains providing a tenuous network for gel structuration thus playing an important role as a crosslinking agent. We believe that ovalbumin, and lysozyme may plays as a crosslinking agent along κ-carrageenan chains, meanwhile other researches must be developed to clarify the mechanism of interaction, and the internal structure of the complexes formed.

4 **CONCLUSIONS**

As the protein/polysaccharide ratio increased from 1:1 to 10:1, the critical pH transitions points shifted to higher pHs with ovalbumin/κ-carrageenan, however, with lysozyme/κ-carrageenan the increased the of the ratio suppressed the critical pH transitions points forming large insoluble complexes from pH 12.0 until 1.0. Addition of salt to the ovalbumin/κ-carrageenan and lysozyme/κ-carrageenan mixtures suppressed the electrostatic interaction between proteins and κ-carrageenan shifting to lowering pHs the critical pH transitions points, meanwhile at the ratio 3:1 with 0.01 mol/L of salt, the complex coacervates yield reached 79.6% ± 0.6 and 93.7% ± 4.8 for the ovalbumin and lysozyme complex. The rheological data of the complexes showed gel-like network structure. When the ratio of ovalbumin reaches 3:1, the maximum yield of the coacervates was achieved, and the maximum shear modulus was observed. Similar behavior was observed with lysozyme; however, maximum shear modulus was found for the ratio 10:1. The state diagram found as well the yield of complex formed can be used to estimate the coacervation for future researches state and also for industry applications. Rheological data associated with microscopy images showed that intrapolymer complexes with heterogeneous structure were formed for both complex, and we suggest that complexes represent a great potential to improve or extent texture, mechanical stability, consistency, and taste of food products.

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CAPÍTULO V

COMPLEXATION OF EGG WHITE PROTEINS AND XANTHAN GUM: EFFECT OF SALT AND PROTEIN/POLYSACCHARIDE RATIO

ABSTRACT

Protein/polysaccharide complex represent great technology to improve texture, mechanical stability, and consistency of food. The complexation between lysozyme (Lyso)/xanthan gum (XG) and ovalbumin (Ova)/xanthan gum was studied in situ by acidification (12.0-1.0) using zeta potential, turbidity, Fourier transform infrared spectroscopy (FTIR), and rheological measurements. The complexes were analyzed in different NaCl concentrations with different protein/polysaccharide ratios. As the protein/polysaccharide ratio increased from 1:1 to 10:1, critical structure forming events (i.e., those associated with soluble, insoluble and large insoluble complexes) shifted to higher pHs with Ova/XG followed by decrease of G' values at ratio 10:1. The increased of the ratio from 2:1 to 10:1 for Lyso/XG complexes suppressed the pH_{φ} forming insoluble at pH 12.0, however, the values of G' decreased at ratio 5:1 and 10:1 indicating that excess of protein impact on compaction of network structures. Addition of salt to the Ova/XG and Lyso/XG mixtures suppressed the electrostatic interaction between proteins and XG shifting to lowering pHs the critical pH transitions points. Although, the electrostatic interaction has been the main mechanism of interaction between the protein and xanthan gum hydrogen bonding was also involved in the interaction between proteins and XG. The rheological data associated with microscopy images showed that interconnected gel-like network structure with heterogeneous structure were formed for both complex, and we suggest that complexes represent a great potential to improve sensorial properties of food products.

1 INTRODUTION

Protein and polysaccharide are biopolymers that are widely used as functional ingredients in industry, which can not only provide nutrition, but also can, affect the sensory and physical chemical properties the final products (TURGEON et al., 2007a). Food proteins are generally recognized as safe (GRAS), which makes them excellent materials for the formulation of food and pharmaceutical products. However, it is also important to mention that their functional properties, such as gelation, water-binding capacity, and ability to establish a wide range of interactions with other compounds establish the proteins as excellent useful ingredient in the formulation of new products (CHEN et al., 2006).

Proteins and polysaccharides are present together in many food systems, and both contribute to the structure, texture, and stability of food. The interesting feature revealed by the formation of complexes is that their functional properties are potentially better than those of proteins are and polysaccharides are alone (BURGESS, 1990; DICKINSON, 2003). Protein/polysaccharide coacervation in an aqueous dispersion often accompanied by either segregative or associative phase separation, depending mainly on the electrical charges on the biopolymers. Several parameters have been shown to exert influence on electrostatic interaction, which include pH, biopolymer ratios, charge density, size and shape of the biopolymers, ionic strength, and ion type (KIZILAY et al., 2011; KLASSEN et al., 2011; SCHMITT e TURGEON, 2011). Among these, pH and ionic strength are one of the most important factors to determine the critical points during coacervation.

The process of coacervation between protein and anionic polysaccharide correspond primary presence of co-soluble biopolymers in the solution ($pH > pH_c$). The critical pH value (pH_c) is traditionally defined as the onset increase of scattered light intensity associated with primary intramolecular soluble complexes (weak interactions) (WEINBRECK et al., 2003a). As far as pH is reduced soluble intramolecular complexes start to aggregate into insoluble protein/polysaccharide complexes at the second critical pH (pH_φ). The pH_φ is traditionally defined as the onset sharply increase of turbidity (WEINBRECK et al., 2003a; WEINBRECK et al., 2004b; JONES e MCCLEMENTS, 2010). It is worth of noting that the maximum of scattered light intensity often coincides with the pH_φ (KAIBARA et al., 2000; WEINBRECK et al., 2003a), nonetheless in some cases it can occur at different pHs (MEKHLOUFI et al., 2005) and some authors have been attributed this pH as pH_{max} (Large insoluble complex) (KAIBARA et al., 2000; CHAI et al., 2014). Eventually, when the pH decrease below the pK_a of polysaccharide the turbidity returns to the baseline (co-soluble biopolymers) and third critical point ($pH_{\varphi 2}$) is reached (WEINBRECK et al., 2003a; TURGEON et al., 2007a). The value of the anionic groups on the polysaccharide molecules loses its charges promoting the dissociation of the protein/polysaccharide complex. This effect is more prevalent with anionic polysaccharides with relatively high pKa values (such as pectin, pH 3.5), rather than those with lower pKa values (such as carrageenan, $pH < 2$).

Proteins can form simple complexes with ligands and gel networks through hydrophobic interactions, covalent and hydrogen bonding; meantime complexes with polysaccharides or polyelectrolytes can be formed through electrostatic and/or non-electrostatic interactions (JONES e MCCLEMENTS, 2010; NICOLAI e DURAND, 2013). Egg white proteins are extensively utilized in processed foods. Chicken egg ovalbumin is the major egg white protein synthesized in the hen's oviduct and accounts for 54% of the total egg white proteins (STADELMAN e COTTERILL, 1995a). The molecular weight of ovalbumin is 45 kDa with 386 amino acids (HUNTINGTON e STEIN, 2001). The amino acid composition of ovalbumin is unique when compared with other proteins: the C-terminal is proline and N-terminal amino acid is acetylated glycine. Ovalbumin contains a carbohydrate group attached to the N-terminal and is also known as a glycoprotein (ABEYRATHNE et al., 2013). Ovalbumin it is an important food ingredient with structural functionality, including emulsifying properties and foam stability. Lysozyme is a ubiquitous enzyme, present in almost all secreted body fluids playing an important role in the natural defense mechanism. The most plentiful source, however, is hens' egg white albumin, containing around 0.3-0.4 g of lysozyme per egg (MINE, 2007). The molecular weight of lysozyme is 14,400 Da with 129 amino acids. In nature, this protein is found as a monomer but is occasionally present as a dimer with more thermal stability. It is considered as a strong basic protein present in egg white (HUOPALAHTI et al., 2007). Lysozyme has four disulfide bridges leading to high thermal stability, and its isoelectric point is 10.7. In the food industry, lysozyme is one of the major bacteriolytic proteins having the capability of controlling foodborne pathogens (LEŚNIEROWSKI e CEGIELSKA-RADZIEJEWSKA, 2012). Xanthan gum is an anionic extracellular polysaccharide secreted by the micro-organism *Xanthomonas campestris*. It is widely used in the industry as a stabilizer (over a wide pH and temperature) and thickener due to its specific physical (viscosity, pseudo- plasticity) and chemical (soluble in cold water and resistant to enzymatic degradation) properties. The primary structure of xanthan gum, is a linear (1-4) linked β-D-glucose backbone with a trisaccharide side chain on every other glucose at C-3, containing a glucuronic acid residue linked (1-4) to a terminal mannose unit and (1-2) to a second mannose that connects to the backbone (MONSANTO, 2009).

The present study has aimed to elucidate the influence of protein, pH, ionic strength and ratio of protein/polysaccharide in the kinetics of the formation of complex coacervates as well the rheology properties of the coacervates.

2 MATERIALS AND METHODS

2.1 Materials

Lysozyme (Lyso purity > 90%), Ovalbumin (Ova; purity > 90%) and Xanthan Gum (XG) were obtained from Sigma Chemicals (St. Louis, USA). Sodium chloride (NaCl, purity > 99%), hydrochloric acid (HCl, 0.5 M), and sodium hydroxide (NaOH, 0.5 M) were purchased from VETEC® Ltda, (Rio de Janeiro, Brazil). The water used was ultrapure with a conductivity of 0.05 µS/cm ± 0.01 (Gehaka-Master P&D – Brazil). Stock solutions of xanthan gum (0.1% w/w), ovalbumin (0.1-1% w/w) and lysozyme (0.1-1% w/w) were prepared by gently stirring the powders in deionized water for 6 hours at room temperature (25 °C).

2.2 Formation of complexes

2.2.1 Preparation of complexes

The concentration of XG used was 0.1% w/w and the concentration of lysozyme or ovalbumin used varied from 0.1-1% w/w. Five ratios of protein:XG (1:1, 2:1, 3:1, 5:1, 10:1) were evaluated. To determine the effect of NaCl on complex formation, Lyso:XG or Ova:XG complexes were formed in five concentrations of NaCl (0.01 M, 0.05M, 0.1M, 0.2M, 0.4 M). The protein:XG mixtures were previously stirred and adjusted to pH 12 for turbidimetric measurements.

2.2.2 Turbidimetric measurements

pH-dependent turbidity was measured at a wavelength of 400 nm using a spectrophotometer (Biochrom mod. LIBRA S12, England) calibrated with ultrapure water to 100% transmittance (T). Turbidity was defined as 100 e %T. With the aid of a magnetic stirrer (Nova Tecnica, NT 101, Brazil) and a pH meter (Tecnopon, mPA-210, Brazil) the pH of the solutions were adjusted (12.0-1.0) with HCl, 0.5 M. Measurements of complexes and the solutions with biopolymers isolates were made at room temperature (25 ºC ± 1), and each sample was measured four times at 1 min intervals.

2.3 Zeta - Potential

A Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK) was used to determine zeta (ζ)-potentials. Lysozyme, ovalbumin and xanthan gum stock solutions and Lyso:XG or Ova:XG complexes formed in 0.01 M NaCl were diluted to 0.01% w/w and

transferred to an MPT-2 autotitrator (Malvern Instruments, Worcestershire, UK) that adjusted pH using 0.5 M NaOH, 0.25 M HCl and 0.025 M HCl solutions. pH was varied from 12.0 to 1.0 by 0.5 unit increments with a confidence interval of ± 0.1 unit. ζ-potentials were calculated using the Smoluschwsky mathematical model. Each experiment was performed three times and sample readings were done in triplicate at 25 °C.

2.4 Characterization of the complexes

2.4.1 Rheological measurements

The rheological properties of complex coacervates were determined using a rotational rheometer Haake™ Mars II Rotational Rheometer (Thermo Scientifi Inc., Alemanha) with cone and plate attachments (20 mm, angle: 1°) and a gap of 0.025 mm between the elements. The ratios of Lys:Ca and Ova:Ca coacervates with different concentrations of NaCl were formed, and after 24 hours the samples were centrifuged under the temperature of 5 ºC (CIENTEC, TC-6000, Brazil) at 6000 rpm for 30 minutes. Before analysis, all coacervates were accommodated in the rheometer plate (10 minutes) along with the protective dome in order to stabilize the temperature and prevent evaporation of water during analysis. Strain sweep tests were carried out to determine the linear viscoelastic range (0,1–100%). The storage modulus (G'), the dissipation module (G'') and apparent viscosity (η^*) were measured while the frequency varied from 0.1 to 100 rad / s. All samples were analyzed at 25 °C with four independent repetitions.

2.4.2 FTIR

The stock solutions of proteins and XG as well as the protein/polysaccharide complexes were freeze-dried, the samples were applied to the FTIR GX System (Perkin-Elmer, Shelton, CT, USA) coupled to an ATR DuraSample II accessory. All the spectra were an average of 16 scans from 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1}.

2.4.3 Scanning electron microscopy

The preparation of samples was adapted from methodology proposed by SOUZA et al. (2013) in which centrifugation of samples containing precipitated (coacervate) was kept under refrigeration for 24 h assuring formation of complexation. The lyophilized samples were analyzed with the aid of a scanning electron microscope (Evo Ma 10, Zeiss, Germany), which was operated in the secondary electron mode with an accelerating voltage of 20 kV.

3 RESULTS AND DISCUSSION

3.1 Effect of pH and the ratio on protein/XG coacervation

The effect of the biopolymer ratio is a critical parameter for controlling the charge balance with the mixed systems (TURGEON et al., 2007a; YE, 2008). The development of the critical pH transitions points (pH_c, pH_φ and pH_{max}) as a function of the biopolymer

mixing ratio was determined by turbidimetric analysis during acid titration, and results are presented in the figure 1.

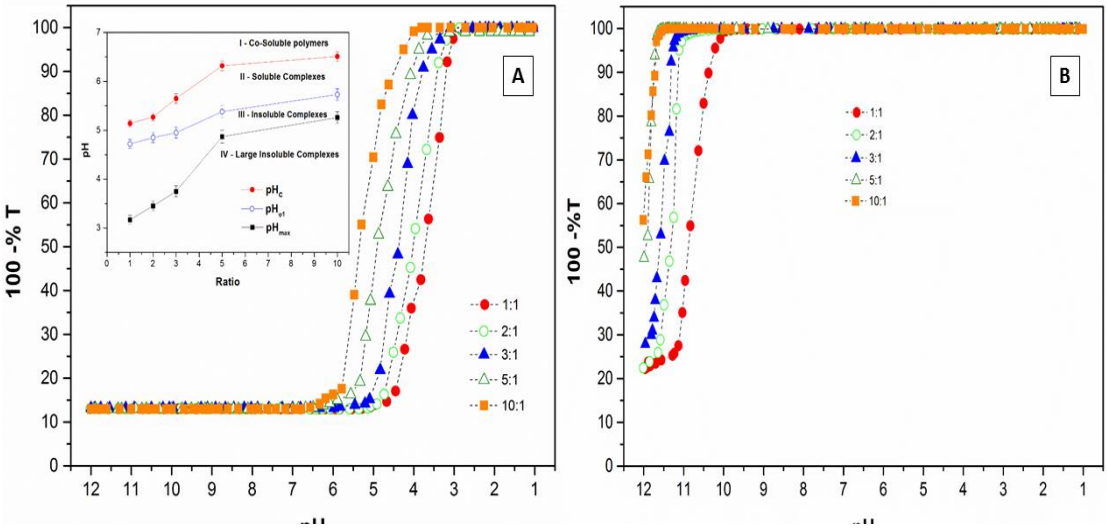

Figure 1: Turbidity (100 –%T) of Ova/XG (A) and Lyso/XG (B) complexes as a function of pH in different ratios in a fixed concentration of 0.01 mol/L NaCL.

In the figure, 1A is possible see that four phases (I, II, III and IV) were obtained with changing of turbidity of the Ova-XG mixtures in the different ratios. The turbidity of mixture remained almost a constant value at pH above of pH_c demonstrating that electrostatic repulsive forces prevent the formation of complexes, and the turbidity remained at the baseline (phase I). With further acidification from neutral pH first led to the formation of soluble complexes (pH_c) characterized by weak electrostatic interaction between protein and polysaccharide (phase II) (JONES e MCCLEMENTS, 2010). Upon continuous further reduction of the pH, another critical value was reached, which we refer to as pH_φ, where the protein and polysaccharide associate to form the insoluble complex (phase III). When the electrostatic attraction between the protein and polysaccharide molecules is sufficiently strong packed, and densely large insoluble complexes are formed (pH_{max}). These complexes scatter light strongly, leading to formation of turbid solutions with highly prone to coalescence (phase IV) (KAYITMAZER et al., 2013). In the figure 1A, is also possible mentioned that ratio of biopolymers influenced directly the critical pH transitions points of Ova/XG complexes. The increase of protein shifted the pH_c, pH_φ and pH_{max} to higher pHs. It occurred because the enlarged of number of the positively charged ovalbumin molecules promotes an early neutralization of negative xanthan gum carboxyl groups (COOH) (KLASSEN et al., 2011). The dependence of critical pHs on mixing ratio was previous reported by WEINBRECK et al. (2003a) and LIU et al. (2009b), where values shifted with the increasing of the ratio, followed by a plateau. The authors mentioned that this behavior occurs due to the greater amount of protein molecules available per polysaccharide chain for bind.

Lyso/XG complexes in the figure 1B exhibited different behavior against the influence of pH and ratio of complexes. Increase of the ratio shifted positively the pH_{max} from 9.09 ± 0.3 at ratio 1:1 to 11.61± 0.16 at ratio 10:1. In the ratio 1:1, the formation of insoluble complexes (pH_φ) occurred in the pH 11.03 ± 0.3 near of isoelectric point (p*I*) of lysozyme 10.7 (ABEYRATHNE et al., 2013). Similar behavior was encountered with Ova/XG in the ratio 1:1 (Figure 1A), meantime in the ratio 2:1 the pH_φ shifted to pH 11.53 ± 0.13 showing that strong electrostatic attraction occurred at a pH > p*I* (DICKINSON, 1998). When the ratio was enlarged from 2:1 to 10:1 was impossible to determine the

pH_{φ} demonstrating that the increased of the electrostatic interaction was stronger enough to form the insoluble complex in pH closer of pH 12.0, but is also worth mention that initial turbidity of the complex in the ratios 1:1 and 2:1 started close of 20 %. We suggest that it occurred due to protonation of lysozyme, even in the pH 12.0, influenced mainly by amino acids arginine (pK_a 12.0) and lysine (pK_a10.5) associated with higher deprotonated xanthan gum (pK_a 2.0) carboxyl groups.

Usually, proteins take on a positive net charge as system pH is lowered below its p*I*, after that they interact more strongly with the anionic polysaccharides. However, with highly charge polysaccharides (e.g., xanthan gum), complexation can occur at a pH > p*I*, assigned to strongly electrostatic attraction to hydrophilic patches on the protein`s surface (DICKINSON, 1998; WEINBRECK et al., 2003a). Similar phenomenon was reported for BSA/pectin coacervate (RU et al., 2012), canola protein isolate/ alginate or ι-carrageenan complexes (KLASSEN et al., 2011), and BSA/κ-carrageenan complex (CHAI et al., 2014). DICKINSON (1998) and DOUBLIER et al. (2000) reported that $-OSO_3^-$ groups have greater attraction to NH_{3+} groups on the protein's surface than have $-COO^-$groups, which may be attributed to earlier complexation (pH > p*I*) for both systems studied.

The ζ-potential of individual components and different ratios studied was conducted in a fixed pH for Ova/XG (pH 3.0) and Lyso/XG (pH 7.0) complexes and results are present at figure 2. The pH was fixed with purpose to suggest the potential pH of application as well to identify the great ratio to investigate the effect of ionic strength on coacervation.

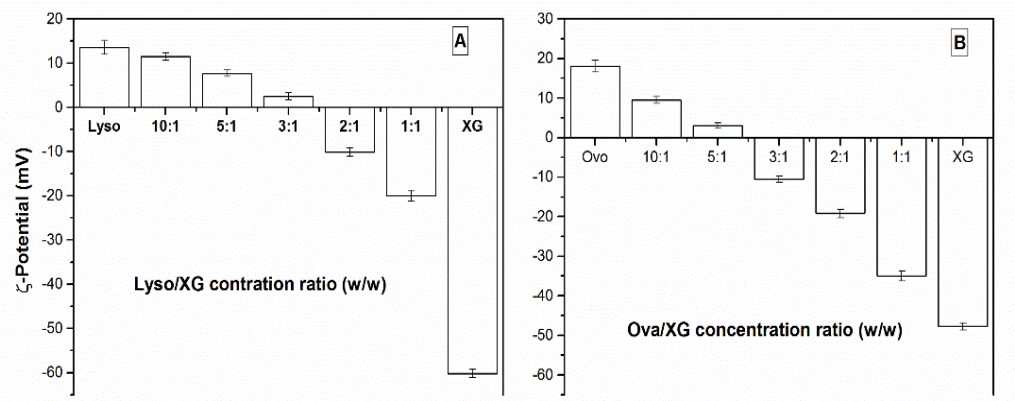

Figure 2: Zeta potential (mV) measurements in a fixed pH for homogeneous (XG, Lyso, and Ova) and mixed (Ova/XG and Lyso/XG) biopolymer systems in different ratios with 00.01 mol/L NaCL. Data represent the means ± on standard deviation, n = 3.

As XG is an acidic polysaccharide having the carboxyl groups on the side chains, the negative zeta potential decreased with decrease of pH from 7.0 (Figure 2A) to 3.0 (Figure 2B). Lysozyme at pH 7.0 (Figure 2A), and ovalbumin at pH 3.0 (Figure 2B) showed net charge positive given the fact that below of electrostatic point proteins carry a net positive (SIM e NAKAI, 1994). When we observe the net charge of Lyso/XG and Ova/XG systems in different ratios, it can be observed that the values found for the mixture containing both polymers are the values of the isolated polymer intermediates. This behavior indicates the interaction between the sulfate groups of the polysaccharide and protein amine groups, featuring electrostatic binding. In figure 2A, at ratios 1:1 and 2:1, the net charge remained negative indicating that positive charged lysozyme molecules was not enough to bind at all the negative xanthan gum carboxyl groups; however, at ratio 3:1 the system became lower positive charge (+2.5 ± 0.8) suggesting

that stoichiometric equivalence ratio occurs at ratio 3:1. Similar phenomenon was encountered for Ova/XG systems (figure 2B). Inasmuch as the concentration of ovalbumin was increased from 1:1 to 5:1 the negative charge of the system decreased reaching stoichiometric equivalence at ratio 5:1. Based on these data is possible conclude that more ovalbumin molecules are necessary to reach the maximum electrostatic interaction with XG when compared with lysozyme. The origin of this phenomenon is not yet fully elucidated, notwithstanding we suggest that the ability of Lyso to form spontaneous electrostatic complexes even with negatively protein may explain the higher electrostatic affinity with XG (STADELMAN e COTTERILL, 1995a; DAMODARAN et al., 1998). Finally, at higher ratios (Lyso/XG $r \geq 5$; Ova/XG $r = 10$), the net charges of all systems were positive and similar to that of the protein, indicating an excess of protein.

3.2 Effect of ionic strength on coacervation

Salts and ionic strength influence protein/polysaccharide complexation by exerting an electrostatic screening effect (YE, 2008). In figure 3, the titration curves of turbidity (100-T%) versus pH for the Protein/XG mixtures as a function of the NaCl concentration showed that increased of NaCl affect directly the turbidity and kinetic of formation of complexes.

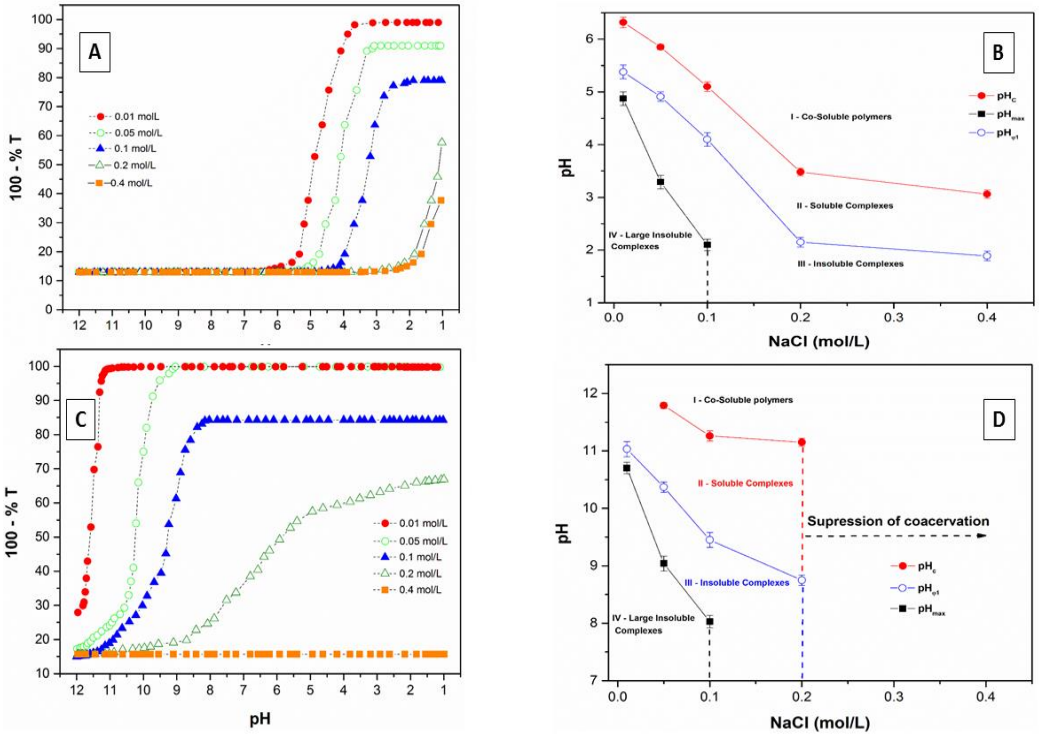

Figure 3: Turbidity (100 –%T) and variation of pH_c, pH_φ and pH_{max} of Ova/XG (ratio 5:1; A and B) and Lyso/XG (ratio 3:1; C and D) complexes as a function of pH in different NaCl concentration. Data represent the means ± on standard deviation, n = 3.

For both systems, the addition of Na^+ and Cl^- ions acted to screen reactive sites at biopolymer`s surface, thus inhibiting gradually complex formation. With the addition of NaCl, turbidity curves in the figure 3A gradually decrease reaching the minimum value of 37 % with 0.4 mol/L in the system. On the another hand, in the figure 3C, the turbidity curves only decreased from 0.1mol/L of NaCl, nevertheless, when the concentration of salt in the system achieved 0.4 mol/L, the contribution of screened suppressed the

interaction between biopolymers, and no difference was found in turbidity during acidification o the system. The results in Figure 3 A and B, indicated that enhanced of NaCl allowed Na^+ competed with the positive charge binding site of protein to bind to polysaccharide while Cl^- also competed with the negative charge binding site of XG to bind with protein, which progressively suppressed the bind affinity (SEYREK et al., 2003; KLASSEN et al., 2011). DONG et al. (2013) investigated the effect of NaCl concentration on the coacervation between soy protein and gum Arabic as a function of pH at a 1:1 mixing ratio and found that at 100 and 500 mmol/L salt concentration, the coacervation was suppressed completely. WEINBRECK et al. (2003a) investigate the effect of salt concentration on the coacervation of whey proteins/gum Arabic and found that the turbidities for all turbidimetric titration curves decreased with the increasing salt concentration. RU et al. (2012) and NIU et al. (2014) also observed similar phenomenon in BSA-pectin and Ova-gum Arabic systems respectively.

The pH_c, pH_φ and pH_{max} values of protein/XG coacervation reduced by the increase of ionic strength as we can see in the figure 3 B and D. At ratio 5:1 with lower NaCl = 0.01 mol/L, the large number of the positively charged ovalbumin molecules promotes an early neutralization of negative xanthan gum carboxyl groups (pH > p*I*). However, the enlarge of NaCl (0.01 - 0.4 mol/L) gradually screened part of the charge groups on Ova and XG surfaces, which promote the formation of soluble complexes (pH_c) only after p*I* at 0.2 mol/L followed by suppression of pH_{max} (0.2 and 0.4 mol/L). Similar behavior was noted for Lyso/XG systems at ratio 3:1, meanwhile the pH_c was, firstly, reached at 0.05 mol/L, and we could see that NaCl screened did not affect the maximum turbidity of the complex formation. The complex coacervation is mainly driven by electrostatic interaction, meantime during coacervation process the rearrangement of the biopolymers may involve non-electrostatic interactions, such as hydrogen bonding, hydrophobic interactions, or even van der Waals forces (DE VRIES e COHEN STUART, 2006; JONES e MCCLEMENTS, 2010; SCHMITT e TURGEON, 2011). We suggest that this behavior may correspond the coexisting of non-electrostatic interactions. KLASSEN et al. (2011) investigate the effect of 0.1 M urea on the coacervation of canola protein isolate- alginate or ι-carrageenan systems and found that disrupt of hydrogen bond and hydrophobic interaction shifted both pH_c, pH_φ to lower pH. Secondary roles of hydrogen bonding during coacervation between protein and polysaccharide have been previously reported for xanthan/gelatin complexes (LII et al., 2002) and , β-lactoglobulin/pectin complex (GIRARD et al., 2002).

3.3 Rheology properties of Protein/XG coacervates

The ionic strength and protein/polysaccharide ratio are one of the factors that can affect the rheological nature of coacervates (DICKINSON, 2003). Figure 4 shows the typical results of small deformation oscillatory measurement of Ova/XG (ratio 5:1) and Lyso/XG (ratio 3:1) complex formed at 0.01 mol/L NaCl. The high complex viscosity of the large insoluble complexes phase correspond the formation of packed coacervate structure (with lower entrapped solvent) induced by higher electrostatic attractive interactions between protein and XG chains (STONE et al., 2014), but is also worth mention that decrease linearly with frequency variation of complex viscosity correspond shear-thinning. The storage modulus (G′) is more than 5 times higher than the loss modulus (G″) for both complexes formed. The significantly higher G′ at all frequencies measured indicates that Ova/Xg and Lyso/XG complex formed a highly interconnected gel-like network structure, which suggest a functional application of those complexes to improve or extent texture, mechanical stability, consistency, and taste of food products.

Similar phenomenon of gel-like network structure was also observed with coacervates phases of canola protein isolate/gum Arabic (STONE et al., 2014), WPI-chitosan

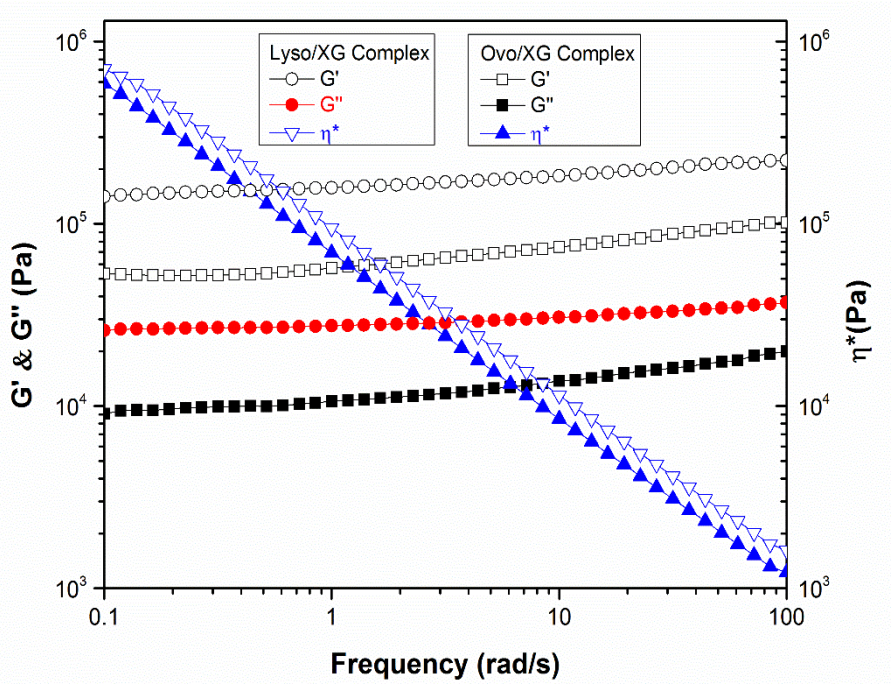

Figure 4: The complex viscosity η*, the storage modulus G', and loss modulus G'' versus angular frequency for Lyso/XG (ratio 3:1) and Ovo/XG (ratio5:1) complexes prepared at a concentration 0.01 mol/L NaCl.

(BASTOS et al., 2010), fish gelatin-laponite (KARIMI et al., 2013), and β-lactoglobulin-pectin (WANG et al., 2007a) mixtures.

In order to identify the influence on the ratio on the rheological properties of complexes formed the dynamic rheological measurements were performed, and the results are presented in the figure 5. Figure 5 A shows that the enlarge of ovalbumin increased the strength of network and the maximum G′ was achieved at ratio 5:1. The higher G′ indicates that stronger and tighter intermolecular Ova/XG network was formed between the interactions protein-polysaccharide and protein-protein molecules (LANEUVILLE et al., 2006). LE e TURGEON (2013) reported for β-lactoglobulin/ XG hydrogels that a tree-dimension network is formed as far as β-lactoglobulin bind along XG suggesting that protein act as crosslinking agents. The authors also mentioned that higher final G′ was reached at ratio 3.5 suggesting that at a high ratio, the excess of protein may reduce the junction zones. As discussed above in the figure 3, at ratio 10:1 the complexes Ova/XG showed positive net charges similar to that of the protein. We believe that this excess of protein may explain the cause of the decrease of G′ at ratio 10:1 (Figure 4A). Similar phenomenon about influence of the ratio was also noted for Lyso/XG meantime, the highest G′ was reached at ratio 3:1. However, further increases in ratios to 5:1 and 10:1 decreased the G′ value of the coacervates. The excess of lysozyme molecules (ratio 10:1) protonated (Figure 3A) reduced the G′ a value less than the ratio 2:1. Last but not least, when we compare both complexes at best ratio is also worth mention that Lyso/XG complex formed strongest gel-like complex compared with Ova/XG complex. We believe that spontaneous electrostatic ability of Lyso (STADELMAN e COTTERILL, 1995a; DAMODARAN et al., 1998) plays a significant role as crosslinking agents when compared with Ova thus less protein is required to form a stronger and tighter intermolecular network.

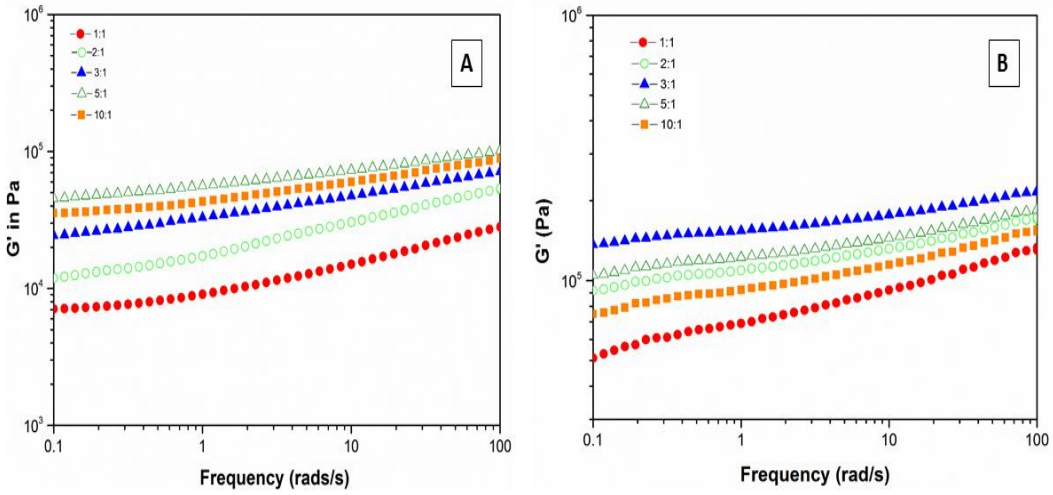

Figure 5: The storage modulus G' curves for the Ova/Ca (**A**) and Lyso/Ca (**B**) complexes versus angular frequency prepared at different ratios at fixed NaCl concentration (0.01 mol/L).

Rheological measurements were also employed to investigate how changes in the salt concentration of the mixtures influence the mechanical properties of the coacervates (Figure 6). In general, the presence of salt in the solution weakens the interaction, and stifles the formation of protein/polysaccharide coacervates (BURGESS, 1990; KRUIF et al., 2004; WEINBRECK et al., 2004b), and as we can see in the figure 6 the values of G′ are inversely proportional to NaCl concentration, mainly because the formation of the weaker binding (weaker gel-like coacervate) between protein and polysaccharide by the added salt. When salt concentration was enlarged (0.01-0.1 mol/L) for Ova/XG or (0.01 to 0.1 mol/L) for Lyso/XG, the screeing effect of the salt on the charges leads to coacervate with weaker intermolecular electrostatic interactions, higher water content and therefore, lower G' (WEINBRECK et al., 2004c). However, at 0.05 mol/L of NaCl Lyso/XG complex not decreased significantly. We suggest that coexistence of non-electrostatic interactions may contribute to maintenance of high affinity of interaction between biopolymers (KRUIF et al., 2004; JONES e MCCLEMENTS, 2010). At higher salt content (0.2 and 0.4 mol/L) Ova/XG was collected at pH 1.5, and complexes exhibit a viscoelastic behavior, which is in line with results on complexes of oppositely charged biological (LIU et al., 2002) and other soft biopolymers (PRIFTIS et al., 2013). Similar phenomenon was found for Lyso/XG (0.2 mol/L) nevertheless, with 0.4 mol/L complex was not formed and system showed viscoelastic behavior of a polymer solution (MONSANTO, 2009). BORAL e BOHIDAR (2010) studied the effect of ionic strength on surface-selective patch binding-induced phase during coacervation between gelatin and agar, and they could observe that increases in salt (0 – 0.1 M) enlarged the G′ of gel-like coacervate phase. The authors implying that the mobile ions established salt-bridges between the biopolymers to enhance the mechanical rigidity of the material. However, the authors concluded that the coacervation transition driven by surface-selective binding is not influenced by the ionic strength of the solution, but the mobile ions participate in the structural organization of the interacting polyions in the coacervate. WANG et al. (2007b) found significant correlations between the rheological properties and the composition of β-lactoglobulin /pectin coacervates: On the other hand, a salt-enhanced effect (increase in G′) was observed at low salt concentration (0.01-0.21) while the reverse occurred at high salt concentration. Similar phenomenon was also described for WPI/chitosan complexes (BASTOS et al., 2010).

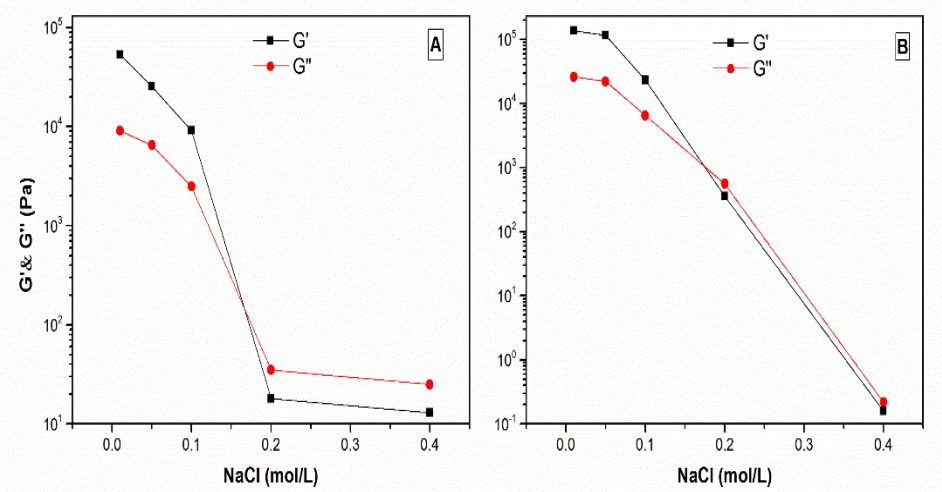

Figure 6: Variation of the storage modulus at 0.1 rad/s frequency as a function of NaCl concentration for the Ova/Ca (ratio 5:1; A) and Lyso/Ca (ratio 3:1; B) complexes.

3.4 Structural characterization of complexes

FTIR spectra of Ova, Lyso, XG, Ova/XG (ratio 5:1) and Lyso/XG (ratio3:1) complex are show in Figure 7. The most distinctive spectral features for proteins are the strong amide I and II bands approximately between 1640 cm^{-1} and 1540 cm^{-1}, respectively (STUART, 2006). The main peaks obtained for native Lyso and Ova are between 1670 and 1390 cm^{-1}, which are mainly associated with amide I, II and III respectively (MILLER et al., 2013). Primary amines display two weak absorption bands at 3500 and 3400 cm^{-1}, which represent the asymmetrical and symmetrical N-H stretching (BARTH e ZSCHERP, 2002; BARTH, 2007). Thus, for native Lyso and Ova, the broad band around 3300 cm^{-1} arises from N-H strength of the free amino groups.

In XG spectra, the vibration peak of hydrogen bonded -OH and C-H stretching were observed at 3436 and 2946 cm^{-1}, respectively. The vibration peak of the acetal was found at 1033 cm^{-1}, but this band can also be assigned to C-C and C-O strength and C-H bending in this polysaccharide (POOJA et al., 2014). Adsorption at 1636 and 1399 cm^{-1} are related to asymmetric and symmetric stretching vibration of the carboxylate anion present in the side chain trisaccharide of XG (CETIN e ERDINCLER, 2004; HAMCERENCU et al., 2007).

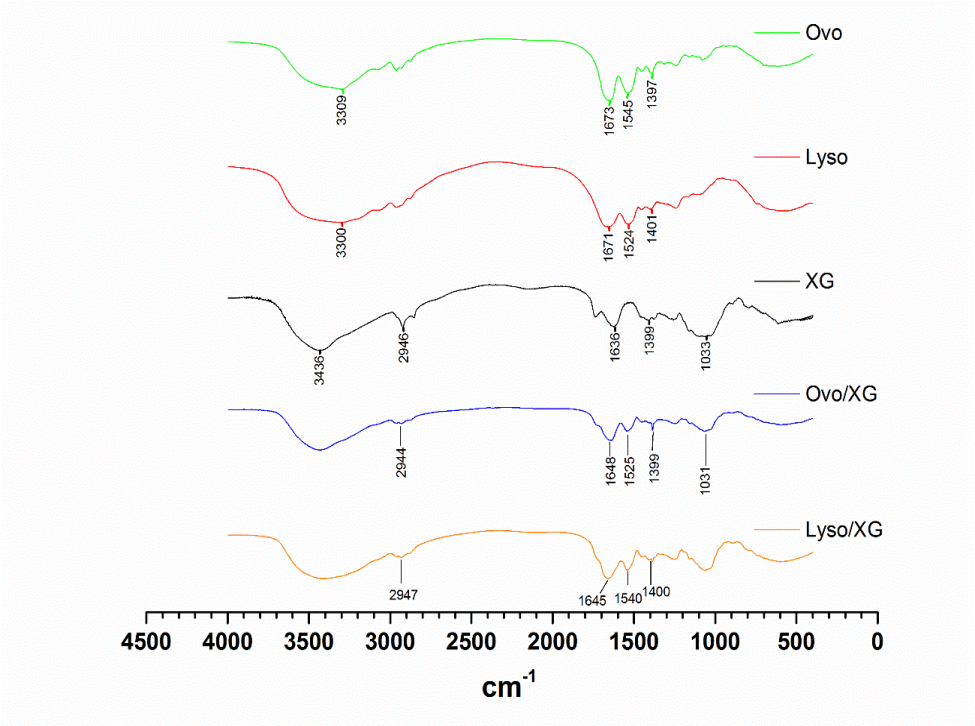

Figure 7: FTIR spectra of Ova, Lyso, XG, Ova/XG (ratio 5:1) and Lyso/XG (ratio3:1) complex. The Ova/XG and Lyso/XG complexes were prepared at pH 3.0 and 7.0 respectively, temperature 25 ºC, and at a concentration 0.01 mol/L NaCl.

The FTIR spectrum of Ova/XG and Lyso/XG complexes some observations are worth analyzing. The intensity of regions between 1650 and 1390 cm^{-1} decreased for both complexes formed. Moreover, disappearance of bands 1636 and 1399 cm^{-1} followed by reduction of band 1033 might be an indication of formation of electrostatic interaction between the carboxyl groups of XG (–COO) and amine groups of proteins ($-NH_3^+$) (BARTH, 2007; WEN, 2007). The formation of electrostatic interaction also promotes a weak shift of the Amide I, for both complexes. This weak shift also observed in Raman spectra (MAITI et al., 2004; HUANG et al., 2006) may be attributed to a particular structural transformation of adsorbed protein from a functional form with predominating α-helix structure to a form with increasing β-sheet and amorphous state (PEREVEDENTSEVA et al., 2011; ABERKANE et al., 2012). Besides, the spectrum of complexes, in special Ova/XG with significant decreased of band 2946 cm^{-1} of XG showed a broad band at around 2940 - 3600 cm^{-1}, indicating enhanced hydrogen bonding compared to that of proteins and XG. This implied that hydrogen bonding was also involved in the interaction between proteins and XG (BARTH e ZSCHERP, 2002).

For better understanding the internal structure of complexes formed, precipitated obtained were separated and dehydrated with the objective of demonstrating the new structures formed (Figure 8).

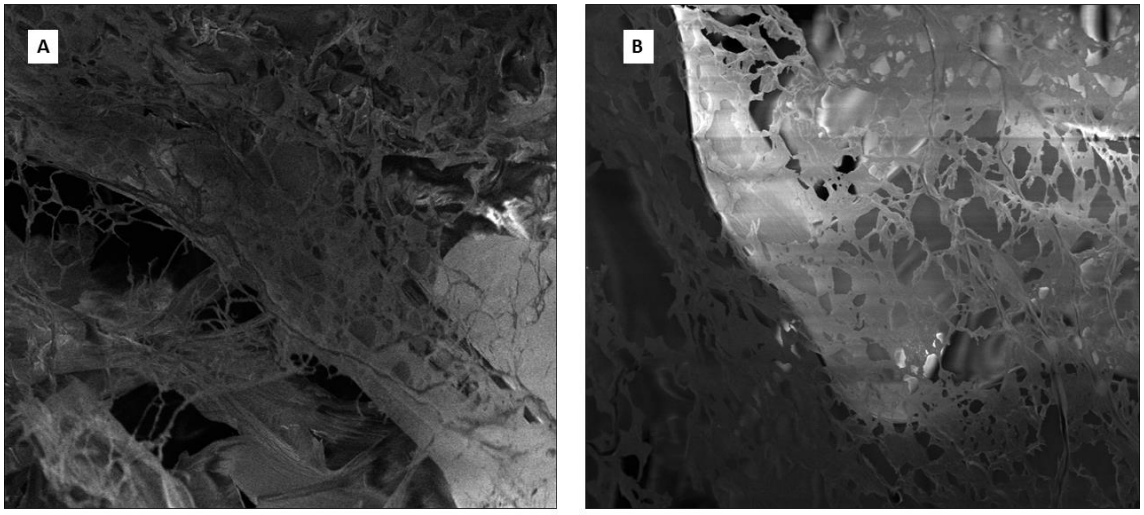

Figure 8: Scanning electron microscopy (100 μm) of Ova/Ca (ratio 5:1; A) and Lyso/Ca (ratio 3:1; B) complexes. The Ova/XG and Lyso/XG complexes were prepared at pH 3.0 and 7.0 respectively, temperature 25 ºC, and at a concentration 0.01 mol/L NaCl.

In the figure 8, A and B is possible to note that three-dimensional network was formed during complexation, and samples revealed that intrapolymer complexes with heterogeneous structure were formed for both complexes (KAYITMAZER et al., 2007a). As discussed above, we believe that ovalbumin, and lysozyme may plays as a crosslinking agent along κ-carrageenan chains, meanwhile other researches must be developed to clarify the mechanism of interaction, and the internal structure of the complexes formed.

4 CONCLUSIONS

The present study reports the effect of pH, biopolymer mixing ratio and ionic strength on the associative phase separation involving lysozyme and ovalbumin with xanthan gum. As the protein/polysaccharide ratio increased from 1:1 to 10:1, critical structure forming events shifted to higher pHs with Ova/XG, however, the increased of the ratio from 2:1 to 10:1 for Lyso/XG complexes suppressed the pH_{φ} forming insoluble at pH 12.0. Addition of salt to the Ova/XG and Lyso/XG mixtures suppressed the electrostatic interaction between proteins and XG shifting to lowering pHs the critical pH transitions points. The rheological data of the complexes showed gel-like network structure directly dependent of the ratio and ionic strength. When the ratio of ovalbumin reaches 5:1, the maximum yield of the coacervates was achieved, and the maximum shear modulus was observed. Similar behavior was observed with lysozyme; however, maximum shear modulus was found for the ratio 3:1. At 0.2 mol/L NaCl the screening effect suppressed the formation of network structure and systems showed viscoelastic behavior. Although, the electrostatic interaction has been the main mechanism of interaction between the protein and xanthan non-electrostatic interactions was also reported in the interaction between proteins and XG. Taken together, these results suggest

that Ova/XG and Lyso/XG complex can be potentially used in formulated food to improve sensorial properties.

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CAPÍTULO VI

RHEOLOGICAL AND STRUCTURAL STUDY OF HYDROGELS INDUCED BY ELECTROSTATIC INTERACTION (PART 1): EFFECT OF LYSOZYME-XANTHAN GUM RATIO

ABSTRACT

Protein-polysaccharide interactions play an essential property to control the structure, texture, and stability of foods and biomaterials. There is an increasing need for food systems using functional ingredients and electrostatic interaction between some proteins and polysaccharides has the property to form low solid gels. Formation of Lysozyme-Xanthan Gum (Lyso-XG) gels was studied with the objective to identify the role of individual biopolymer as well as the influence of their mixing ratio on gelation mechanism, texture properties, pores, and syneresis of the resulting gels. The Lyso-XG ratio modulates the gelation kinetic and is the main factor controlling the gelation process and the gel structure. The excess of protein affected the compaction of gel, which resulted in gels with dense clusters, with poorer water-bolding capacity, though, in the ratio 2:1 the excess of protein was correlated with the most discontinuous and heterogeneous network with large strands and porous, high waterloss, and low hardness. The gel strength mainly depended on the XG content, hence, at the ratio 1:2 the hardness and held-water were elevated and final G' was almost five times stronger than ratio 1:1. Lyso-XG gels produced without heat treatment and with low water content presents a great potential application for the food and pharmaceutical industry

1 INTRODUCTION

Gelation is an important functional property of protein in the food industry; however, also there is a large variety of applications in the cosmetics, pharmaceutical, biomedical and coating industries (DRURY e MOONEY, 2003; VAN VLIET et al., 2004b; LI et al., 2014a). A better understanding of protein functionality and exchangeability of one source for another is the key to the discovery of new products or protein functionality commonly used for other purposes.

The use of electrostatic interaction between proteins and polysaccharides is commonly encountered in food products to control the structure, texture, and stability of the products (TURGEON e BEAULIEU, 2001; SCHMITT e TURGEON, 2011). However, one of the functional properties of this interaction is a formation of different types of gel structures depending on the characteristics of the proteins and polysaccharides used, the methodology of gelation and on the environmental conditions (ZASYPKIN et al., 1997; BEAULIEU et al., 2001; PICONE e CUNHA, 2010). Cold gelation or electrostatic gel corresponds to the major ways of gel formation by electrostatic interaction between the protein and polysaccharide. The process of gel formation by cold gelation is divided into two consecutive steps. In the first step, the protein is heated in a solution with low ionic strength at neutral pH until form aggregates (CAVALLIERI e CUNHA, 2009; DE JONG et al., 2009). These aggregates are subsequently cooling remaining soluble, and the gel is not formed due to predominant electrostatic repulsive forces among the aggregates. In the second step, the gelation is induced by changing the solvent characteristic, for example, by the addition of calcium chloride. (CHUNG et al., 2013) or by lowering the pH with the addition of glucono-δ-lactone (GDL) (BRAGA et al., 2006; URBONAITE et al., 2014) or by enzymatic hydrolysis (OTTE et al., 1999; MIWA et al., 2013). On the other hand, the electrostatic gel is formed by associative interaction between native proteins and polysaccharide induced by lowering the pH with the

addition of GDL (LANEUVILLE et al., 2006; LE e TURGEON, 2013). Comparing the two methods is possible confirm that both are able to form functional gels with lower concentration of protein and/or polysaccharide meantime electrostatic gels can be formed without any thermal, enzymatic or other denaturing treatments to the protein or the mixture which thereby conserving the useful properties of polymers (LANEUVILLE et al., 2006). These gels presenting a great potential use in food industry to enhance the stability of foods and to protect micronutrients (e.g. products with delicate texture or compounds) even as in the pharmaceutic industry to deliver and to protect drugs or to prevent postsurgical adhesions (LANEUVILLE et al., 2006; LI et al., 2014a).

Xanthan gum is an extracellular polysaccharide secreted by the microorganism *Xanthomonas campestris*. Xanthan gum is soluble in cold water and solution exhibit highly pseudoplastic flow. Its viscosity has excellent stability over a wide pH and temperature range, and the polysaccharide is resistant to enzymatic degradation. The primary structure of xanthan gum is a linear (1-4) linked β-D-glucose backbone (as in cellulose) with a trisaccharide side chain on every other glucose at C-3, containing a glucuronic acid residue linked (1-4) to a terminal mannose unit and (1-2) to a second mannose that connects to the backbone (URLACHER e NOBLE, 1997). In the food industry, its use is greatly valued due to some characteristics such as elevated solubility in both hot and cold water, to produce high viscosity solutions even at low concentrations, not cause noticeable changes in the viscosity of solutions in a wide temperature range, from 0 to 100 ° C (which makes it unique among edible gums), being soluble and steady in acid solutions, to be quite compatible salt, to be stabilizing suspensions and emulsions and provide fixedness for products submitted to freezing and thawing (MONSANTO, 2009; DAMODARAN et al., 2010). Lysozyme is an important protein found in egg white. The molecular weight of lysozyme is 14,400 Da and consists of a single polypeptide chain with 129 amino acids. In nature, this protein is found as a monomer but is occasionally current as a dimer with more thermal stability. It is considered as a strong basic protein present in egg white (HUOPALAHTI et al., 2007). Lysozyme has 4 disulfide bridges leading to high thermal stability, and its isoelectric point is ~10.7-11.3. It has a tendency of binding to negatively charged proteins such as ovomucin in egg white; however, this tendency can be used to form complex or interpolymeric complex with anionic polysaccharides (STADELMAN e COTTERILL, 1995b). Recently, lysozyme has shown a wide range of application in the food and pharmaceutical industry (ABEYRATHNE et al., 2013). In the pharmaceutical industry, lysozyme has been shown to exert antiviral activity, reportedly associated with its charge, rather than its lytic ability (NAIDU, 2010). Oral and topical applications of lysozyme were found to be effective in preventing and controlling several viral skin infections, including herpes simplex and chicken pox, as well as acting as exerting anti-inflammatory action (SAVA, 1995). It has also been shown to normalize humoral and cellular responses in patients with chronic bronchitis (BLANKENVOORDE et al., 1998). In the food industry, lysozyme is one of the major bacteriolytic proteins having the capability of controlling foodborne pathogens such as *Listeria monocytogens* and *Clostridium botulinum* (RADZIEJEWSKA et al., 2008; LEŚNIEROWSKI e CEGIELSKA-RADZIEJEWSKA, 2012), which are considered 2 main pathogens that cause problems in the food industry. The World Health Organization and many countries allow the usage of lysozyme in food as a preservative, and it is currently used in sushi, cheese, and wine production (LIBURDI et al., 2014).

Dynamic testing, which involves an oscillatory applied strain or stress, can provide very useful information on the gel formation process. In the strain controlled the sample is subjected to a sinusoidally oscillating strain generating an oscillating stress response, which is, to some extent, out of phase with the strain. Some of the main parameters that are usually determined from these responses include the elastic or storage modulus (G'), which is a

measure of the energy stored per oscillation cycle, the viscous or loss modulus (G"), which is a measure of the energy dissipated as heat per cycle (CLARK et al., 1983; NORMAND et al., 2000). The elastic modulus of the gel is governed by the number of the elastic effective junctions between strands thereby during small-deformation the elastic modulus allows monitor the process of organizing and structuring the gel during acidification (LUCEY, 2008). Gel hardness is another good parameter to determine the number of the effective strands in the gel and the modulus of the protein strands and polysaccharide, nevertheless, syneresis data of gels also can help to understand if factors such as concentration of the polymer or methodology of gelation affected the final structure of the gel (e.g. density and pores) given the fact, e.g. most of the water in milk gels is not chemically "bound" to proteins but rather is physically entrapped in the network structure (VAN VLIET et al., 2004b; LUCEY, 2008).

The objective of this study was to investigate the role of lysozyme and xanthan gum in gel formation as well as the influence of the ratio of both in gelation mechanism, texture properties and syneresis of gels. The framework images of gel were used to discover the rol e of lysozyme and xanthan in the network development as a cross-linking agent.

2 MATERIALS AND METHODS

2.1 Material

Lysozyme (Lyso with purity > 90%, molecular mass of 14.307 Da and isoelectric point 11.35) were obtained from Sigma Chemicals (Oakville, CA). Xanthan gum (XG) (Keltrol RD, 96.36 wt% total sugar, 3.02 wt% protein) was provided by CP. Kelco Ltd. (Chicago, USA). Glucono-δ-lactone (GDL, lot # 70H0163, USA) and rhodamine B isothyocyanate (lot # G39595, USA) were purchased from Sigma (St. Louis, MO, USA) and J.T. Baker (Philipsburg, NJ, USA), respectively.

2.2 Sample preparation

The powders of lysozyme and xanthan gum were dissolved at 0.3% in deionized water (Modulab Analytical, Fisher Scientific) with continuous stirring at room temperature during overnight, and pH of this solution was previously adjusted to pH 12.0 using 0.1 M NaOH. For gel formation research, three ratios of Lyso-XG were systematically studied: 1:1; 2:1 and 1:2 in fixed solid total of 0.3 wt%. The gelation of the system was induced by addition of GDL on solution containing Lyso-XG and pH of the system was continuously monitored during 16 hours at room temperature using a DL53 titrator (Mettle Toledo, Switzerland) to obtain the final pH 7.06 ± 0.03

2.3 Turbidimetric measurements

pH-dependent turbidity was measured at a wavelength of 600 nm using a spectrophotometer (Biochrom mod. LIBRA S12, England) calibrated with ultrapure water to 100% transmittance (*T*). Turbidity was defined as 100 - %*T*. With the aid of a magnetic stirrer (Nova Técnica, NT 101, Brazil) and a pH meter (Tecnopon, mPA-210, Brazil) the pH of the solutions were adjusted (from 12.0 to 7.0) with HCl, 0.5 M. Measurements of were made at room temperature (25 ºC ± 1), and each sample was measured four times at one minute intervals.

2.4 ζ- Potential measurement

The Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was used to determine ζ-potentials. Lysozyme and Xanthan gum stock solutions and Lyso:XG complex (ratio 1-2) formed were diluted to 0.01% w/w and transferred to an MPT-2 autotitrator (Malvern Instruments, Worcestershire, UK) that adjusted pH using 0.5 M NaOH, 0.25 M HCl and 0.025 M HCl solutions. pH was varied from 12.0 to 7.0 by 0.5 unit increments with a confidence interval of ± 0.1 unit. To measure of ζ-potential of gels Lyso:XG (ratio 1:1; 2:1, 1:2) the GDL was added to the solutions and the ζ-potential was measured for 16 hours with interval of 5 minutes between analyzes. Each experiment was performed three times and sample readings were done in triplicate at 25 °C and ζ-potentials were calculated using the Smoluschwsky mathematical model.

2.5 Dynamic Oscillatory Measurements

The small-deformation properties of the mixed gels during gelation were determined in dynamic oscillation mode with a controlled-strain rheometer (ARES-G2, TA-Instrument, Piscataway, NJ, USA) equipped with a circulating water bath temperature controller. A Couette device with a cup (30.0 mm diameter) and bob system (27.05 mm diameter, 42.0 mm length) was used. Ten seconds after GDL addition, the samples were transferred to the rheometer. The development of the storage modulus (G') and the loss modulus (G') during gelation was recorded for 16 h at 25 C at a frequency of 0.1 Hz and a strain of 0.1%. Following oscillation, a strain sweep test was recorded at increasing strains from 0.1 to 100% to verify the linear region. The gelation point was defined as the point when G´ was greater than 1 Pa (LUCEY et al., 1998; LE e TURGEON, 2013). Samples were covered with a thin 3 lm of castor oil to prevent evaporation during measurement.

2.6 Texture Properties

Gel texture parameters were determined by a Texture Analyzer TA XT-2 (Texture Technologies Corporation, NY). The gels formed in the 70 mL-beaker were penetrated with 12-mm diameter cylinder probe and force – time curve was obtained at a crosshead speed of 0.50 mm/s for 20 mm displacement (LE e TURGEON, 2013). The resulting force-time curves were studied using the texture profile analysis (TPA) method in order to determ ine hardness and deformation at fracture (TURGEON e BEAULIEU, 2001). All texture measurements were performed after 24 h of gelation.

2.7 Syneresis

The released water and waterbinding were quantified using Millipore Ultrafree-CL centrifugal filter units with microporous membranes of 0.45 µm pore size (EMD Milipore, Ontario, Canada). The three ratios of gels were formed *in situ* in six filter cups of 2 mL with a pinhole obstructed during the gelation process. These filter cups were placed in a filtrate collection tubes with caps. After 24 h of gelation, the pinhole was freed for 4 h, and then the released water was measured. After this process, the samples were centrifuged at 1000 rpm for 4 min on a fixed 45º angle rotor microcentrifuge (KOCHER e FOEGEDING, 1993) for measure waterbinding. The waterloss and waterbinding were calculated as:

$$Waterloss\ (\%) = \frac{(weight\ of\ water\ released)}{weight\ of\ sample}\ x\ 100 \qquad (1)$$

$$HW = \frac{(Total\ g\ water\ in\ the\ sample - g\ water\ released)}{total\ g\ biopolymers\ in\ sample} \quad (2)$$

2.8 Confocal Laser scanning microscopy

The structural features of the gels were investigated using a microscope confocal live-cell Zeiss (Toronto, Canada) equipped with an inverted microscope. The objective lens used was 60X WI, NA 1.20. The excitation wavelength was 543 nm, with an emission maximum at 605/675 nm. The solutions with lysozyme were stained by adding rhodamine B isothyocyanate (RITC) to protein dispersions under magnetic stirring for 1 h before mixing with XG dispersions. XG was stained by fluoresceinamine (FA). The FA-XG prepared according to the method of Donato et al., (DONATO et al., 2005) was provided by Laneuville and was used in mixture with RITC-Lyso in order to determine the location of Lyso and XG in the gel. Mixtures of RITC-Lyso/FA-XG were prepared as described above in three different ratios. After GDL addition, the sample was poured between a 0.5-mm-deep well concavity slide and a cover slip, which was hermetically sealed with nail enamel. Micrographs were taken 24 h after GDL addition. Digital images were acquired at a pixel resolution of 1024 x 1024.

The average pore size (μm) was estimated by image analysis using ImageJ (v1.49r; http://rsb.info.nih.gov/ij/) following the procedure detailed by (LE e TURGEON, 2012). For pore size measurement, the "watershed" command was performed on 8-bit binary images in order to separate the pores. The mean pore size (μm) was estimated from the average area of the pores which was calculated from the count of pores and the total area corresponding to pore.

2.9 Statistical analysis

Final elastic modal (G'), pore size, final pH, waterloss, hardness and held-water data were analysed using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). The means for each condition were compared with a Duncan test using a significant level of $p < 0.01$.

3 RESULTS AND DISCUSSIONS

3.1 Gelation process

In order to characterize the process of gelation formed between lysozyme and xanthan gum the turbidity, storage (G´) and loss (G´´) modulus were monitored as a function of pH (Figure 1). In figure 1a is possible observe that at pH 12 the net charge of protein is more negative and values of G´ and G´´ show that solution presents sol state with translucent aspect (Figure 1b) meantime, when pH starts decrease, we observe that G´ and turbidity start increase as far as pH approaches the isoelectric point of Lysozyme (p*I* 11.3), and this is due to non-covalent attractions between positive patches on the Lyso and negative charge of XG forming soluble complexes.

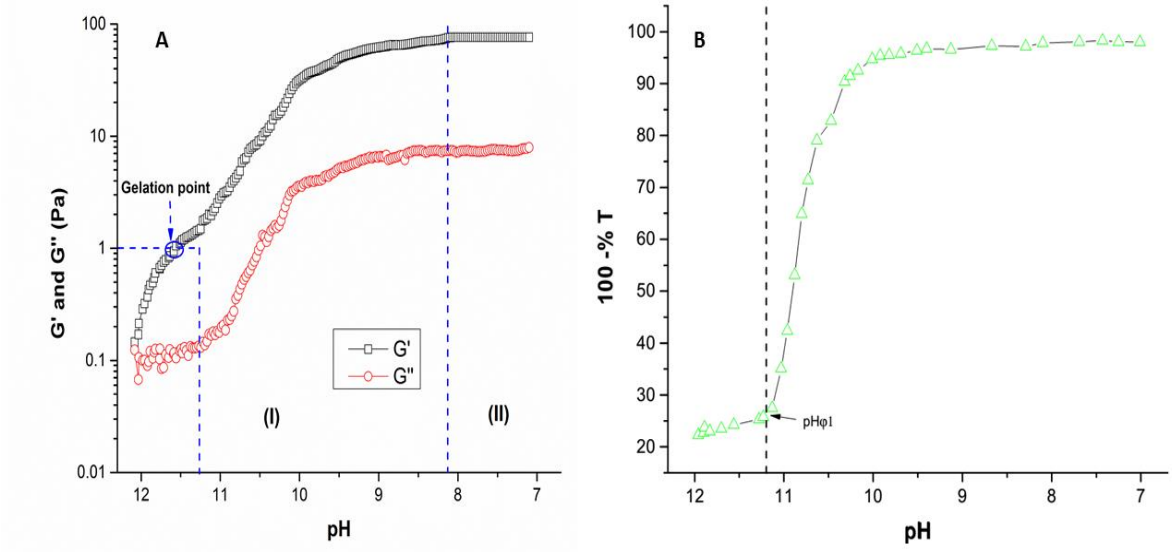

Figure 1: pH dependence of (a) storage modulus and loss modulus, and (b) absorbance for the Lyso–XG mixture ratio 1:2.

At figure 1b G' appear to be higher than G" at pH around 12.0. However, as far as interaction between Lyso and XG was promoted by more positive protonation of protein the values of module storage increased rapidly evidencing gel formation, nevertheless, crossover between G´ and G´´ was not observed during beginning of the gelation process. Previous studies (LANEUVILLE et al., 2005a; LANEUVILLE et al., 2006) have shown that crossover always happens around the proteins p*I*, with a tendency to come about at slightly pH values. However, the structuration process currently begins well above the p*I*. We believe that behavior occurs mainly because of two influences. Firstly, is possible that mixture was highly structured before gelatin point, and this behavior may be derived from pseudo-plastic properties of XG that can exhibit dominant elastic responses to the wide range of pH (MONSANTO, 2009). The Second reason can be the structuration of the tenuous network between Lyso-XG influenced by the formation of soluble complexes. Previous studies have noted that gels obtained between β-Lactoglobulin-XG demonstrated the same formation of soluble complex in the region above of p*I* of β-Lactoglobulin. However, when pH further decrease interbiopolymer complexes were formed, which explain begin of the gelation process before p*I* (LANEUVILLE et al., 2006; LE e TURGEON, 2013).

When we observe the gelation point in figure 1a we note that G´exceeds the value of 1 Pa at pH 11.47 ± 0.2 however, is accomplishable to note that after pH 11.90 the values of G´ increased much faster them G´´. This swift exponential growth of storage modulus corresponds to the rapid initial aggregation of protein molecules as a result of reduced electrostatic repulsion to form the tree-dimensional network. In the pH 12.0, the XG (p*K*a 2.9) was highly negative charged, and Lyso exposes some positive patches. As far as pH was decreased the soluble complex formed was rapidly interconnected begetting dense interpolymeric complexes. Similar behavior was mentioned recently by DE FARIA et al. (2013) when they evaluated the effect of protein composition (β-Lg, α-La and GMP) on gelation. They could observe that gels containing mostly α-La the gelation happened at lower pH values suggesting that it may occurs primarily because of net negative density of α-La which reduced interaction between proteins around pH 7.0 until pH 6.2, meantime mixed gels containing more GMP gelled at higher pH levels (pH 6.4 – 6.1). At pH 7.0, weak electrostatic interactions can occur between positive patches of β-Lg and negative patches of GMP. However, it was sufficient to promote β-Lg aggregation and consequently, shorter gelation time in higher pH (MARTINEZ et al., 2010). When we compare the gelation point presented

at figure 1a and region of pHφ1 (pH 11.25 ± 0.1) at figure 1b is possible to note also that both gelation point and complex coacervate formation happened around the same pH which permits suggests that gels formed between Lyso-XG present the gelation point directly associated with pHφ1 of complex formed among even biopolymers.

During the process of gel formation, the storage modulus and loss modulus presented growth totally dependent on decrease of pH; however, the final microstructure was obtained at pH 8.31 where the region of the plateau was formed, and storage modulus did not exhibit alterations within this region indicating that gel structuration attainment of an electrostatic quasiequilibrium in the system (LANEUVILLE et al., 2006). At this region, the repulsion electrostatic is quasi-null and thereby the three-dimensional network of gel is formed by maximum interconnections between protein and polymer. Therefore, the structuration process of gel cease

3.2 Effect of ratio Lyso-XG

The gelation process of three ratios of Lyso-XG is show at figure 2. As possible see the concentration of lysozyme and xanthan affected directly the gelation kinect of storage modulus.

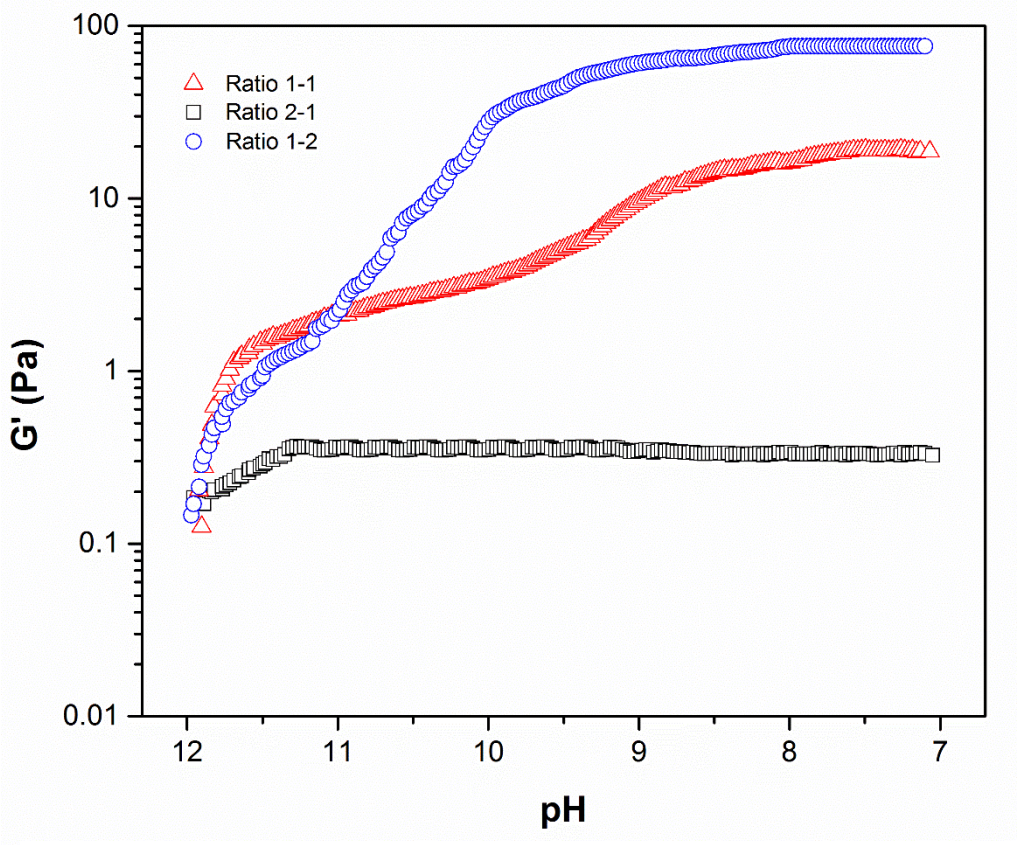

Figure 2: Evolution of storage modulus as a function of pH for Lyso-XG in different ratios.

In the ratio 1:1 is feasible to see that gelation process occurred in two stages of structuration, and the gel was second stronger of the three ratios studied. Firstly, was viable to see that gelation point occurred rapidly (pH 11.80 ±0.07) followed the region of slow increased of G´ meanwhile, at pH 9.47 ±0.04 the G´ started increase more stabilizing at pH 7.88 ±0.04. When the protein concentration is elevated the gelation process befalls faster mainly because more protein is available to develop crosslinking function (LE e TURGEON,

2013), nevertheless, after gelation point is possible see that G´ increase slowly reflecting the incorporation of additional protein clusters and/or molecules in the gel network as well as the continued rearrangement of bonds and strains in the (DICKINSON, 1997; VAN VLIET et al., 2004a). The presence of a secondary stage is not clear. However, we believe that behavior may have been caused for presence of two different stages of rearrangement of bonds and strains in the network. LANEUVILLE et al. (2006) studied formation of gel through electrostatic interaction between β-lactoglobulin – Xanthan gum and when they compared the internal structure development of gels, They could see that at ratio 2:1 the structuration occurred in two stages. The first one showed a slow compression indicating a diffusion-limited cluster aggregation mechanism induced by primary, complex and second was followed by densification of clusters caused by the tendency of molecules to reorganize due to the increase in opposite net charges and protein cooperative binding. We believe that amount of protein associated with the viscosity of the system could contribute to the difficulty to compaction of the system after the faster gelation of this system.

The compaction of gel is further favored when more protein is available to fill all gaps of the developing structure (LOPEZ-LOPEZ et al., 2006; ZHU et al., 2013), meantime when we increase the amount of lyso at ratio 2:1 we could see that gelation process was directly affected. At the ratio 2:1 gelation process occurs faster if we compare with other ratios finishing after p*I* of lysozyme. Is also possible note that G´ started at pH 11.95 about 0.04 ± 0.01 meanwhile, after gelation process the G´ increased to 0.27 ±0.05 and subsequently a slow decrease of G´ is noted, even though the precipitation could not be related since the system was gelled showing behavior of weakness gel. This behavior occurred because of effect of excess of Lyso, which associated with individual molecules of XG formed complexes that reduced the junction zones of the gel network meantime when we increase the amount of lyso at ratio 2:1 we could see that gelation process was directly affected. At the ratio 2:1 gelation process occurs faster if we compare with other ratios finishing after p*I* of lysozyme. Is also possible note that G´ started at pH 11.95 about 0.04 ± 0.01 meanwhile, after gelation process the G´ increased to 0.27 ±0.05 and subsequently a slow decrease of G´ is noted, even though the precipitation could not be related since the system was gelled showing behavior of weakness gel. This behavior occurred because of effect of excess of Lyso, which associated with individual molecules of XG formed complexes that reduced the junction zones of the gel network (LUCEY, 2008; DE JONG et al., 2009). Finally at ratio 1:2 was possible see that gelation point occurred at pH 11.47 ± 0.2 followed by exponential growing of G´ exhibiting a gel almost 5 times stronger than ratio 1:1 suggesting them XG plays one important role in the formation and structuration of gel network in systems with lysozyme. LANEUVILLE et al. (2006) suggested that protein played one determinant role in the structuration of gel formed between β-lactoglobulin – Xanthan gum showing that high concentration of β-lactoglobulin (ratio 15 and 20) affected stoichiometry of system and consequently, the formation and stability of gel. However, in lower ratios (2 and 5) presented higher G´values arguing this result was due to optimum balance of attractive and repulsive forces between biopolymers The same system was studied by LE e TURGEON (2013), however, the effect of polysaccharide concentration was evaluated. The authors could also observe that the ratio 10 the stoichiometry of the system was affected by excess of β-lactoglobulin meantime when XG concentration was increased from 0.03 wt% to 0.06 wt% in the best ratio of protein (5) the G´ increased almost 7 times stronger comparing ratio 10 with XG 0.03 wt% concluding then that gel networks are based mostly in XG chains. Grounded on these results we can suggest that XG was base of gel network formed with lysozyme and when we compare the ratio of lysozyme with ratio of β-lactoglobulin described above we may as well suggest that is possible form quality gels with low concentrations of lysozyme.

In order to identify the density of charge of individual biopolymers and three ratios of gel formed the ζ-potentials as function of pH was conducted and results are present at figure 3. As it is possible to notice the Lyso has a change in the electrical charge on the individual protein solution, ranging from a negative charge at pH 11.9 to a positive charge at pH 6.9 with a zero load point (p*I*) near 11.3 as reported in the literature (STADELMAN e COTTERILL, 1995b) however, when we observe XG may note which polysaccharide charge patches remained considerably negative during all pH range studied. It occurred because XG is an anionic polysaccharide with p*K*a 4.5, for this reason, have charge patches highly negative in basic pHs (12.0-7.0). It can be observed that the values found for the mixture containing both biopolymers are the values of the isolated polymer intermediates. This behavior indicates the interaction between the carboxyl groups of the polysaccharide and protein amine groups, featuring electrostatic binding. In the ratios 1:1 and 2:1 are possible behold that both system present a slight decrease of negative charge patches at pH range studied. We believe that behavior could correspond the excess XG highly negative charge that did not bind with protein. In the ratio 1:2 is possible see that after p*I* of Lyso the negative charge patches decrease faster with final value of -19.0 ± 1.5 and it can indicate that in high concentration of Xg the excess of protein can neutralize more carboxyl groups of XG increasing density of gel. Similar behavior is observed in complex formation where the interaction between protein-polymer and complex—complex was almost able to neutralize the electrical charge in the system at pH 7.0.

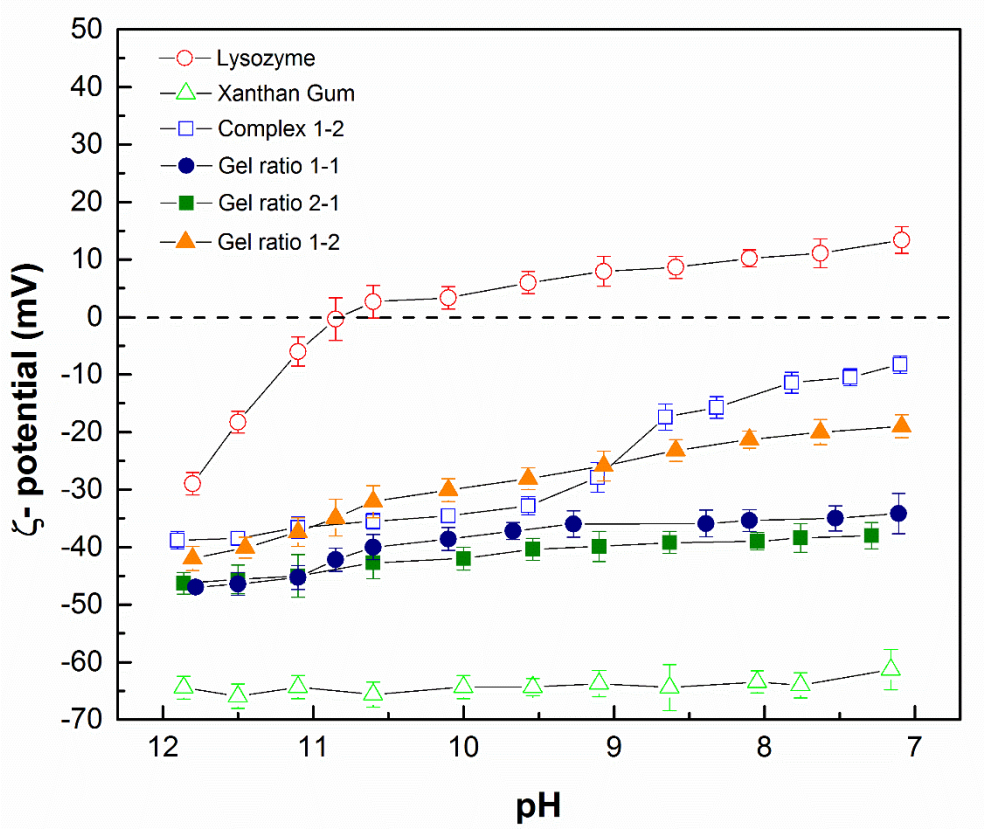

Figure 3: Zeta potential for individual biopolymers, Lyso-XG complex and Lyso-XG gels in three ratios as a function of pH.

3.3 Structure of Gel

In the figure 4 is possible to see networks formed between Lyso and XG in the 3 ratios studied. As was suggested above the gel network was based on the initial weak network of xanthan gum and Lyso aggregated along xanthan gum chains and played the role of cross-linking agent by electrostatic interactions with other xanthan gum chains. In the ratio 1:1 (figure 4a) we noted a compact network with a good and dense distribution the interconnected network meanwhile, when we compare that structure with ratio 1-2 (figure 4c) we confirm that more XG could form a network more homogeneous and stronger with smaller pores in more interconnected network confirming the rheological results previously discussed. At the ratio 1:1 note that process of gelation occurred in two different stages being a process influenced by excess protein and other influenced by stage of rearrangement of bonds and strains in the network. In the figure 4d notice the distribution of Lyso along XG chain, and we can mention that it is possible observe the presence of some protein aggregation along zones of interconnection points. We suggest that aggregation may have been formed during first process of gelation influenced by excess of protein, which formed condensed interpolymeric complex. These interpolymeric complexes formed dense clusters which hampered compactation of system resulting porous larger as we can see on network superposition in figure 4e. When concentration of protein was increased at ratio 2:1 the gel obtained presented the most discontinuous and heterogeneous network with large strands and porous indicating that excess of protein increased repulsive, which result in a weakness gel. In the figure 4 is possible to see networks formed between Lyso and XG in the 3 ratios studied. As was suggested above the gel network was based on the initial weak network of xanthan gum and Lyso aggregated along xanthan gum chains and played the role of cross-linking agent by electrostatic interactions with other xanthan gum chains. (BEAULIEU et al., 2001; LAKEMOND e VAN VLIET, 2008). Bigger pores were qualitatively observed in systems with lower total biopolymers concentration, and polysaccharide content by LE e TURGEON (2012) and large pores were also associated with excess of β-lactoglobulin in gels formed between β-lactoglobulin- xanthan gum by LE e TURGEON (2013) and LANEUVILLE et al. (2006).

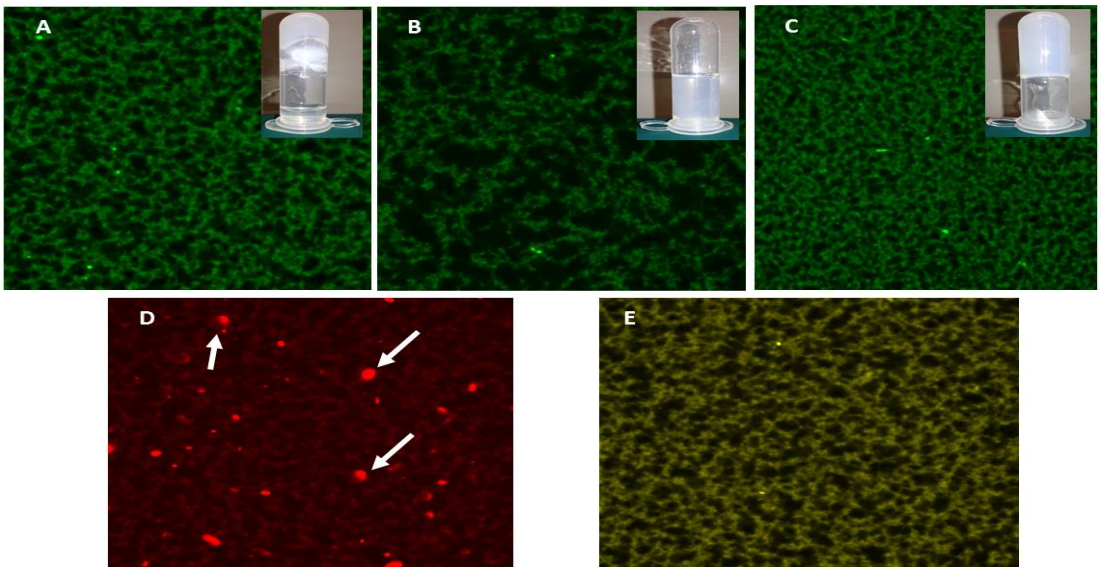

Figure 4: Confocal micrograph of RITC-Lyso-XG gels obtained at ratios 1-1 (A), 2-1 (B) and 1-2 (C) and localization of Lysozyme (D) and network superposition (E) in the ratio 1-1 observed by confocal laser scanning microscopy. Scale bar corresponds to 30 µm.

In the ratio 1:2 note a highest dense network of the three ratios studied, which suggest that density is proportional to the XG concentration. The formation of interconnection points in the network is directly favored at higher XG content due to high molecular weight and entanglement of this polysaccharide, which promoted less space between molecules. Therefore, smaller pores were formed, and stronger gels were obtained confirming results obtained in figure 2 and suggesting also that this ratio may correspond the best ratio for the gel formation between Lyso-XG by electrostatic interaction.

3.4 Texture and waterbinding properties of gels

In the table 1 is possible to see the texture, waterbinding properties even as pHgel, final storage modulus and pore size of all ratios studied.

Table 1: Pore size, held-water, waterloss, Hardeness, final elastic modulus (G') and pH_{gel}[a]

Ratio	**pH_{gel}**	**Final G'**	**Hardness (N)**	**Waterloss (%)**	**Held-Water**	**Pore size (µm)**
1-1	7.08 ± 0.02^{a}	0.273 ± 0.05^{a}	0.08 ± 0.01^{a}	29.98 ± 1.33^{a}	75.15 ± 0.48^{a}	12.06 ± 0.24^{b}
2-1	7.06 ± 0.05^{a}	19.830 ± 0.42^{b}	0.13 ± 0.05^{b}	16.67 ± 0.36^{b}	115.38 ± 0.70^{b}	7.63 ± 0.04^{a}
1-2	7.01 ± 0.04^{a}	76.211 ± 0.43^{c}	0.22 ± 0.07^{c}	4.27 ± 0.09^{b}	324.07 ± 0.79^{c}	4.68 ± 0.11^{c}

[a] Different letters in each column indicate statistical significant differences in mean values ($p<0.01$);

The variation of ratio did not affect significantly ($p<0.01$) the pHgel even in high concentration of protein or polysaccharide and GDL hydrolysis also did not show the influence of the pH during acidification. CAVALLIERI e DA CUNHA (2008) described that GDL hydrolysis showed a strong influence of the pH during acidification if low acidification rates were used, and when the final pH was achieved the properties remained stable. However, when the rate of acidification increased by adding larger amounts of GDL to the system, the effect of molecular rearrangements at the final pH was fundamental to network strengthening. Recently LANEUVILLE e TURGEON (2014) suggested higher pHgel were related to protein excessive self-aggregation in gels formed between skim milk and xanthan gum or κ-carrageenan. It is worth mentioning that Lyso-XG gels exhibited versatility also be formed at pHgel 4.0 (results not shown) which may further expand the applicability or functionality of these gels. However all parameters discussed in this article should be taken into account in assessing the quality and functionality of these gels.

Gel hardness is determined by both the number of the effective strands in the gel and the modulus of the protein strands and polysaccharide (VAN VLIET et al., 2004b). The elastic modulus of the gel measured in rheological measurement, on the other hand, is governed by the number of the elastic effective junctions between strands (LUCEY, 2008). The hardness and final G´ of Lyso-XG gels was directly dependent of the ratio showing a significant difference ($p<0.01$). The lowest value of gel hardness was obtained at ratio 2:1 followed by intermediate value in the ratio 1:1 and finally the highest hardness corresponded to the ratio 1:2. As mentioned above, this behavior is related to the density of the network. At high concentration of XG the gel´s network became denser influenced by greater electrostatic interactions between protein and XG presenting gel with higher hardness and final G´. In the ratio 1:1 the influence of formation of some dense interpolymeric complex caused by excess of protein hampered compactation of system resulting in a gel less strong with final G´ intermediate. Finally when concentration of protein was increased at ratio 2:1 the higher availability of proteins favored the formation of discontinuous and heterogeneous network with large strands, which may hinder gel hardness and final G´.

The changes in the gel structure at different ratios can be also explained observing the changes in waterbinding properties and pore size, and as we can see the ratios studied affected significantly ($p<0.01$) these properties and pore size. Generally, syneresis increase with the increase degree of aggregation, resulting in a coarse network with large pores; consequently, it results in poorer water-holding capacity comparing with a homogeneous network structure with small pores (BEAULIEU et al., 2001; LANEUVILLE e TURGEON, 2014; ZHANG e VARDHANABHUTI, 2014). Higher Waterloss and pores even as low held-water was associated with high protein in the ratio 2:1. The heterogeneous network with large strands affected directly the held-water which contributed instability gel 48 hours after being formed inside of test-tube (figure 4b). In the ratio 1:1 the presence of two different steps of gelification (figure 2) may have contributed to the 16.67 ± 0.36 waterloss (test-tube figure 4a) and 7.63 ± 0.04 of pore size respectively. We suggest that rearrangements of the network may have results in large dense coarser aggregates susceptible to shrinkage and serum expulsion (VAN VLIET et al., 2004a; BRENNAN e TUDORICA, 2008). Finally, in the ratio with higher polysaccharide content (ratio 1:2) more junction zones were formed inducing the formation of smaller pores and thus low waterloss as we can see in the figure 4c. Recently, (ZHANG et al. (2014a)) suggested that heating of complex WPC-pectin may result in more hydrophilic sites exposed on the surface of protein, further favoring the water binding during gelation. From our results, is not possible suggest that electrostatic interaction between lyso and XG may interfere or modify the structural conformation of biopolymers meantime, additional analyzes are needed to understand the link structure between them and the influences in their structures.

4 **CONCLUSION**

The gelation kinetics and structural evolution of electrostatic Lyso-Xg gels are followed by rheological measurement. The gelation process followed two different stages: induction stage faster linked to gelation process, and quasiequilibrium stage. The formation of the soluble complex was faster converted to interaction between interpolymeric complexes before isoelectric point of lysozyme, nevertheless, the final microstructure was obtained at pH 8.31 where the region of the plateau was formed. It was found that ratio of biopolymers is a factor determinant of gelation and microstructure of gels. The excess of protein affected the compaction of gel, which resulted in gels with dense clusters, with poorer water-bolding capacity, though, in the ratio 2:1 the excess of protein was correlated with heterogeneity of gaps, large pores, high waterloss, and low hardnes. The gel strength mainly depended on the XG content, thence, at the ratio 1:2 the hadness and held-water were elevated and final G´ was almost five times stronger than ratio 1:1.

These experimental findings suggesting that gels Lyso-Xg presenting a great potential to enhance the stability semi-solid foods for the food industry, is possible develop gels enriched with other proteins, and pharmaceutical industry may use to deliver and/or to protect drugs

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CAPÍTULO VII

RHEOLOGICAL AND MICROSTRUCTURAL STUDY OF TWO HYDROGELS INDUCED BY ELECTROSTATIC INTERACTIONS BETWEEN PROTEIN AND POLYSACCHARIDE (PART 2): EFFECT OF PROTEIN AND WEIGHT TOTAL

ABSTRACT

Hybrid hydrogels from biopolymers have been applied for various indications across a wide range of biomedical, pharmaceutical, and functional food industries. In particular, hybrid hydrogels synthesized from two biopolymers have attracted increasing attention. The objective of this study was investigate the influence of lysozyme and β-lactoglobulin on network formation induced by electrostatic attractive interaction. In general, the critical strain enlarged as well as the solid total of the system was increased, and gels became more strength and rigid. Comparing the value of G' and G" between gels produced with lysozyme, and β-Lactoglobulin we noted that Lyso-XG gels are less stronger than BLG-XG, and it occurred because the small size of Blg introduces fewer steric hindrances to polysaccharide and also was possible speculate that self-association of Blg during formation of interpolymer complexes may foment formation of protein clusters, in turn, increased further the zone junctions of the gels. The increased of solid total in the systems also promoted the network become more dense and compact which, decrease the pore size entailed higher hardness, and better waterbinding capacity of gels. The average estimated amount of water retained in lyso-XG and Blg-XG gels at 0.9 wt% achieved 726.5g and 874. 1g water per g biopolymer respectively. These study experimental findings should enable biomedical, pharmaceutical, and food industries to develop new functional semi-solid products and also may be considered as a great model for the interpretation of the behavior of other protein-polysaccharide networks.

1 INTRODUCTION

Hydrogels, are three-dimensional (3D) networks composed of cross-linked hydrophilic polymer chains, can be cast into practically any shape, size, or form and can absorb from 10% to thousands of times of the weight of the dry polymer network (OSADA e KHOKHLOV, 2001). The water holding capacity of the hydrogels arises mainly due to the presence of hydrophilic groups (amido, amino, carboxyl, hydroxyl groups etc.) in polymer chains (SELIKTAR, 2012) or even from hydrophilic amino acids in surface of protein (PACE et al., 2004; ZHANG et al., 2014a).

Hydrogels generally can be classified in two groups depending on cross-linking nature. When cross-linking reaction involves the formation of covalent bonds between polymer chains (LAU e KIICK, 2015), hydrogels are termed as permanent ones, for example, hydrogels formed with hyaluronic acid via Diels−Alder “click” chemistry (NIMMO et al., 2011); However, if hydrogels are formed due to physical interactions (hydrogen bonding, ionic interaction, van der Waals interactions, and molecular entanglement) among the polymeric chains, the hydrogels are termed as physical hydrogels (e.g. β-lactoglobulin - Xanthan gum) (LANEUVILLE et al., 2006).

Hydrogels can also be classified as conventional and stimulus responsive ones (KASHYAP et al., 2005). Conventional hydrogels are the cross-linked polymer chains which absorb water from aqueous mediums without any change in the equilibrium swelling with the change in pH, temperature, electric field or other external stimuli of the environment; while the stimulus responsive (smart or intelligent) hydrogels are polymeric networks, which rapidly change their equilibrium swelling with the change of the

environment such as temperature, pressure, light, electric, magnetic, pH, ionic strength, ions or specific molecular recognition events (BUWALDA et al., 2014). Depending ability the hydrogels them can be useful in applications such as controlled drug delivery (KASHYAP et al., 2005), separation/concentration process (FREDOLINI et al., 2008), biomedical applications (SELIKTAR, 2012), cosmetics (PARENTE et al., 2015), microvalves (LIN et al., 2014), bioreactors (CHAIKASEM et al., 2014), agricultural applications (RUDZINSKI et al., 2002) and, last but not least, in functional foods (LIU et al., 2012).

LANEUVILLE et al. (2006) for the first time find out that interpolymeric electrostatic complexes between β-lactoglobulin (Blg) and xanthan gum (XG) could form clusters and junction zones that resulted in the freeze-in of the whole structure at the point of gelation. Gelation was induced by in-situ acidification, at room temperature, to a pH where both molecules carry net, opposite charges and gel structure is stabilized by electrostatic interaction. They are so-called electrostatic gels or coupled gels due to the involvement of two different molecules to form the junction zones. (LE e TURGEON (2013), 2015)) examined for the first time the role of XG and Blg in network formation induced by electrostatic attractive interaction, as well as, the relationships between structure, texture, and waterbinding properties of hydrogels between Blg-XG under different ratios and total solid concentrations. They found that the decreasing repulsive interaction between XG chains and the increasing attractive interaction between Blg and XG with decreasing pH resulted in the formation of soluble complexes followed by the formation of interpolymer complexes suggesting that Blg aggregated along the XG chains as a crosslinking agent, and more elastic gels were obtained at high XG concentrations. They also found that a decrease in the pore size and the heterogeneity of gaps, by increasing the concentration or reducing the ratio, resulted in an increase in gel hardness, and a decrease in the water loss of gels under the weak centrifugation conditions concluding that these hydrogels an attractive material for biomedical applications, including cell scaffolds, matrices for bioreactor systems and for bio-separation processes.

In previous study, formation of Lysozyme-Xanthan gum hydrogels was studied with the objective to identify role of lysozyme and xanthan gum in gel formation as well as the influence of the ratio of both in gelation mechanism, texture properties, pores, and syneresis of gels (DE SOUZA et al., 2015). The Lyso-XG ratio strongly affects the gelation kinect being the main factor controlling the gelation and structuring of gels. The excess of protein affected the compaction of gel, which resulted in gels with dense clusters, with poorer water-bolding capacity, though, in the ratio 2:1 the excess of protein was correlated with the most discontinuous and heterogeneous network with large strands and porous, high waterloss, and low hardnes. The gel strength mainly depended on the XG content, thence, at the ratio 1:2 the hadness and held-water were elevated and final G´ was almost five times stronger than ratio 1:1. The Lyso-XG gels presenting a great potential application for the food and pharmaceutical industry.

The goal of this study is investigate the influence of lysozyme and β-lactoglobulin on network formation induced by electrostatic attractive interaction. The Mechanical properties, texture, water-holding capacity, and pore size will be studied as a function of protein-polysaccharide hydrogels in three different solid total concentrations.

2 MATERIALS AND METHODS

2.1 Material

Lysozyme (Lyso with purity > 90%, molecular mass of 14.307 Da and isoelectric point (p*I*) 11.35) were obtained from Sigma Chemicals (Oakville, CA). Whey protein isolate (High-Beta, 98.2 wt% protein, 85 wt% of which is βlg, 1.8 wt% minerals, 4 wt% moisture,) was obtained from Davisco Foods International Inc. As mentioned by LE e TURGEON (2013) and LANEUVILLE et al. (2006) the powder was assumed as β-lactoglobulin (βlg) with molecular mass of 18.300 Da, and isoelectric point of 5.1. Xanthan gum (XG) (Keltrol RD, 96.36 wt% total sugar, 3.02 wt% protein) was provided by CP. Kelco Ltd. (Chicago, USA). Glucono-δ-lactone (GDL, USA) and rhodamine B isothyocyanate (lot, USA) were purchased from Sigma (St. Louis, MO, USA) and J.T. Baker (Philipsburg, NJ, USA), respectively.

2.2 Sample preparation

The powders of proteins and xanthan gum were dissolved at 0.3% in deionized water (Modulab Analytical, Fisher Scientific) with continuous stirring at room temperature during overnight, and pH of this solution was previously adjusted to pH 12.0 and 6.60 respectively for lyso and βlg solutions using 0.1 M of HCL or NaOH. For gel formation research, the ratio of Lyso-XG and βlg-XG were fixed of 1:2 and solid total were systematically studied: 0.3, 0.6 and 0.9 wt%. The gelation of the system was induced by addition of GDL on solutions containing Lyso-XG or βlg-XG to reach a final pH of 7.06 ± 0.03 and 4.4 ± 0.08 respectively.

2.3 ζ- Potential measurement

The Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was used to determine ζ-potentials. Lysozyme, β-lactoglobulin and Xanthan gum stock solutions were diluted to 0.01% w/w and transferred to an MPT-2 autotitrator (Malvern Instruments, Worcestershire, UK) that adjusted pH using 0.5 M NaOH, 0.25 M HCl and 0.025 M HCl solutions. pH was varied from 12.0 to 7.0 to lysozyme solution or from 6.6 to 4.4 to β-lactoglobulin by 0.5 unit increments with a confidence interval of ± 0.1 unit. To measure of ζ-potential of gels Lyso:XG and βlg-XG (solid total 0.3, 0.6 and 0.9 wt%) the GDL was added to the solutions and the ζ-potential was measured for 16 hours with interval of 5 minutes between analyzes. Each experiment was performed three times and sample readings were done in triplicate at 25 °C and ζ-potentials were calculated using the Smoluschwsky mathematical model.

2.4 Dynamic Oscillatory Measurements

The Strain sweeps properties of the mixed gels after gelation were determined in dynamic oscillation mode with a controlled-strain rheometer (ARES-G2, TA-Instrument, Piscataway, NJ, USA) equipped with a circulating water bath temperature controller. A Couette device with a cup (30.0 mm diameter) and bob system (27.7.05 mm diameter, 42.0 mm length) was used. Ten seconds after GDL addition, the samples were transferred to the rheometer. After gelation (16 h at 25 C) the dynamic strain sweep from 0.1 to 250%

at a frequency of 0.1 Hz was recorded at a constant temperature of 25 ºC. Samples were covered with a thin three lm of castor oil to prevent evaporation during gelation process.

2.5 Texture Properties

Gel texture parameters were determined by a Texture Analyzer TA XT-2 (Texture Technologies Corporation, NY). The gels formed in the 70 mL-beaker were penetrated with 12-mm diameter cylinder probe and force – time curve was obtained at a crosshead speed of 0.50 mm/s for 20 mm displacement (Le, et al., 2013). The resulting force-time curves were studied using the texture profile analysis (TPA) method in order to determine hardness and deformation at fracture (Turgeon & Beaulieu, 2001). All texture measurements were performed after 24 h of gelation.

2.6 Syneresis

The released water and waterbinding were quantified using Millipore Ultrafree-CL centrifugal filter units with microporous membranes of 0.45 µm pore size (EMD Milipore, Ontario, Canada). The three ratios of gels were formed *in situ* in six filter cups of 2 mL with a pinhole obstructed during the gelation process. These filter cups were placed in a filtrate collection tubes with caps. After 24 h of gelation, the pinhole was freed for 4 h, and then the released water was measured. After this process, the samples were centrifuged at 1000 rpm for 4 min on a fixed 45º angle rotor microcentrifuge (KOCHER e FOEGEDING, 1993) for measure waterbinding. The waterloss and waterbinding were calculated as:

$$Waterloss\ (\%) = \frac{(weight\ of\ water\ released)}{weight\ of\ sample}\ x\ 100 \qquad (1)$$

$$HW = \frac{(Total\ g\ water\ in\ the\ sample - g\ water\ released)}{total\ g\ biopolymers\ in\ sample} \qquad (2)$$

2.7 Confocal Laser scanning microscopy

The structural features of the gels were investigated using a Nikon Eclipse TE2000-E Confocal Laser Scanning Microscope (Tokyo, Japan) equipped with an inverted microscope. The objective lens used was 60X WI, NA 1.20. The excitation wavelength was 543 nm, with an emission maximum at 605/675 nm. The solutions with lysozyme were stained by adding rhodamine B isothyocyanate (RITC) to protein dispersions under magnetic stirring for 1 h before mixing with XG dispersions. XG was stained by fluoresceinamine (FA). The FA-XG prepared according to the method of Donato et al., (DONATO et al., 2005) was provided by Laneuville and was used in mixture with RITC-Lyso in order to determine the location of Lyso and XG in the gel. Mixtures of RITC-Lyso/FA-XG were prepared as described above in three different ratios. After GDL addition, the sample was poured between a 0.5-mm-deep well concavity slide and a cover slip, which was hermetically sealed with nail enamel. Micrographs were taken 24 h after GDL addition. Digital images were acquired at a pixel resolution of 1024 x 1024.

The average pore size (µm) was estimated by image analysis using ImageJ (v1.49r; http://rsb.info.nih.gov/ij/) following the procedure detailed by (LE

e TURGEON, 2012). For pore size measurement, the “watershed” command was performed on 8-bit binary images in order to separate the pores. The mean pore size (µm) was estimated from the average area of the pores which was calculated from the count of pores and the total area corresponding to pore.

2.8 Statistical analisys

The data were subjected to one-way analysis of variance and correlations between the results were carried out using Statistical Analysis System Software (SAS version 9.0, SAS Institute, Cary, NC); significant differences were determined by Duncan's multiple range test and accepted at $P < 0.05$

3 RESULTS AND DISCUSSION

3.1 Gel rheology

3.1.1 Strain sweep

Stress sweep test of Lyso-XG and BLG-XG gels is shown in the Figure 1. During the stress test with increasing strain, two different regions namely linear viscoelastic region where G´ and G´´ were almost constant and nonlinear region in which G' and G" started to decrease were distinguished. The storage modulus, G", is indicative of the energy conserved through elasticity during a cycle of deformation. The loss modulus, G", is indicative of the energy dissipated as heat during a cycle of deformation. Both G' and G" shown in Figure 1 are relatively independent of the applied strain at low strains, which indicates the materials are in the linear viscoelastic (LVE) regime. The strain at which G' decreased sharply is defined as the critical strain value (γ_c) (SHIH et al., 1990).

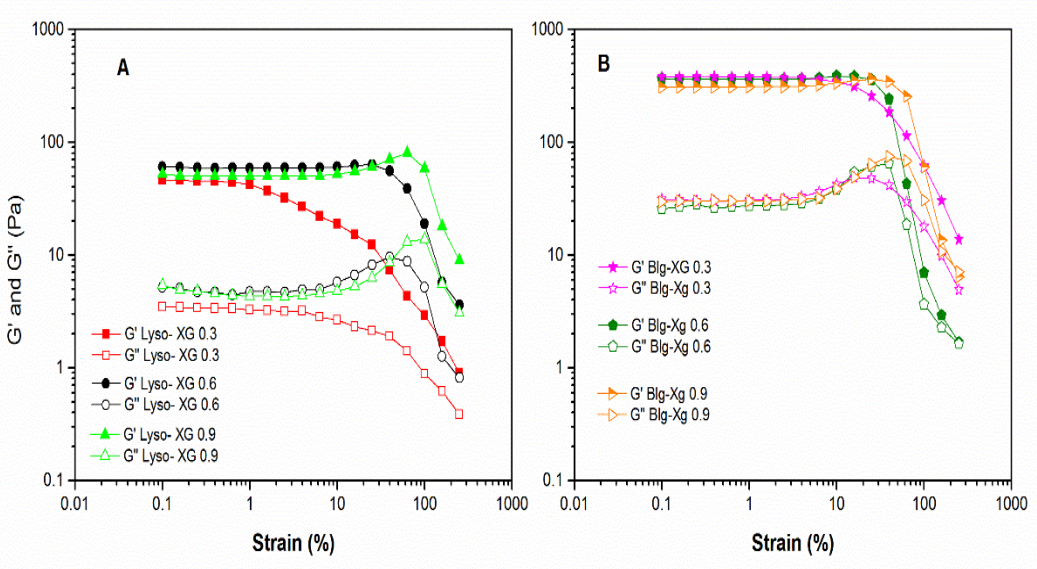

Figure 1: Strain sweep of Lyso-XG (A) and BLg –XG (B) gels after 16 hours formed on ratio 1-2 as function of total solid concentration (0.3, 0.6 and 0.9 wt %)

In general, is the possible note that the storage modulus was greater than the loss modulus at all concentration in LVE and that region as well as critical strain was directly dependent of weight total to both gels studied. In the figure 1A, is the feasible note that at the weigh total of 0.3% the LVE is the lower among variation studied with γ_c 1.23 ± 0.05 % nonetheless, when concentration was enlarged to 0.6 and 0.9% the γ_c increased to 39.85 ± 0.13 and 86.61 ± 0.41 respectively. Similar behavior has been observed with gels formed with Blg. However, different of Lyso-XG gels the LVE was almost the same for both concentrations. At the concentrations 0.3, 0.6 and 0.9 wt % the γ_c increased from 15.78 ± 0.36, 62.65 ± 0.26 and 89.41± 0.26 respectively. This significant increase of the linear viscoelastic region indicates that increasing of weight total of the system increased the strength of the system and got more rigid (STEFFE, 1996). As far as concentration of the system increase the zone junctions enlarge in number and orientation with the clusters, for this reason, the zone junctions became less rigid than clusters, thus increasing the LVE (SHIH et al., 1990; ROMER et al., 2014).

All the above-mentioned results indicate that weight total of the system is influential in the formation and maintenance of elastic behavior of Lyso-XG and Blg-XG gels nonetheless; it is noteworthy that storage modulus and loss modulus did not increase significantly with an increase weight total of the system. It is also interesting to note that for all samples in figure 1 except Lyso-XG with 0.3 wt % the G" has a maximum value at the onset where G' begins to decline. Yielding of colloidal gels is known to occur first by localized gel rupture followed by the eventual breakup of the coarse gel fragments into finely dispersed particles or smaller clusters (VERMANT e SOLOMON, 2005; MASSCHAELE et al., 2009; GIBAUD et al., 2010; RAJARAM e MOHRAZ, 2010). We believe that this phenomenon is caused by viscous properties being enhanced as far as the dense zone junctions were ruptured. We speculate viscous properties were enhanced by the formation of dense flocs rich in clusters with gradual strain elevation. Similar behavior has been observed and elucidated in other physical gels (SIM et al., 2003; HYUN et al., 2011; SHAO et al., 2013; ZHANG et al., 2014b).

3.1.2 Influence of chemical structure

When we compare the value of G' and G" in LVE between gels produced with lysozyme, and β-Lactoglobulin is suitable to note that Lyso-XG gels are less strong than BLG-XG. In the table 1, we observed that chemical structural of protein present significant differences (e.g., charged and hydrophobic amino acids), and we believe that this lack foment to the different structure of the gels. Comparing the size and length of molecules is achievable to note although Blg is greater than Lyso the size of Blg is three-quarter of the Lyso. We believe that small size of Blg introduces fewer steric hindrances to polysaccharide chains to bind more (LI e HUANG, 2013) and as a consequence, the Blg-XG gels introduced better distribution of protein molecules along the chains that formed the junction zones and clusters of the gel. GIRARD et al. (2003a) reported that two XG molecules may share the same Blg molecule to support electrostatic interactions. Grounded on this phenomenon we suggest that fewer steric hindrances to polysaccharide chains to bind with Blg provide formation of network more dense and stronger than Lyso-XG gels, which corroborate with results presented in the figure 1. Comparing the kind of patches in the protein surfaces is suitable realize that lysozyme and Blg shows distribution heterogeneous of patches meantime, Blg has an amount even greater of positive and hydrophobic patches. XU et al. (2011) investigate the effect of polyelectrolyte binding affinity on selective coacervation of proteins (BSA, Blg-A and Blg-B) with the cationic polyelectrolyte, poly(diallyldimethylammonium chloride) (PDADMAC). The authors

found that Blg had a stronger affinity than BSA to interact with polycation, and form complex coacervate. They concluded that the difference comes from the anisotropic distribution of surface in these proteins. We believe that similar stronger affinity may have occurred with Blg comparing with Lyso. The greater positive patches of Blg associated with final pH of gel formation (4.4 ± 0.08) provided the higher positive charger comparing with Lyso which in turn, bring a better distribution of positive charges on protein surface, thereby Blg-Xg gels may be stronger than Lyso-XG (FOGOLARI et al., 2000). When we compare the number of positive patches of the Lyso is suitable imply that during acidification of systems from pH 12.0 until 7.0 some of the main positive amino acids were above or exactly at pKa (e.g., arginine and lysine, 12.0 and 10.5 respectively) which reduced the positive charge of the system thus affecting the affinity of protein. It must be emphasized that amino acids, arginine and lysine may be found along structure of protein eleven and six times respectively and together, they represent about 63% of positive amino acids. Still talking about the differences of the viscoelastic storage and loss storage between Lyso-XG and Blg-XG is also possible speculate that self-association of Blg during formation of interpolymer complexes may promote the formation of protein clusters, in turn, increased further the zone junctions of the gels. LI e HUANG (2013) studied the complex coacervation of pectin with BSA and two isomers of beta-lactoglobulin (Blg-A and Blg-B). The authors could note that Blg-A and Blg-B displayed 4 and 7 bound proteins respectively along polysaccharide chains. The enrichment may be contributed from two aspects. One is the size of BLG, which is one-eighth of that of BSA which, as was explained above, allow polysaccharide chains to bind more molecules of protein. The second contribution may be that the strong self-association of Blg, influenced by high hydrophobic patches, which makes the proteins cluster. Pectin chains binding to one protein also tends to bind others in the same cluster.

Table 1: Parameters of the protein crystal structures[a]

Protein	PDB[b]	Length	Size (nm)	Positive	Negative	Hydrophobic
Lysozyme	1lyz	129	2	27	45	57
Beta-lactoglobulin	2q2m	162	1.5	46	38	78

[a]Positive, negative, and hydrophobic provide the number of residues in proteins
[b]Protein Data Bank

In aim to identify, the density of charge of individual biopolymers and three total solid concentration of gel formed the ζ-potential as function of pH was conducted and results are present at figure 2. When we observe XG notices that polysaccharide charge patches remained considerably negative during all pH range studied, however, behavior different was observed for both proteins studied. In the figure 2a, lyso has a change in the electric charge, ranging from a negative charge at pH 11.9 to a positive charge at pH 6.9 with a zero load point (p*I*) near 11.3 (STADELMAN e COTTERILL, 1995b). Similar behavior the transition of the charges was noted in the figure 2b with Blg in the pH range studied meanwhile, at pH 5.0 the net charge on Blg was close to zero indicating the p*I* of the protein (FOGOLARI et al., 2000). It can be observed that the values found for the mixture containing both biopolymers are the values of the isolated polymer intermediate. This behavior indicates the interaction between the carboxyl groups of the polysaccharide and protein amine groups, featuring electrostatic binding. When we compare the influence of the total solid in gels Lyso-XG is achievable to note that at 0.6 wt% the system was the closer to the electrical charger equivalence followed by the systems with 0.3 and 0.9 wt % nonetheless, the gels made with Blg showed that variance of the total solids did not affect considerably the neutralization of the negative charge of

polysaccharide. LE e TURGEON (2013) studied the stoichiometric equivalence of gels (Blg-XG) at different ratios in fixed polysaccharide concentration of 0.03 wt% at pH 4.4, and they could note that at ratio 2, the negative charge of XG dominated the charge of the Blg; However, at the ratio 3.5, the maximum of electrical charge equivalence was reached. We also believe that behavior showed at the figure 2a correspond the excess of negative charge of the XG.

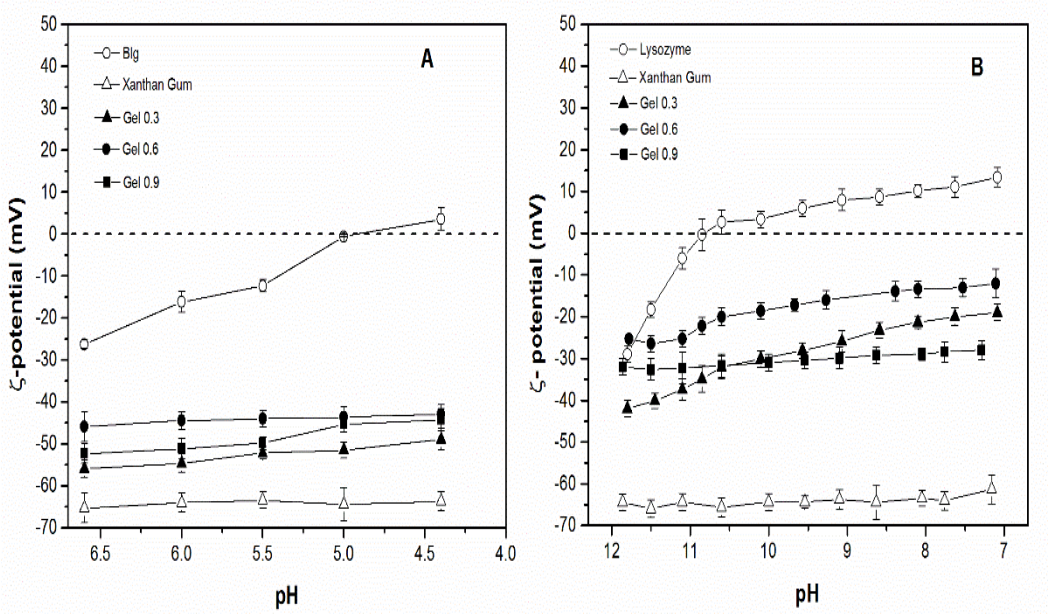

Figure 2: Zeta potential for individual biopolymers, Lyso-XG and BLg-XG gels as function of total solid concentration (0.3, 0.6 and 0.9 wt %) on the fixed ratio of 1-2 as a function of pH (mean ± SD, n = 3).

3.2 Structure of gel

Figure 3 presents the final CLSM images of gels at different total solid concentrations. Le, et al. (2013) LE e TURGEON (2013) in a previous study showed that proteins are aggregated on XG chains through electrostatic interactions. Therefore, red zones correspond to zones enriched in proteins and dark zones correspond to zones, which may contain little free proteins. In the figure 3a, is possible see that Blg-BG gel presented a homogeneous structure. However, when the total solid concentration is enhanced in the figure 3b, and c is a suitable note that network become more dense and compact, and it reflects directly in the pore size of gels. In the figure 3e, we see that pore size of Blg-XG at 0.3 wt% correspond 3.77 ± 0.25 µm. Nonetheless, increasing total solid concentration to 0.6 and 0.9 wt% the pore size reduced to 2.87 ± 0.10 and 2.36 ± 0.13 µm respectively. The formation of interconnection points in the network was favored with the increased of total solid concentration, which caused less space between these molecules. Therefore, smaller pores were formed, and stronger gels were obtained; which coincided with the rheological results discussed above (Figure 1b). When we compare the structure between Blg-XG and Lyso-XG (Figure 1a and 1d respectively) is suitable to note that Lyso-XG network is coarser than Blg-XG exhibiting some large pores isolated, which contributed to the large pore's population (Figure 1e) comparing with Blg-XG. The structure presented in the figure 1d coincided with the rheological results discussed above (Figure 1a) nonetheless; we were not able to us discuss the change in structure of the gels based in CLSM images as the total solid concentration was increased, considering, that was

impossible collects the samples still in the fluid state. In our previous study (DE SOUZA et al., 2015) we showed that gelation point of Lyso-XG gels at ratio 1-2 in 0.3 wt% occurred at pH 11.47 ± 0.2 meantime, believed that as far as the total solid concentration was increased the gelation point decreased considerably, which prevented the preparation of samples for the CLSM images.

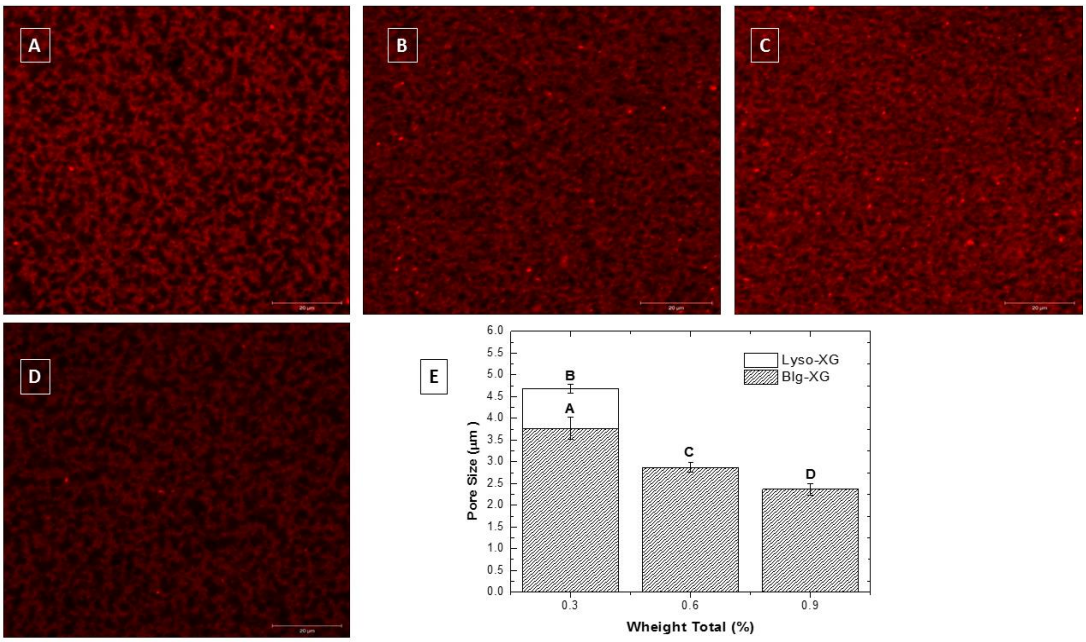

Figure 3: Confocal micrograph of RITC-Blg-XG gels obtained at total solid concentration of 0.3 % (A), 0.6 % (B), 0.9% (C), RITC-Lyso-XG at total solid concentration of 0.3 % (D) and pore size (E).. The different letters in each bars indicate

3.3 Textural and waterbinding properties of gels

3.3.1 Textural property

The deformability of gels as well as the appearance of the gels 24 hours after textural analysis is presented in the figure 4. In general, we can note that the hardness of gels is directly dependent of total solid concentration. In the concentration of 0.3 wt% is possible to see in microscopic view that gels have a translucent aspect indicating that networks were not compacted. However, lyso gels were weaker than Blg gels presetting hadness of 0.22 ± 0.012 comparing with 0.43± 0.017 of Blg gels. We believe that this difference corresponds to the ability of Blg molecules to induce fewer steric hindrances to bind polysaccharide chains (LI e HUANG, 2013) which affected directly the number of the effective strands in the gel and the modulus of the protein strands and polysaccharide (VAN VLIET et al., 2004b). As far as the solid total of the systems were increased to 0.6 and 0.9 wt%, we observed that gels became more turbid and firm, indicating formation of a dense coupled network induced mainly by the stronger electrostatic interactions between protein and XG (LE e TURGEON, 2015) which, coincided with the rheological measurements (Figure 1) and image analysis (Figure 3a, b and c). Nevertheless, increase in the concentration of total solids did not increase the difference of hardness between Lyso and Blg gels, on the contrast; a slight difference of hardness was noticed, which indicates that although Blg-XG has the larger storage modulus compared with Lyso-XG the final structure (e.g., pore) and texture between gels were not greatly different.

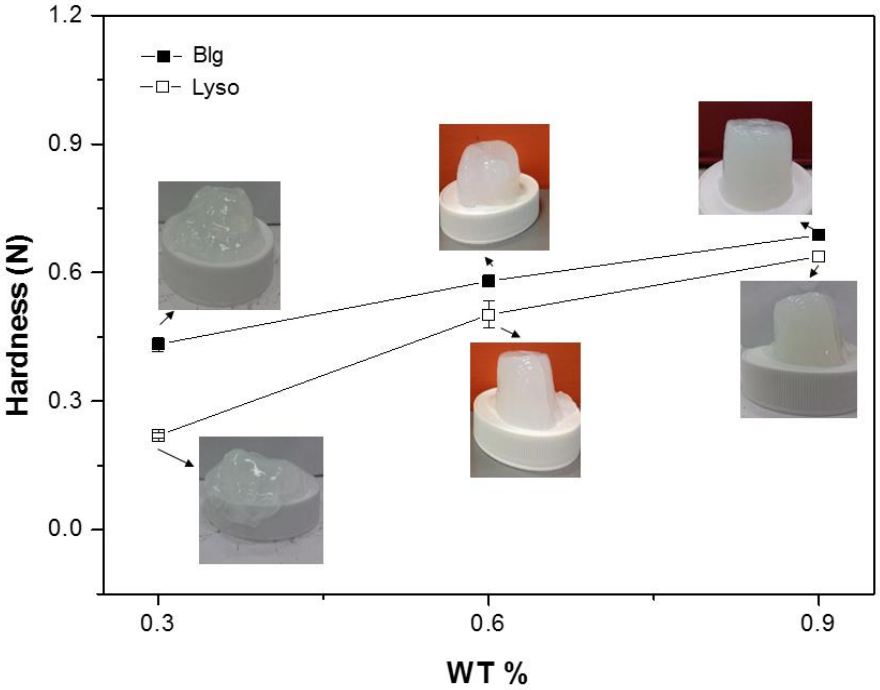

Figure 4: Hardness of Lyso-XG (A) and BLg –XG (B) gels on ratio 1-2 as function of total solid concentration

3.3.2 Waterbinding property

The waterbinding property of gels was directly affected by protein and solid total of systems as we can see in figure 5a. In the systems with 0.3 wt% Lyso gels showed higher waterloss compared with Blg gels indicating that presence of some larger pores of Lyso-XG gels decreased the water-holding capacity. However, is necessary emphasize that both gels produced with Lyso and Blg showed lowest water loss when compared with Blg-XG gels formed at ratio 2-1 in the system with 0.3 wt% (LE e TURGEON, 2015) or even with rennet-induced skim milk gels formed xanthan gum at different concentration (0.025, 0.050, 0.075 and 0.100 wt%). In the systems with 0.6 wt% is suitable see that waterloss decreased significantly for both gels formed Lyso and Blg, influenced majorly by dense network developed during the gelation process. Nonetheless, in the systems with 0.9 wt% is the waterloss values are close to zero indicating that Lyso-Xg and Blg-XG could form gels with smallest pores that hindered almost completely the release of water.

The waterbinding of gels was estimated by the amount of water (gram) held by 1 g biopolymer and results are presented in figure 5b. As expected as far as the solid total of systems was raised the held-water increased exponentially seeing that, more molecules of biopolymer could be held to water, notwithstanding when we compare the gels formed between lyso and Blg is achievable to note that Blg-XG present high held-water even in the systems with 0.3 wt%. Whey proteins are extremely hydrophobic molecules since they are formed by polar amino acids (Table1) (PICONE e CUNHA, 2010); However, as discussed above the self-association of Blg (LI e HUANG, 2013) molecules during formation of interpolymer complexes may foment more hydrophilic sites exposed on the

surface of protein, which explain the higher favoring the water binding during gelation (ZHANG et al., 2014a).

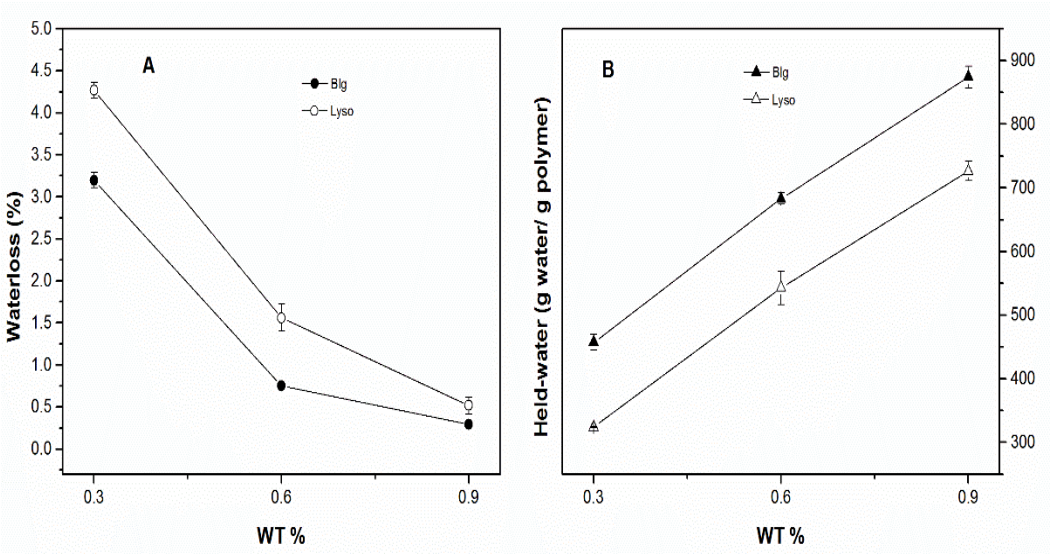

Figure 5: Waterloss (A) and Held-Water (B) of Lyso-XG and BLg –XG gels on ratio 1-2 as function of total solid concentration (mean ± SD, n = 3).

4 CONCLUSIONS

Relationships between aspects rheological, microstructure, textural, and waterbinding properties among Blg-XG and Lyso-XG hydrogels were investigated at different solid total concentrations. In general, the critical strain enlarged as well as the solid total of the system was increased, and gels became more strength and rigid. That indicates that variation impact the formation and maintenance of elastic behavior of gels. The results reveal that the size and chemical structure of Blg contributed to fewer steric hindrances to polysaccharide followed by formation of proteins clusters, which, in turn, increased the zone junctions of the gels. The increased of solid total in the systems also promoted the network become more dense and compact which, decrease the pore size entailed higher hardness, and better waterbinding capacity of gels. The average estimated amount of water retained in lyso-XG and Blg-XG gels at 0.9 wt% achieved 726.5g and 874. 1g water per g biopolymer respectively and as the consequence, the waterloss was close to zero after the centrifugation of gels. These study experimental elucidated fundamental aspects about texture formation of Lyso-XG and Blg-XG hydrogels and we suggest that those results can be used by industry biomedical, pharmaceutical, and food industries to develop new functional semi-solid products with high water and low protein. In case of Lyso-XG gels, the presence of lysozyme in the system makes use of these gels, especially interesting for biomedical and pharmaceutical industry, bearing in mind, their functional properties such as antiviral and bacteriolytic activity. The investigated system of Blg-XG and Lyso-XG may be considered as a great model for the interpretation of the behavior of other protein-polysaccharide networks.

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CONCLUSÕES GERAIS

A interação entre ovoalbumina e pectina foi diretamente afetada pela concentração de proteína e de NaCl. Concentrações elevadas de proteínas e de NaCl promoveram a formação de agregados de proteína o que afetou diretamente na cinética de formação dos complexos assim como na dissociação da interação em pHs muito ácidos. Influencia semelhante da concentração de NaCl foi observada nossos complexos formados entre Lisozima e pectina, porém o excesso de NaCl suprimiu por completo a formação dos complexos na concentração de 0.4 mol/L. As partículas dos complexos Lyso / pectina aqui descritas representam possuem um potencial de aplicação nas indústrias de biotecnologia, farmacêutica e de alimentos

Os complexos formados entre Ova/ Ca e Lyso/ Ca foram diretamente dependentes da concentração das proteínas e NaCl. Podemos perceber que a concentração de ovoalbumina foi entorno de 3:1 enquanto que para lisozima as razões de proteína entre 3:1 e 5:1 não apresentaram diferença significativa. A faixa de pH de formação dos complexos Lyso/Ca demonstrou uma potencial gama de aplicação. Os resultados reológicos associados aos resultados de microscopia demonstraram que complexos inter-poliméricos podem ser formados pela interação entre estes dois biopolímeros.

Os complexos inter-poliméricos formados entre Ova/XG e Lyso/XG apresentaram comportamento semelhante aos formados com Carragena, porém a concentração de NaCl impactou, mais diretamente na formação e estruturação dos mesmos. Todavia, os complexos inter-poliméricos formados entre Lyso/XG apresentaram valores de G´ muito maiores, quando comparados com os outros complexos inter-poliméricos estudados neste tese. O que sugeriu a propriedade funcional de formação de Hidrogéis.

A formação de hidrogeis através da interação eletrostática entre lisozima e goma xantana foi diretamente dependente da concentração de proteína, haja visto que a razão de 2:1 formou uma estrutura mais fraca e hetorogênica quando comparada a razão 1:2. Quando foi estudado a influência da proteína (Lisozima ou β-lactoglobunina) na formação e estruturação dos hidrogéis, pode-se perceber que o tamanho da molécula assim como a distribuição dos aminoácidos da β-lactoglobunina afetou diretamente na estruturação e formação das zonas de junção dos géis. Contudo, os géis de Lyso/XG e BLG/XG formados à 0.9% de massa total no sistema apresentaram textura, capacidade de retenção e água e diâmetro de poro únicos no tange a possiblidade de utilização dos mesmo na pela indústria.