PRISCILA CARDOSO FIDELIS

EMULSÕES SIMPLES E MULTICAMADAS ESTABILIZADAS POR LISOZIMA E β-LACTOGLOBULINA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 30 de outubro de 2017.

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RESUMO

FIDELIS, Priscila Cardoso, D.Sc., Universidade Federal de Viçosa, outubro de 2017. **Emulsões simples e multicamadas estabilizadas por lisozima e β-lactoglobulina.** Orientador: Luis Antonio Minim. Coorientadoras: Ana Clarissa dos Santos Pires e Valéria Paula Rodrigues Minim.

β-lactoglobulina (BLG) é uma proteína presente no soro de leite, com propriedades emulsificantes, nutricionais e de transporte, amplamente utilizada como ingrediente. Esta proteína tem um ponto isoelétrico (PI) baixo, o que permite a sua interação eletrostática com proteínas de alto PI, como a lisozima (LIS), uma proteína da clara de ovo. Essas proteínas são usadas como emulsificantes, de forma a aumentar a estabilidade cinética de emulsões. A estabilidade das emulsões é afetada pela composição das fases contínua e dispersa. Emulsões óleo em água (O/A) de misturas de LIS e BLG foram avaliadas quanto ao efeito do pH e da adição de sal nas suas propriedades emulsificantes (tamanho, potencial ζ, floculação, concentração e composição interfacial proteica) e estabilidade cinética. Posteriormente, emulsões multicamadas formadas por uma emulsão primária de BLG ou LIS, seguida da adição de uma segunda camada de BLG, LIS ou pectina, com carga inversa, foram obtidas e avaliadas quanto a suas propriedades de emulsificação (floculação, diâmetro hidrodinâmico médio e potencial ζ) e estabilidade cinética. Emulsões formadas pelas proteínas puras apresentaram diâmetros menores. A BLG apresentou uma adsorção preferencial na interface em relação a LIS. O pH influenciou principalmente a carga superficial de emulsões, o que afetou a interação entre proteínas, o tamanho final e a estabilidade de emulsões. O aumento da força iônica influenciou negativamente a estabilidade das emulsões formadas pela mistura das proteínas, levando a um aumento dos tamanhos das gotículas, com posterior separação macroscópica de fases. As emulsões estabilizadas por múltiplas camadas, contendo pectina, apresentaram menores diâmetros e maior potencial ζ, em comparação com emulsões formadas apenas por proteínas. A adição de pectina, um polímero carregado e estabilizante, favoreceu a resistência de emulsões multicamadas ao armazenamento e à adição de NaCl. As emulsões podem ser usadas para dispersar compostos lipofílicos, como o β-caroteno (BC), que pode ainda interagir com as proteínas da interface, aumentando a capacidade de carreamento da emulsão. A interação de BLG e LIS com BC foi determinada por meio de espectroscopia de fluorescência, de forma a explorar a possibilidade de uso dessas

proteínas em sistemas para a incorporação desse composto. A fluorescência da LIS e BLG foi suprimida pela presença do BC por um processo de supressão estático. As constantes de velocidade de supressão e as constantes de interação calculadas indicaram o mecanismo de supressão estática e a força de ligação média. A capacidade de ligação do BC a BLG diminui significativamente ($p<0,05$) com a desnaturação, indicando uma inativação parcial dos sítios de ligação. Esses resultados são importantes para embasar o uso dessas proteínas para incorporação do BC em alimentos, o que pode ser alcançado utilizando emulsões simples ou multicamadas de até duas camadas, utilizando pectina.

ABSTRACT

FIDELIS, Priscila Cardoso, D.Sc., Universidade Federal de Viçosa, October, 2017. **Simple and multilayers emulsion stabilized by lysozyme and β-lactoglobulin.** Adviser: Luis Antonio Minim. Co-advisers: Ana Clarissa dos Santos Pires and Valéria Paula Rodrigues Minim.

β-lactoglobulin (BLG) is a protein present in whey, with emulsifying, nutritional and transport properties, widely used as an ingredient. It has a low isoelectric point (IEP), which allows its electrostatic interaction with high IEP proteins, such as lysozyme (LYS), an egg white protein with high IEP. These proteins can be used as emulsifiers in order to increase the kinetic stability of emulsions. The stability of the emulsions is affected by continuous and dispersed phase composition. Oil-in-water (O/W) emulsions of LYS and BLG mixtures were evaluated for the effect of pH and salt addition on their emulsifying properties (oil droplet size and potencial ζ, flocculation, protein concentration and interfacial composition) and kinetic stability. Subsequently, multilayer emulsions formed by a primary emulsion of BLG or LYS, followed by the addition of a second layer of BLG, LYS or pectin, with reverse charge, were obtained and also evaluated for their emulsification properties and kinetic stability. Emulsions formed by pure proteins showed smaller diameters. BLG showed a preferential adsorption at the interface comparing to LYS. The pH mainly influenced the surface charge of emulsions, which affected the interaction between proteins, final size and stability of emulsions. The increase in ionic strength negatively influenced the stability of the emulsions formed by the mixture of proteins, leading to an increase in droplet size, with subsequent macroscopic phase separation. The multilayer stabilized emulsions containing pectin had smaller diameters and higher potential ζ, compared to multilayer emulsions formed only by proteins. The addition of pectin, a charged polymer and stabilizer, favored the resistance of multilayer emulsions to the storage and addition of NaCl. Emulsions, composed of two slightly soluble phases, can be used to disperse lipophilic compounds, such as β-carotene (BC), which may further interact with interface proteins, increasing the emulsion load capacity. The interaction of BLG and LYS with BC was determined by fluorescence spectroscopy in order to explore the possibility of using emulsified systems containing those proteins in the incorporation of this compound. The quenching rate constants and binding constants calculated indicated the static quenching mechanism and medium binding

force. The binding ability of the BC to both proteins decreased significantly ($p<0.05$) with denaturation, indicating partial inactivation of the binding sites. Those results are important to support the use of proteins for incorporation of BC in foods, which can be used using single or multilayer emulsions up to two layers, using pectin.

INTRODUÇÃO GERAL

A busca por novos produtos, com compostos mais saudáveis, tem aumentado o desafio na formulação de alimentos, nos quais a substituição de ingredientes deve ser realizada sem afetar a estabilidade e a aceitação do produto. A redução da gordura de alimentos diminui a solubilidade e disponibilidade de compostos lipofílicos e também altera a textura final do produto. O uso de emulsões é desejável, nesse cenário, de forma a auxiliar na incorporação de compostos e melhoria da textura.

Emulsões são utilizadas como meio excipiente de compostos bioativos lipofílicos a fim de carrear, proteger e liberar esses componentes, dentre os quais podem-se citar: ácidos graxos, vitaminas lipofílicas, carotenoides e fitoesteróis. Esses sistemas de encapsulamento podem utilizar emulsificantes e estabilizantes sintéticos, no entanto, o uso de compostos naturais como proteínas e gomas extraídas de fontes naturais tem sido amplamente estudado como alternativa com maior apelo comercial.

Emulsões são dispersões de dois líquidos de baixa miscibilidade, nas quais um dos líquidos está disperso como pequenas gotículas esféricas no outro. Para manter as gotículas de fase dispersa uniformemente distribuídas no seio da mistura durante processamento, armazenagem e consumo, são usados tensoativos, que reduzem a tensão interfacial. O estudo do tipo e do comportamento desses tensoativos é necessário de forma a melhorar a estabilidade cinética das emulsões, que por sua vez depende do tipo e concentração de ingredientes contidos na emulsão, assim como as condições de processamento e armazenamento.

As proteínas do soro de leite, assim como as da clara do ovo, são frequentemente usadas como tensoativos naturais para formulações obtidas por meio de emulsões.

A β-lactoglobulina (BLG) é a principal proteína do soro do leite, com massa molar de 18,3 $kg.mol^{-1}$ e ponto isoelétrico em pH≈5,2. Esta proteína apresenta propriedades interfaciais interessantes e amplamente estudadas. A lisozima (LIS) é uma proteína extraída da clara de ovo com massa molecular em torno de 14 $kg.mol^{-1}$ e ponto isoelétrico em pH≈10,7. Dada sua natureza básica, afeta as propriedades de outras proteínas, interagindo com elas.

O uso de mistura de surfactantes, tanto em camadas simples ou multicamadas proporciona uma amplitude de opções e pode melhorar a estabilidade das emulsões

geradas contornando limitações de compostos individuais. O fato de as duas proteínas apresentarem cargas opostas em pH neutro possibilita ainda a interação entre elas, o que pode aumentar a concentração proteica na interface. A adição de um polissacarídeo carregado às emulsões pode aumentar a resistência a alterações de pH, força iônica e temperaturas elevadas de emulsões estabilizadas por proteínas. A pectina é um polissacarídeo solúvel em água e apresenta carga negativa em pH neutro.

Alguns trabalhos avaliaram as propriedades interfaciais, emulsificantes e de formação de espumas de misturas de β-lactoglobulina ou lisozima com diferentes proteínas ou hidrocolóides. No entanto, até o presente momento pouco se conhece sobre as capacidades emulsificante e estabilizante de misturas de lisozima e β-lactoglobulina em diferentes condições de pH e força iônica, nem o efeito da adição de pectina a essas emulsões. O β-caroteno é um pigmento hidrofóbico e a principal fonte de vitamina A na dieta humana. Vários modelos de carreados tem sido propostos para melhorar sua estabilidade e dispersibilidade em água.

O objetivo principal desse trabalho foi obter emulsões cineticamente estáveis, que possam ser utilizadas para incorporação de compostos. Para tal, avaliou-se a formação e estabilidade de emulsões O/A estabilizadas por misturas de lisozima e β-lactoglobulina, em diferentes condições de pH e concentrações de NaCl; avaliou-se a formação e estabilidade de emulsões multicamadas de lisozima, β-lactoglobulina e pectina, determinando as concentrações de saturação na interface; e estudou-se a interação dessas proteínas com um composto lipofílico modelo, o β-caroteno.

Este trabalho é apresentado em quatro capítulos. O Capítulo 1 consiste em uma revisão bibliográfica geral sobre emulsões e sua caracterização, aplicações e obtenção de emulsões multicamadas, propriedades gerais dos emulsificantes utilizados (lisozima e β-lactoglobulina) e de pectina e β-caroteno, interações entre proteínas, hidrocolóides e compostos hidrofóbicos/caroteno e supressão da fluorescência. Na sequência são abordados, na forma de artigos, os seguintes temas: Capítulo 2: Effect of pH and NaCl addition on emulsifying properties of Lysozyme and β-lactoglobulin mixtures, Capítulo 3: Emulsifying properties of Lysozyme and β-lactoglobulin multilayers formation with pectin e Capítulo 4: Binding of β-carotene to β-lactoglobulin and Lysozyme - a spectroscopic study.

CAPÍTULO 1 - REVISÃO BIBLIOGRÁFICA

1. Emulsões

Na área de alimentos, tem-se buscado o desenvolvimento de sistemas de proteção que possam carrear, proteger e liberar componentes bioativos lipofílicos, como ácidos graxos, vitaminas lipofílicas, carotenoides e fitoesteróis. Diferentes sistemas de encapsulamento de nutrientes destinados à aplicação em alimentos têm sido desenvolvidos empregando proteínas, como as do soro de leite (CHEN et al., 2006; EGAN et al., 2013; WANG et al., 2016; CHEN et al., 2017) da soja e do trigo (CHEN & SUBIRADE, 2009), caseína (LESMES et al., 2010; ZIMET et al., 2011); ou polissacarídeos, como alginato, carragena, pectina e quitosana (GUZEY & MCCLEMENTS, 2006; BENGOECHEA et al., 2011; BENJAMIN et al., 2012; WEI & GAO, 2016). No entanto, há vários desafios para o desenvolvimento e produção de sistemas de liberação de grau alimentício, tais como evitar a toxicidade do produto, assegurar a estabilização química dos ingredientes bioativos, as características sensoriais, digestibilidade e liberação de bioativos (TOKLE et al., 2010).

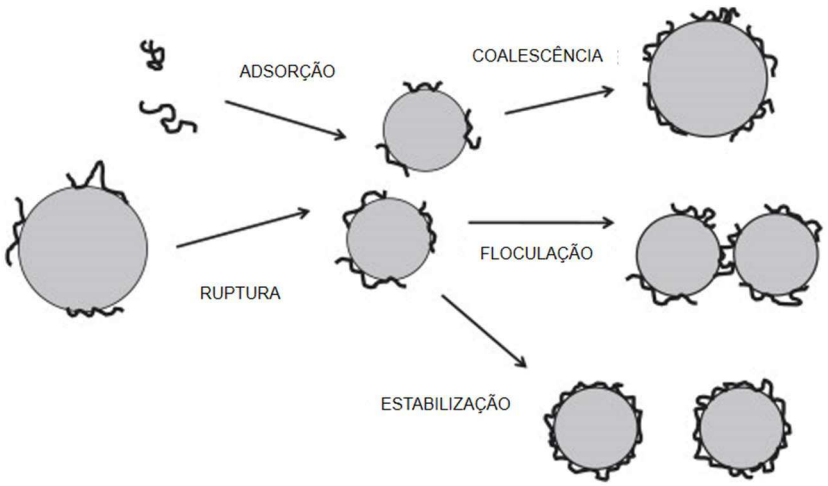
Essas demandas podem ser atendidas pelo uso de sistemas emulsionados, que podem (i) dissolver e dispersar o composto lipofílico, (ii) protegê-lo no interior da fase dispersa da emulsão e (iii) liberar o composto por meio da destruição/desestabilização da interface da emulsão (SHCHUKINA & SHCHUKIN, 2012; WANG et al., 2016). O uso de proteínas de ampla ocorrência, de fácil obtenção e de inocuidade garantida é incentivado.

Emulsões são dispersões de dois líquidos parcialmente imiscíveis entre si, nas quais um dos líquidos está disperso como pequenas gotículas esféricas no outro (WALSTRA, 2003). A adição de moléculas anfipáticas, ou tensoativos reduz significativamente a tensão interfacial, gerada pela assimetria das interações intermoleculares de moléculas das duas fases (MASON et al., 2006). Quando a fase dispersa é composta de solução aquosa e a fase contínua consiste em uma solução oleosa a emulsão é chamada água em óleo (A/O), o inverso corresponde a uma emulsão óleo em água (O/A).

Emulsificantes são moléculas tensoativas que adsorvem à superfície das gotículas recém-formadas. Após a adsorção, os emulsificantes podem atuar diminuindo a tensão interfacial e formando uma camada protetora que retardará agregação posterior das gotículas (WALSTRA, 2003; MCCLEMENTS et al., 2007).

Os emulsificantes tipicamente estabilizam as gotículas contra a agregação gerando interações repulsivas estéricas e/ou eletrostáticas. Os emulsificantes que formam interfaces abertas relativamente espessas (tais como polissacarídeos e tensoativos não iônicos com grupos hidrofílicos de cabeça grande) podem gerar uma repulsão estérica suficientemente forte e de longo alcance para superar as forças atrativas de van der Waals e assim estabilizar o sistema contra agregação. Os emulsificantes que formam interfaces altamente carregadas (tais como proteínas e surfactantes iônicos) podem gerar uma forte repulsão eletrostática entre gotículas que impedem sua agregação (DICKINSON, 2009). No entanto, emulsificantes que só podem estabilizar emulsões devido a interações eletrostáticas estão susceptíveis a instabilidades quando o pH ou a força iônica é alterada.

Um emulsificante ideal deve reduzir rapidamente a tensão interfacial na interface recém-formada de óleo-água, ligar-se fortemente à interface, uma vez adsorvido, e proteger as gotículas recém formadas contra floculação ou coalescência (Figura 1.1) (DICKINSON, 2009). Esta proteção contra coalescência ocorre em primeiro lugar pela diminuição da tensão superficial (tensão superficial dinâmica) e mais tarde através de interações repulsivas coloidais (mecanismos de estabilização eletrostática e estérica) (TADROS, 2013).



Adaptado de DICKINSON (2009).

Figura 1.1 - Processos físico-químicos envolvidos na formação e estabilização de emulsões.

Considerando uma mesma taxa de energia dissipada durante a emulsificação, a distribuição final do tamanho das gotículas será determinada pelo tempo necessário para que a interface seja coberta com o emulsificante, em comparação com o intervalo médio de colisões entre gotículas. Quando o emulsificante adsorve muito lentamente, ou está presente em uma concentração muito baixa, a maioria das gotículas individuais formadas durante a emulsificação não são retidas na emulsão final. Isto pode ser devido à ruptura da película fina entre gotículas devido a colisões (coalescência) ou à interação da camada adsorvida de duas gotículas (floculação em ponte) (TADROS, 2013).

Uma vez que uma emulsão de pequenas gotículas tenha sido preparada com êxito, considerações de atividade superficial ou gradientes de tensão interfacial já não são relevantes. O importante para a estabilidade a longo prazo passa a ser como esse emulsificante contribuirá para a formação de uma barreira robusta na interface contra a desestabilização da emulsão. Os processos físico-químicos envolvidos na prevenção da agregação das gotículas ou coalescência são basicamente: estabilização estérica e estabilização eletrostática (DICKINSON, 2009).

De um modo geral, quanto menor a tensão interfacial, maior a extensão à qual as gotículas podem ser quebradas durante o cisalhamento intenso ou fluxo turbulento. Durante a homogeneização a alta pressão, por exemplo, os processos ocorrem em escalas de tempo de milissegundos ou menos: deformação de gotículas, adsorção e espalhamento de emulsificante e colisão de gotículas. O ideal é que durante a emulsificação, o tempo entre colisões de gotículas seja longo comparado com o tempo de adsorção do adsorvente na nova interface O/A para que seja criada uma camada estabilizadora transitória.

As misturas de proteínas do leite são amplamente utilizadas como ingredientes em vários produtos alimentares porque são excelentes emulsificantes. A homogeneização é um passo essencial na formação da emulsão, na qual as grandes gotas deformáveis são quebradas pela vigorosa aplicação da energia mecânica. Durante a emulsificação, as proteínas do leite são capazes de adsorver rapidamente na superfície das gotas de óleo recém formadas, reduzindo a tensão interfacial e formando camadas espessas que impedem a coalescência ou floculação das gotículas através de mecanismos de estabilização estérica e eletrostática. A adsorção de proteínas na interface O/A é também termodinamicamente favorável porque os resíduos hidrofóbicos da proteína são removidos da fase aquosa e são orientados para a fase oleosa, seguindo o rearranjo

estrutural da proteína na interface, diminuindo dessa forma a energia livre de Gibbs do sistema. Como resultado, a estrutura da proteína adsorvida encontra-se em algum ponto intermediário entre o estado nativo e o estado totalmente desnaturado (CHANG & LI, 2002).

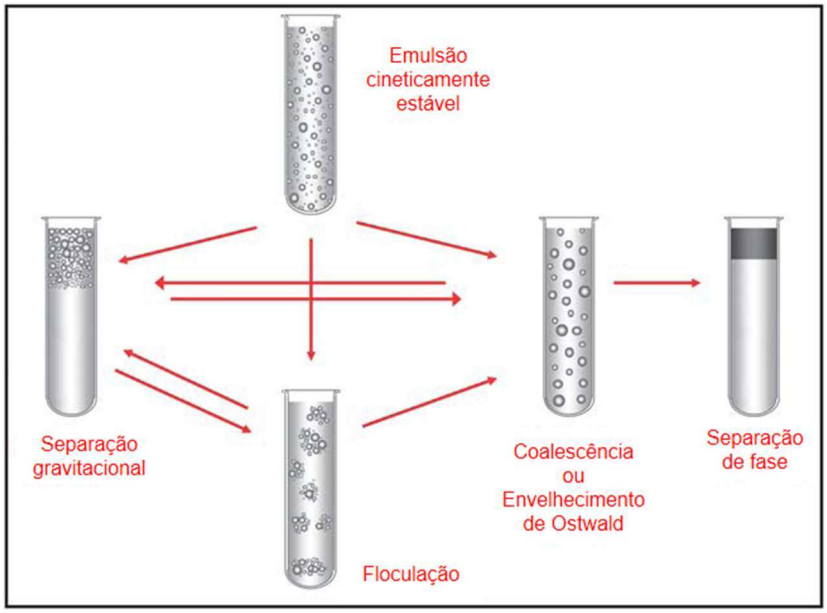
Homogeneizadores de alta pressão são os mais utilizados para a produção de emulsões de alimentos, proporcionando emulsões de menor diâmetro, com propriedades de textura desejadas e maior estabilidade. Cisalhamento, cavitação e condições de escoamento turbulento conduzem ao rompimento das gotículas de óleo durante a homogeneização (MCCLEMENTS, 2005). A diminuição do tamanho médio das gotículas de gordura leva a uma redução na velocidade de formação de creme e aumenta a estabilidade da emulsão (FRIBERG et al., 2004).

1.1 Sistemas de estabilização e desestabilização de emulsões

Todas as emulsões são termodinamicamente instáveis, ou seja, eventualmente irão sofrer separação macroscópica de fases. No entanto, o tempo que levará para que isso ocorra é determinado pela estabilidade cinética da emulsão (MCCLEMENTS, 2005). Uma emulsão cineticamente estável consiste em um sistema sem variações significantes no tamanho das gotículas, no estado de agregação ou de sua distribuição espacial ao longo de determinado tempo. Esse tempo é definido pelas caracteristícas do produto e seu uso.

Uma das principais preocupações em relação a emulsões é manter as gotículas que constituem a fase dispersa uniformemente distribuídas no seio da mistura durante o processamento, armazenagem e consumo (CHANAMAI & MCCLEMENTS, 2002). Isto tem levado ao desenvolvimento de novas tecnologias visando melhorar a estabilidade cinética das emulsões, que por sua vez depende do tipo e concentração de ingredientes contidos na emulsão, assim como as condições de processamento e armazenamento.

Emulsões à base de proteínas podem se tornar instáveis devido a diferentes fenômenos físicos e químicos. A instabilidade física refere-se a modificações no arranjo espacial ou distribuição do tamanho das gotículas de emulsão, tais como agregação, floculação e coalescência (Figura 1.2). Já a instabilidade química inclui alterações na composição das próprias gotículas de emulsão, tais como oxidação e hidrólise (SINGH, 2011).



Adaptado de MCCLEMENTS (2011).

Figura 1.2 – Representação esquemática dos tipos de desestabilização de emulsões.

O estado de agregação das gotículas numa emulsão é importante porque influencia a estabilidade do produto à separação gravitacional e depende de interações entre camadas estabilizadoras, que por sua vez depende de fatores tais como a cobertura de superfície do emulsificante, espessura da camada, densidade de carga superficial e condições da solução aquosa (especialmente pH, força iônica e íons bivalentes). A manifestação visual inicial mais evidente de instabilidade em uma emulsão O/A é a cremeação, que tipicamente conduz à separação de fases macroscópica em regiões separadas discerníveis de creme e soro. Pode-se seguir então a coalescência de gotículas dentro do creme e separação do óleo no topo da amostra (TADROS, 2013).

A separação gravitacional ocorre quando existe diferenças de densidade entre as fases contínua e dispersa. Na cremeação, as gotículas da fase dispersa apresentam um movimento ascendente, enquanto na sedimentação um movimento descendente, pela ação da força gravitacional (SÁNCHEZ & PATINO, 2005; CHEN et al., 2017). A velocidade de equilíbrio de sedimentação ou cremeação é determinada pela lei de Stokes, por meio do balanço de forças que atuam sobre uma partícula em movimento em um meio (forças gravitacionais e de fricção). Essa velocidade é proporcional a diferença de densidade da partícula e do fluido, ao quadrado do raio e inversamente a viscosidade do fluido. Assim, a taxa de cremeação deve diminuir à medida que o tamanho da gotícula ou a diferença de

densidade diminuem ou a viscosidade da fase contínua aumenta. Uma emulsão cujas gotículas tenham elevada velocidade de Stokes apresentará uma menor vida de prateleira (DAMODARAN et al., 2010).

Em geral, emulsões O/A são susceptíveis a cremeação enquanto emulsões A/O são susceptíveis a sedimentação, uma vez que a fase oleosa tem menor densidade que a fase aquosa. Deve-se observar, no entanto, que a densidade da fase dispersa deve englobar também a densidade da membrana interfacial, que terá maior influência quanto menor forem as gotículas da emulsão (MCCLEMENTS, 2005). As gotículas da emulsão tendem a se mover até a superfície da emulsão formando assim uma camada de creme. Aquelas de maior tamanho apresentam maiores velocidades e, pela distribuição de tamanho de gotículas, dentro de uma mesma emulsão podem existir diferenças na velocidade de cremeação, gerando um gradiente tanto de concentração quanto de tamanho de gotículas.

A floculação, uma vez que aumenta o tamanho das gotículas, resulta em maior taxa de cremeação. Se as gotículas forem suficientemente pequenas, o movimento Browniano irá dominar e o sistema permanecerá estável à cremeação ou à sedimentação. A separação gravitacional pode ser inibida aumentando a viscosidade da fase aquosa, por exemplo, por adição de agentes espessantes ou gelificantes, abordagem essa nem sempre viável, uma vez que também irá influenciar a textura do produto final (TADROS, 2013).

As gotículas em emulsões estão em movimento contínuo por causa dos efeitos da energia térmica, da gravidade ou das forças mecânicas aplicadas e, à medida que se movem, colidem frequentemente com os seus vizinhos. Além da distribuição do tamanho das gotículas, o fator mais importante que afeta a cinética de cremeação é o estado de floculação da emulsão. Após uma colisão, as gotículas de emulsão podem se separar ou permanecer agregadas, dependendo da magnitude relativa das interações atrativas e repulsivas entre elas. A floculação de gotículas é o processo pelo qual duas ou mais gotículas se juntam para formar um agregado no qual as gotículas retêm a sua integridade individual, sem interrupção da camada protetora estabilizadora na interface (MCCLEMENTS, 2005).

A floculação ocorre quando não há repulsão suficiente para manter as gotículas separadas a distâncias onde a atração de van der Waals é fraca. A floculação pode ser "forte" ou "fraca", dependendo da magnitude da energia atrativa envolvida (WALSTRA, 2003).

O mecanismo de floculação pode ocorrer pelo aumento da atração de depleção, onde a presença de entidades coloidais não adsorventes na fase contínua de uma emulsão, tal como biopolímeros ou micelas de surfactante, gera um aumento na força de atração entre as gotículas devido a um efeito osmótico associado à exclusão das entidades coloidais de uma região estreita ao redor de cada gotícula (ISRAELACHVILI, 2011). A partilha de moléculas de proteína ou agregados entre gotículas adjacentes pode ocorrer quando a quantidade de proteína disponível durante a emulsificação é insuficiente para cobrir completamente toda a interface O/A recém-criada. Este tipo de floculação em ponte irreversível ocorre durante a formação de emulsões preparadas com misturas binárias de macromoléculas de atividade superficial muito diferente, por exemplo.

Outro mecanismo de desestabilização é a maturação de Ostwald, processo pelo qual o tamanho das gotículas de óleo numa emulsão O/A aumenta ao longo do tempo devido à difusão de moléculas de óleo de pequenas a grandes gotículas através da fase aquosa circundante (DICKINSON, 2009). Esse fenômeno ocorre de forma espontâneo devido a uma maior estabilidade das gotículas maiores, que apresentam menor energia livre, e é importante principalmente em emulsões cuja fase dispersa apresenta alta solubilidade na fase contínua (HAN et al., 2018).

A coalescência refere-se ao processo de ruptura do filme líquido entre as gotículas como o resultado da fusão de duas ou mais gotículas em gotas maiores (TADROS, 2013). Um pequeno orifício pode se formar no filme, pelo movimento natural das gotículas (movimento browniano) e a depender da pressão de Laplace no local o fluido interno da gotícula pode fluir pelo orifício, aumentando-o e levando a união de duas gotículas. Esse mecanismo é influenciado pela magnitude relativa das forças entre gotículas, pela resistência da interface à ruptura, duração do contato entre gotículas e do processo de rompimento das interfaces. A coalescência será menos provável para gotículas pequenas, filmes espessos entre as gotículas e maiores tensões na superfície (SINGH, 2011).

Em geral, a susceptibilidade das gotículas de óleo à coalescência é determinada pela natureza das forças que atuam entre as gotículas (i.e. forças gravitacionais, coloidais, hidrodinâmicas e mecânicas) e a resistência da camada interfacial à ruptura. A estabilidade das emulsões para coalescência pode ser melhorada evitando que as gotículas se aproximem por períodos prolongados, por exemplo, impedindo a floculação de gotículas, impedindo a formação de uma camada de creme ou tendo concentrações de

gotículas relativamente altas (MCCLEMENTS, 2005). Alternativamente, pode-se controlar as propriedades da camada interfacial que envolve as gotículas de óleo para torná-la mais resistente à ruptura, por exemplo, selecionando um emulsificante apropriado ou outros aditivos que alterem as propriedades de superfície.

Torna-se de suma importância também reconhecer os fatores que levam a um aumento da instabilidade das emulsões (DICKINSON, 1997). Uma redução substancial da densidade de carga superficial das gotículas revestidas de proteínas conduz à perda de estabilização eletrostática, o que ocorre, por exemplo, perto do ponto isoelétrico ou na presença de íons cálcio. Assim, com o aumento da força iônica, os eletrólitos podem diminuir a repulsão de dupla camada e, portanto, reduzir a estabilização eletrostática.

1.2 Emulsões multicamadas

As proteínas são largamente utilizadas em emulsões alimentícias como emulsificantes devido ao seu caráter anfipático. A capacidade das proteínas em gerar interações repulsivas entre as gotículas de óleo, e, ao mesmo tempo, formar uma membrana interfacial resistente à ruptura, desempenha um papel importante na estabilização das gotículas contra a floculação e a coalescência durante o armazenamento a longo prazo (MCCLEMENTS, 2005).

As proteínas adsorvidas numa interface O/A podem interagir com moléculas vizinhas adsorvidas na mesma gotícula, ou em diferentes gotículas através de uma combinação de ligações não covalentes ou covalentes de ligação dissulfeto, aumentando a estabilidade à coalescência.

As emulsões estabilizadas com proteínas são sensíveis a condições ambientais tais como pH e força iônica e tendem a flocular a valores de pH próximos ao PI das proteínas adsorvidas ou quando a força iônica excede um determinado nível. A interação entre proteína e polissacarídeos pode melhorar as propriedades tecnológicas das proteínas em sistemas coloidais, tais como suspensões, emulsões ou espumas. A floculação é normalmente evitada em emulsões estabilizadas por proteínas-polissacarídeo devido à repulsão eletrostática relativamente forte entre as gotículas carregadas em valores de pH longe do PI (LEROUX et al., 2003).

A intensidade da complexação entre proteína e polissacarídeos, em geral, depende da distribuição de grupos ionizáveis na superfície da proteína, da facilidade de desdobramento da estrutura nativa da proteína e da flexibilidade da estrutura e distribuição de carga no polissacarídeo (DICKINSON, 2003). Biopolímeros carregados tendem a associar-se através de interações eletrostáticas. A formação de complexos se dá como consequência de alterações conformacionais dos biopolímeros e da liberação de contra-íons e moléculas de água para a solução (TURGEON et al., 2003; BENGOECHEA et al., 2011).

Apesar de serem menos ativos na superfície, os polissacarídeos, tais como pectina, produzem emulsões que são frequentemente mais resistentes a alterações de pH, força iônica e temperaturas elevadas do que as estabilizadas por proteínas (MCCLEMENTS, 2004). Isto se deve a uma membrana polissacarídica interfacial mais porosa e mais espessa do que a produzida pelas proteínas, o que aumenta a repulsão estérica e reduz a atração de van der Waals entre as gotículas de óleo (DICKINSON, 2003).

No entanto, devido à sua baixa atividade de superfície, os polissacarídeos devem ser utilizados em concentrações muito superiores às proteínas. Assim, ao combinar os atributos positivos de proteínas e polissacarídeos usando complexos proteína-polissacarídeo como emulsificante, pode-se obter emulsões com características desejáveis e com menores concentrações dos agentes de superfície (BENGOECHEA et al., 2011; BURGOS-DÍAZ et al., 2016).

A mistura de polissacarídeos e proteínas pode levar à formação de entidades supramoleculares capazes de retardar a cremeação, a floculação e a coalescência, a depender da aplicação pretendida. As propriedades de complexos proteína-polissacarídeo, tais como a hidratação (solubilidade, viscosidade), estruturação (agregação, gelificação) e de superfície (formação de espuma, emulsificantes) podem ser exploradas na formulação de novos alimentos (LORENZIS et al., 2008).

Em uma emulsão contendo proteína e polissacarídeo, a proteína geralmente forma a camada interfacial primária por adsorção direta à superfície do óleo. O polissacarídeo eventualmente forma uma camada espessa secundária de estabilização estérica, no lado de fora da proteína adsorvida às gotículas da emulsão, desde que a interação proteína-polissacarídeo seja suficientemente atrativa (SINGH & SARKAR, 2011; BURGOS-DÍAZ et al., 2016).

Biopolímeros eletricamente carregados são capazes de adsorver na superfície de gotículas de emulsão com carga oposta (MOREAU et al., 2003; GUZEY & MCCLEMENTS, 2006; LORENZIS et al., 2008) (BENGOECHEA et al., 2011) (BURGOS-DÍAZ et al., 2016). Essas gotículas revestidas por membranas interfaciais multicamadas apresentam melhor estabilidade a fatores ambientais (como tratamentos térmicos, secagem, congelamento e agitação mecânica) do que aquelas estabilizadas por membranas únicas devido ao aumento na espessura da interface e modificações em suas características reológicas (MCCLEMENTS, 2004; LESMES et al., 2010). A estabilidade da emulsão primária, assim como a correta concentração dos componentes das camadas subsequentes (concentração de saturação), é essencial para obtenção de emulsões multicamadas cineticamente estáveis e reprodutíveis (MCCLEMENTS, 2005). Emulsões multicamadas deste tipo podem ser obtidas usando o método de saturação para um melhor controle da concentração relativa dos reagentes adicionados (LORENZIS et al., 2008).

Emulsões de múltiplas camadas podem ser formadas de duas maneiras principais: misturando todos os ingredientes em conjunto ou pela adição de um componente de cada vez. Neste método, chamado de deposição camada-a-camada, uma emulsão primária é formada pela homogeneização de óleo, água e surfactante, resultando em pequenas gotículas revestidas por uma camada de emulsificante. Em seguida, uma emulsão secundária é formada pela mistura, à emulsão primária, de um polieletrólito de carga oposta promovendo, assim, a inversão de carga (GUZEY & MCCLEMENTS, 2006). Esta técnica permite a adsorção passo a passo de vários componentes (polieletrólitos, nanopartículas, proteínas, enzimas, etc), e a camada de crescimento é regulada por atração eletrostática, levando à formação de multicamadas com espessura controlada à escala nanométrica. Além da atração eletrostática, outras interações, como ligações de hidrogênio ou interações hidrofóbicas podem estar envolvidas na montagem camada por camada.

Um dos principais problemas práticos na utilização de emulsões multicamada consiste da prevenção da floculação em ponte que pode ocorrer durante a preparação dos sistemas multicamadas, o que normalmente significa que devem ser usadas concentrações de gotículas bastante baixas para assegurar que a adsorção do polímero ocorra mais rapidamente do que as colisões de gotas-gotas. Além disso, é importante selecionar emulsificantes e combinações de polímeros que proporcionem as características

interfaciais necessárias para o produto em particular onde a tecnologia multicamada será utilizada, por exemplo, sensibilidade ao pH, a sais e à temperatura (GUZEY & MCCLEMENTS, 2006).

O preparo de emulsões com camadas formadas por proteínas e polissacarídeos é interessante, uma vez que seriam estabilizadores estéricos ideais por se ligarem firmemente a uma interface como uma proteína e ser capazes de solvatar num meio aquoso como um polissacarídeo para formar uma película carregada em forma de gel de espessura significativa, com cadeias que se estendem extensivamente da interface. A resistência mecânica da camada adsorvida, juntamente com a repulsão eletrostática (das mesmas gotículas de emulsão carregada) e efeitos estéricos (devido à camada espessa como uma barreira) são os fatores mais importantes que afetam a estabilidade cinética de emulsões de óleo em água (CHO & MCCLEMENTS, 2009).

1.3 Características das emulsões estabilizadas por proteínas

As proteínas são adsorvidas espontaneamente em interfaces O/A, de forma a diminuir o contato de suas cadeias laterais não polares do ambiente desfavorável da solução aquosa. Durante o processo de adsorção há uma diminuição da atividade termodinâmica (isto é, a concentração) das moléculas de água na região interfacial. A fração de cada molécula de proteína acessível à região interfacial diminui com o aumento do tamanho molecular (DAMODARAN, 2005). A principal contribuição para a energia livre de adsorção de proteínas é de caráter entrópico, uma vez que o processo envolve o deslocamento de moléculas de água ordenadas do ambiente desfavorável da interface. Uma força motriz secundária está associada ao desdobramento da molécula de proteína na adsorção. Isto leva a uma nova alteração no equilíbrio das interações proteína-proteína.

A adsorção da proteína à interface é determinada pelo equilíbrio de dois fatores opostos: a barreira ao desprendimento de moléculas de proteína da solução e a atração das moléculas adsorvidas devido à força superficial hidrofóbica. A atração hidrofóbica pode explicar por que uma maior cobertura superficial leva a uma adsorção mais rápida (ALEXANDROV et al., 2012).

Para a adsorção da proteína, ela deve inicialmente se difundir da solução para a interface. A taxa de transferência de massa para a interface é determinada pela

concentração de proteína na fase aquosa e pelo tamanho das moléculas de proteína (ou agregados). Exceto em concentrações muito baixas, esta não é normalmente a etapa predominante ou limitante da taxa. Posteriormente ocorre a adsorção efetiva, uma vez que a molécula de proteína que atinge a interface não se torna imediatamente adsorvida. Há tipicamente algum tipo de energia de barreira, relacionada ao equilíbrio das interações envolvendo a vizinhança da molécula proteica nativa e a interface e que pode ser manipulada alterando características físico-químicas da proteína, como por exemplo alterando a exposição de grupos hidrofóbicos (DICKINSON, 2011).

Uma vez localizada na interface, a proteína apresenta tipicamente uma reorganização conformacional. Isto permite a exposição adicional de locais hidrofóbicos acessíveis à fase não aquosa. O desdobramento irreversível de uma proteína globular adsorvida é às vezes chamado de "desnaturação superficial" (ZHAI et al., 2013). Quanto maior o tempo e espaço disponível, maior a extensão desse desdobramento.

A fase final é o estabelecimento de uma camada concentrada de moléculas de proteína densamente associadas, que podem influenciar as propriedades mecânicas a longo prazo, como demonstrado através de medições reológicas de superfície dependentes do tempo (DICKINSON, 2008).

Como regra geral, a proteína que chega primeiro na interface é a que predomina. No entanto, a capacidade de uma proteína ser a principal espécie numa interface O/A durante ou após a emulsificação depende principalmente da sua flexibilidade molecular e da sua hidrofobicidade superficial. Além disso, uma vez que as proteínas se tornam adsorvidas na interface O/A, existe baixa migração de moléculas de proteínas da interface para a fase aquosa e vice-versa (DAMODARAN, 2005).

A desnaturação parcial das proteínas antes da emulsificação costuma melhorar as propriedades emulsificantes. Isso se deve ao aumento da flexibilidade molecular e da hidrofobicidade de superfície. No estado desordenado as proteínas que contém grupos sulfidrila livres e pontes dissulfeto sofrem lenta polimerização por meio da reação de troca dissulfeto-sulfidrila, o que leva a formação de uma película altamente viscoelástica na interface óleo-água. A desnaturação excessiva pode prejudicar as capacidades emulsificantes por tornar a proteína insolúvel (RAIKOS, 2010).

O número e a distribuição dos resíduos de aminoácidos não polares na superfície de uma proteína afetam a hidrofobicidade efetiva superficial da proteína, que influencia sua adsorção na interface. Uma proteína com maior hidrofobicidade apresenta maior afinidade por interfaces anfipáticas, incluindo as interfaces O/A e A/A. A hidrofobicidade de uma proteína pode ser avaliada por meio de cromatografia de interação hidrofóbica ou pela supressão da fluorescência, por exemplo (MORO et al., 2001; SCHRÖDER et al., 2017).

As proteínas flexíveis com uma proporção mais elevada de grupos não polares são mais eficazes na redução da tensão interfacial do que as proteínas rígidas com menos grupos. Esta é presumivelmente a origem da boa correlação entre a atividade superficial e a hidrofobicidade superficial. Quando uma proteína globular se desdobra, sua hidrofobicidade superficial aumenta. Isto explica, por exemplo, a melhora da atividade superficial de uma proteína como a β-lactoglobulina (BLG) após tratamento térmico, dependendo da concentração (DICKINSON, 2011).

A Lisozima (LIS) é reconhecidamente uma proteína altamente hidrofóbica e estável, indicando que a hidrofobicidade superficial domina a dinâmica de tensão desta proteína, que adsorve fortemente à interface, mas mantém uma estrutura mais compacta (BEVERUNG et al., 1999). Essa maior estabilidade proteica está relacionada também a um maior tempo de diminuição da tensão interfacial. A hidrofobicidade superficial apresenta menor importância em tempos mais elevados, uma vez que as alterações conformacionais tanto na camada inicial como nas cadeias que se estendem dessa camada promovem a agregação adicional e determinam a tensão interfacial (ALEXANDROV et al., 2012).

O balanço entre a flexibilidade e a hidrofobicidade de uma proteína parece ser o fator determinante para as propriedades emulsificantes. Proteínas pouco rígidas, com propriedades espumantes com superfície hidrofóbicas exibem taxas de adsorção rápidas, enquanto proteínas rígidas e com superfícies anfipáticas exibem taxas de adsorção lentas (TRIPP et al., 1995).

1.4 Tamanho médio e carga superficial de partículas de emulsão

Gotículas menores costumam produzir emulsões mais estáveis. No entanto, a energia e a quantidade de emulsificante aumentam com a diminuição do tamanho. Em virtude da grande dependência da estabilidade em relação ao tamanho da gotícula, é de suma importância a determinação do intervalo de distribuição de tamanho (PATTY & FRISKEN, 2006).

O tamanho e a distribuição do tamanho de emulsões pode ser determinado por espalhamento de luz dinâmico (DLS), que se baseia na espectroscopia de correlação de fótons (PCS) para medir o tamanho das partículas em movimento aleatório constante browniano ou térmico. Este movimento faz com que a intensidade da luz dispersa a partir de partículas varie com o tempo. Partículas grandes movem-se mais lentamente que as pequenas, de modo que a taxa de variação da luz dispersa seja também mais lenta. O PCS utiliza a taxa de alteração destas flutuações de luz para determinar a distribuição do tamanho de partículas dispersando a luz (YE & SINGH, 2007).

O DLS mede as flutuações dependentes do tempo na intensidade de dispersão para determinar o coeficiente de difusão de translação (DT) e subsequentemente o raio hidrodinâmico (RH). O diâmetro z-médio das partículas é calculado a partir do seu movimento Browniano através da equação de Stokes-Einstein tendo em conta as viscosidades da fase aquosa corrigida (SCHEIBELHOFER et al., 2016).

O potencial zeta (ζ) é uma medida da carga sobre uma superfície de partícula num meio líquido específico. É o potencial da superfície carregada no plano de cisalhamento entre a partícula e a solução circundante à medida que a partícula e a solução se movem uma em relação à outra. O plano de cisalhamento pode ser definido como a distância da superfície da gotícula abaixo da qual os contra-íons permanecem fortemente ligados à partícula quando se movem em um campo elétrico (MCCLEMENTS, 2005). Os íons no meio e a concentração iônica total podem afetar a velocidade de difusão da partícula, alterando a espessura da dupla camada elétrica.

Este valor de carga superficial é útil para compreender e prever interações entre partículas em suspensão. A manipulação do potencial ζ é um método para aumentar a estabilidade da suspensão, ou acelerar a floculação de partículas em aplicações tais como tratamento de água.

Ao medir o potencial ζ, o foco é a magnitude do potencial ζ e não se é positivo ou negativo. Quanto maior a magnitude do potencial ζ, maior repulsão as partículas terão entre si, e menor agregação ocorrerá, o que gera estabilidade cinética da emulsão. Se o potencial ζ é baixo, então não há forças para repelir as partículas umas das outras e a instabilidade da emulsão dará lugar à agregação. Uma linha típica que divide estabilidade e instabilidade está em +30 mV ou -30 mV. Quanto mais altas forem as magnitudes além desses valores, mais estável será o sistema coloidal quanto à estabilização estérica (MCCLEMENTS, 2005; JI et al., 2015).

As características elétricas da superfície de uma gotícula dependem do tipo, concentração e organização das espécies ionizadas presentes, bem como da composição iônica e propriedades físicas da fase aquosa circundante. A carga elétrica nas gotículas de óleo pode ser importante, pois determina a estabilidade das gotículas à agregação devido à sua influência pela magnitude, alcance e sinal de interações eletrostáticas; determina as interações de gotículas com outras espécies carregadas numa emulsão, por exemplo, íons (tais como cálcio ou ferro), ou polieletrólitos (tais como proteínas ou polissacarídeos); influencia a maneira com que as gotículas interagem com superfícies eletricamente carregadas, tais como recipientes de armazenamento e na boca; e influencia o comportamento das gotículas em um campo elétrico, o que é importante para medir sua carga usando eletroforese.

Medidas de potencial ζ podem ser realizadas por meio de um instrumento de eletroforese de partícula. O potencial ζ é determinado por meio da medida da direção e velocidade do movimento de uma gotícula em um campo elétrico bem definido (HOLMBERG et al., 2002).

A mobilidade eletroforética ($V.\ m^2.s^{-1}$) pode ser calculada conforme a equação Eq. 1, para partículas esféricas.

$$\mu = \frac{UE}{\omega} \qquad \text{Eq. 1}$$

em que U é a velocidade da partícula ($m.s^{-1}$), ω a frequência (s^{-1}) e E o módulo do vetor campo elétrico efetivo ($V.m^{-1}$). O potencial ζ (mV) é calculado usando a equação de Smoluchoski (Eq. 2).

$$\zeta = \frac{\eta\mu}{\varepsilon} \qquad \text{Eq. 2}$$

Em que ε é a permissividade elétrica ($F.m^{-1}$) e η a viscosidade do solvente (Pa.s).

2. Componentes das emulsões

Proteínas que tem menor insolubilidade no ponto isoelétrico, ou seja, são menos afetadas pela mudança interna de cargas, apresentam alta capacidade emulsificante nesse pH. Como as interações repulsivas eletrostáticas são diminuídas pela baixa carga líquida, a carga proteica na superfície é aumentada, promovendo a formação de uma película altamente viscoelástica que contribui para a estabilidade da emulsão. No entanto, justamente pela falta de carga líquida, essas emulsões estarão mais susceptíveis a floculação e coalescência, o que diminuiria a estabilidade da emulsão (DAMODARAN et al., 2010).

As propriedades emulsificantes das proteínas relacionam-se, ainda, com a sua hidrofobicidade de superfície e sua rigidez. LIS é menos hidrofóbica que BLG e apresenta mais ou menos a mesma tensão superficial e metade do índice de atividade emulsificante (DAMODARAN et al., 2010).

A natureza anfipática das proteínas, com os seus aminoácidos polares, não polares e carregados, também contribui para a atividade superficial. Embora os aminoácidos polares e carregados hidrofílicos estejam geralmente localizados no exterior da molécula e os resíduos hidrofóbicos no interior, isto não é absoluto. Assim, os aminoácidos hidrofóbicos podem estar disponíveis na superfície da proteína para interação com substratos. Adicionalmente, a desnaturação de proteínas pode expor regiões hidrofóbicas e permitir a interação com a superfície (IDENYI et al., 2006).

As proteínas do soro de leite, assim como as da clara do ovo, são frequentemente usadas como tensoativos naturais para formulações obtidas por meio de emulsões.

2.1 Lisozima

A lisozima (LIS) é uma proteína extraída da clara de ovo com massa molecular em torno de 14 $kg.mol^{-1}$, raio de giro de 15 Å e ponto isoelétrico em pH≈10,7 (TYN & GUSEK, 1990). A LIS tem quatro ligações dissulfeto intramoleculares, não possuindo

grupos tiol livres. Dada sua natureza básica, afeta as propriedades de outras proteínas, interagindo com elas (LI-CHAN et al., 1995). A LIS é uma proteína mais compacta, enquanto a BLG tem uma estrutura mais flexível, o que faz com que a BLG tenha maior afinidade a adsorver na interface óleo/água (ZHU et al., 2007).

A LIS é uma proteína secretora que catalisa a hidrólise de tipos específicos de polissacarídeos de paredes celulares bacterianas. É uma proteína básica excepcionalmente abundante na clara de ovo e contém uma única cadeia polipeptídica constituída por 129 resíduos de aminoácidos (SATO et al., 1998) e dobrada para uma conformação globular com dois domínios (α e β) reticulados com quatro pontes dissulfeto (Figura 1.3).

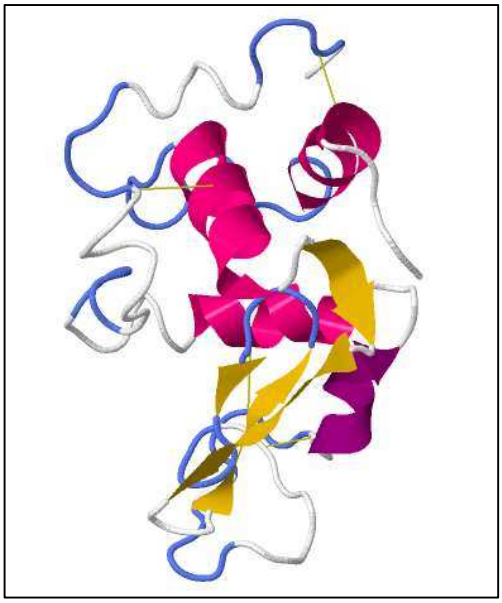


Figura 1.3 – Estrutura do monômero de Lisozima (PDB ID: 2LYZ). α-hélices em rosa, folhas-β em amarelo e alças e voltas em branco (DIAMOND, 1974).

As proteínas da clara do ovo são usadas como ingrediente para a indústria de alimentos devido às suas propriedades, que incluem a capacidade de gelificação, formação de espuma e propriedades emulsificantes (HANDA et al., 1998).

LIS existe na sua forma monomérica em soluções aquosas ácidas (pH <5), mas forma dímeros na região de pH de 5 a 10 e agregados a pH> 10 (SATO et al., 1998). A lisozima monomérica é uma proteína relativamente pequena com as dimensões aproximadas 3x3x4,5 nm, e consiste em uma elevada porção de resíduos de aminoácidos aromáticos incluindo seis resíduos de triptofano (Trp) e três resíduos de tirosina (Tyr). Três dos resíduos Trp estão localizados nos locais de ligação do substrato, dois na caixa

de matriz hidrofóbica, enquanto um é separado dos outros. Trp62 e Trp108 são os fluoróforos mais dominantes (> 80%) e estão localizados nos locais de ligação ao substrato (DING et al., 2009).

A efetividade da clara de ovo em estabilizar emulsões é atribuída em parte aos benefícios da adsorção cooperativa entre suas proteínas constituintes. Em ordem de abundância, estas proteínas (glicoproteínas) são ovalbumina, conalbumina, ovomucóide, (ovo) globulinas, lisozima e ovomucina. As globulinas de superfície provavelmente contribuem mais para a capacidade de espuma. A lisozima pura tem baixa capacidade espumante, mas a associação com outras proteínas ou carboidratos melhora sua capacidade emulsificante (HOLMBERG et al., 2002).

Além do ovo, a LIS é encontrada em quase todos os organismos vivos, de insetos e nematóides a répteis e mamíferos, incluindo vírus e plantas, e em diversos tecidos e secreções nos mesmos, como lágrimas, saliva, leite, músculos e órgãos (CHANG & LI, 2002).

2.2 β-lactoglobulina

A β-Lactoglobulina (BLG) é encontrada naturalmente no soro de leite e possui massa molar de 18,3 $kg \cdot mol^{-1}$ e ponto isoelétrico em pH≈5,2 (CHEFTEL et al., 1985). Pertence à família das Lipocalinas (proteínas com função de transporte), cuja principal característica é sua habilidade de se ligar a pequenas moléculas lipofílicas em sua cavidade central hidrofóbica de forma a minimizar o contato dessas moléculas com solventes polares (FLOWER, 1996). Dentre essas moléculas, pode-se citar retinol, ácidos graxos, vitaminas lipossolúveis e colesterol (FLOWER et al., 2000; KONTOPIDIS et al., 2002).

A BLG contém duas ligações dissulfeto e um grupo tiol livre muito reativo que possibilita um intercâmbio entre as ligações dissulfeto durante mudanças conformacionais associadas com o tratamento térmico e alterações de pH (BOTTOMLEY et al., 1990).

A estrutura terciária do BLG é composta por 19% de α-hélices, 41% de folhas-β e 40% de estruturas aleatórias (CROGUENNEC et al., 2004a). A estrutura do monômero de BLG (Figura 1.4) é composta de nove folhas-β antiparalelas (A a I), oito (A a H) dos

quais envolvem-se para formar um barril cônico achatado (barril-β) também chamado de cálice central, e uma α-hélice localizada na superfície exterior do barril-β.

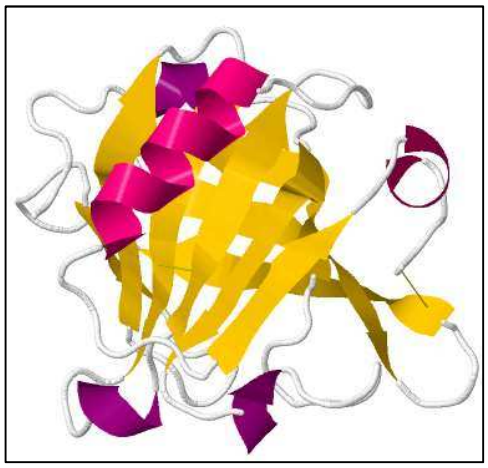


Figura 1.4 – Estrutura do monômero de β-lactoglobulina (PDB ID:1BEB). α-hélices em rosa, folhas-β em amarelo e alças e voltas em branco (BROWNLOW et al., 1997).

Cada monômero tem duas ligações dissulfureto, em Cys66-Cys160 e Cys106-Cys119, responsáveis pela estabilização da estrutura terciária de BLG e um grupo sulfidrila livre em Cys121. Este último encontra-se ocultado dentro da estrutura proteica nativa a pH <7,5 (CROGUENNEC et al., 2004b).

Mudanças críticas na conformação de BLG envolvem exposição à superfície da proteína tanto dos resíduos hidrofóbicos interiores como do grupo sulfidrila que se tornam disponíveis para interações intermoleculares. Em pH baixo (<7), o cálice central está numa posição fechada, e a ligação no seu interior é inibida ou impossível. Em pH elevado (> 7), este ciclo está aberto, permitindo que os ligantes entrem no núcleo hidrofóbico (KONTOPIDIS et al., 2004).

A BLG existe como um dímero na maior parte das condições, uma vez que as condições para a formação do monômero são bastante extremas (pH<3,0 ou pH> 9,0). Os valores de pH em torno do ponto isoelétrico favorecem a formação de agregados de ordem elevada (YAN et al., 2013). Na faixa de pH de 4 a 7, a proteína é normalmente encontrada como dímeros de diâmetro hidrodinâmico de cerca de 6 nm (ZÚÑIGA et al., 2010).

2.3 Pectina

Pectina é um polissacarídeo solúvel em água amplamente utilizado na fabricação de alimentos para o seu espessamento e propriedades gelificantes. Este hidrocoloide, essencialmente constituído por resíduos D-galacturônicos, tem uma carga negativa devido à presença de grupos ácidos carboxílicos ionizados ao longo do seu esqueleto que têm valores de pKa de cerca de 3,6 (BAYARRI et al., 2014). As pectinas nativas são pectinas de alta metoxilação, em que a maioria dos grupos de ácido carboxílico são esterificados por metanol e consequentemente não ionizáveis. Uma proporção mais elevada de grupos ácido carboxílico livres ionizados favorece interações eletrostáticas com proteínas positivamente carregadas.

As pectinas de baixa-metoxilação (LM) são carboidratos complexos considerados como heteropolisacarídeos de cadeias de monossacáridos não repetitivos. As pectinas de baixo teor de metoxilação são definidas como sendo inferiores a 50%, enquanto as pectinas de elevado teor de metoxilação são superiores a 50%. A pectina LM utilizada na indústria alimentar pode contribuir para a fabricação de produtos tais como geleias e marmeladas (LEROUX et al., 2003).

Diferentes tipos de pectina contêm diferentes densidades de grupos ácido carboxílico ionizáveis, dependendo do grau de esterificação (DE). Em princípio, a ação estabilizadora da pectina envolve a adsorção da pectina na superfície das gotículas de óleo de uma emulsão sob a influência de interações atrativas entre proteína-polissacarídeo, estabilizando assim as partículas dispersas por um mecanismo eletrostático e estérico. Contudo, na prática, a eficácia da estabilização e a natureza das interações proteína-pectina são ambas sensíveis a muitos diferentes fatores, especialmente a extensão da esterificação (e amidação) da amostra de pectina e o pH do meio aquoso (LEROUX et al., 2003).

2.4 β-caroteno

Carotenoides são pigmentos orgânicos encontrados no cloroplasto de plantas e outros organismos fotossintéticos como algas, algumas bactérias e fungos. A função fisiológica principal dos carotenoides é como precursores da vitamina A, mesmo assim eles têm atraído atenção considerável também devido a uma miríade de benefícios para a

saúde (GUL et al., 2015). A vitamina A é encontrada na dieta humana, quer como vitamina A pré-formada em alimentos de origem animal, ou como carotenoides provitamina A em alimentos de origem vegetal.

O β-caroteno (BC) é um membro importante da família dos carotenoides e é um pigmento vermelho-alaranjado abundante em plantas e frutos. BC (Figura 1.5) é considerado como o principal carotenoide presente na dieta humana e é por sua vez a principal fonte de vitamina A em humanos (GUL et al., 2015). Tem uma cadeia de 40 carbonos com anéis terminais de β-ionona, onze ligações duplas conjugadas e um massa molar em torno de 536 $g.mol^{-1}$.

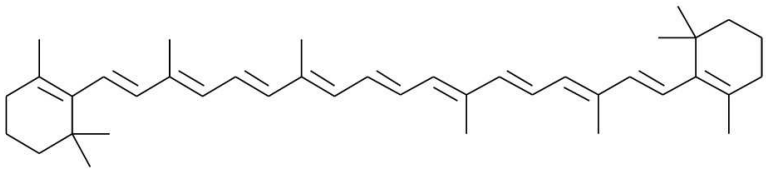


Figura 1.5 – Estrutura do β-caroteno.

BC tem forte atividade antioxidante com capacidade de extinção de até 1.000 radicais livres por molécula. Embora uma molécula de BC possa ser quebrada em duas moléculas de retinol, cerca de 2 mg de BC absorvido equivalem a 1 mg de retinol no corpo humano (VAN LOO-BOUWMAN et al., 2014).

A fraca estabilidade ao calor e a solubilidade extremamente baixa limitam a sua biodisponibilidade e a sua utilização como ingrediente funcional em vários alimentos e formulações de fármacos. Uma outra desvantagem do BC é a sua susceptibilidade à oxidação, devido à elevada quantidade de ligações insaturadas, podendo ser prontamente degradado por exposições à luz na presença de oxigênio/ar e a degradação de BC podendo, ainda, gerar compostos com atividade pró-oxidante (LOVEDAY & SINGH, 2008; MENSI et al., 2014).

Assim, novas tecnologias que aumentam a estabilidade de BC em alimentos foram investigadas e muitas partículas de transporte e proteção foram desenvolvidas de modo a melhorar a estabilidade e a dispersibilidade destas moléculas lipofílicas bioativas instáveis. Entre os sistemas desenvolvidos para a proteção do BC podem ser citados: sistemas de emulsão, nanopartículas lipídicas sólidas, lipossomas, cocleato, complexos

de inclusão de ciclodextrina, encapsulação de polímeros e ligação a proteínas (LOVEDAY & SINGH, 2008).

3. Supressão da fluorescência

A supressão da fluorescência pode ser definida como a diminuição no rendimento quântico da fluorescência de uma molécula fluoróforo induzida por interações moleculares com moléculas supressoras. Estas interações moleculares podem ser uma reação de estado excitado, rearranjos moleculares, transferência de energia, formação de complexos de estado fundamental e supressão por colisão. Durante uma supressão por colisão, o supressor se difunde para o fluoróforo durante o tempo de vida do estado excitado e após o contato, o fluoróforo volta ao estado fundamental, sem emissão de um fóton. Na situação de supressão estática um complexo não fluorescente entre o fluoróforo e o supressor é formado (LAKOWICZ, 2007).

Analisando o valor da intensidade de fluorescência de uma molécula no comprimento máximo de emissão, podem ser obtidos diferentes parâmetros, tais como a eficiência de transferência de energia, duração e polarização de fluoróforo. A partir destes parâmetros, podem ser extraídas informações relacionadas com as alterações estruturais e microambiente dos fluoróforos em uma macromolécula (XIAO et al., 2008).

O microambiente exerce uma forte influência sobre a emissão de fluorescência de moléculas fluorescentes, que podem ser utilizadas como sondas para investigação de sistemas físico-químicos, bioquímicos e biológicos. As sondas fluorescentes podem ser divididas em sondas extrínsecas ligadas covalentemente, sondas de associação extrínsecas e sondas intrínsecas (ALARCÓN et al., 2012).

As sondas intrínsecas à molécula não necessitam de preparo e permitem o estudo das moléculas em condições nativas. No entanto, esse tipo de sonda depende da presença de fluoróforos na estrutura da molécula, condição de elevada especificidade que não ocorre em muitos grupos de compostos. Um exemplo é a fluorescência do triptofano em proteínas (QU et al., 2009).

A fluorescência intrínseca de proteínas tem sido amplamente utilizada para obter informação sobre o equilíbrio de ligação entre proteína e ligantes (BI et al., 2004; WANG et al., 2009; LI et al., 2013), para avaliar o efeito de condições adversas (p. e. aquecimento, desnaturação, adição de íons) na estrutura de proteínas (MANDERSON et al., 1999;

BUSTI et al., 2005; SCHRÖDER et al., 2017) e para determinar sítios de ligação (LANGE et al., 1998; COLLINI et al., 2000).

A fluorescência intrínseca da proteína é devida a aminoácidos aromáticos (Figura 1.6): triptofano (Trp), tirosina (Tir) e fenilalanina (Fen), embora a contribuição de Fen para a fluorescência intrínseca da proteína é negligenciável em virtude de sua baixa absortividade, além de um rendimento quântico muito baixo (DAMODARAN et al., 2010).

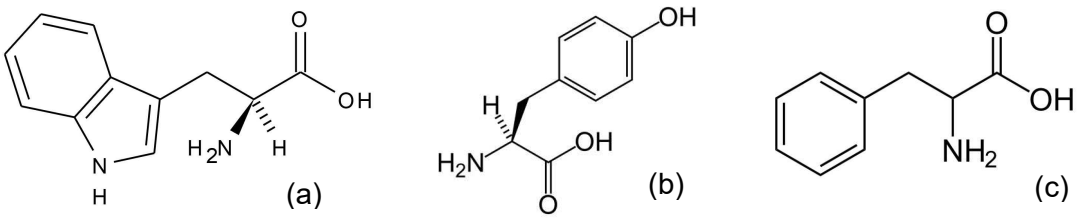


Figura 1.6 – Estrutura química de resíduos de aminoácidos: (a) triptofano, (b) tirosina e (c) fenilalanina.

Os resíduos de triptofano e tirosina possuem diferentes comprimentos de onda de absorção e emissão, diferindo grandemente nos seus rendimentos quânticos e tempos de vida. No comprimento de onda de excitação de 280 nm, os resíduos Trp e Tyr têm uma emissão de fluorescência, mas a um comprimento de onda de excitação de 295 nm, apenas os resíduos Trp apresentam emissão de fluorescência (LIANG et al., 2008).

Estes resíduos de aminoácidos são sensíveis ao ambiente do fluoróforo e, portanto, potenciais indicadores de alterações na conformação das proteínas. Alterações nos espectros de emissão do triptofano são geralmente empregadas para avaliar mudanças estruturais de proteínas, que afetam o ambiente local envolvendo o anel indol, causando deslocamentos no comprimento de onda de fluorescência máxima e na intensidade de fluorescência(QU et al., 2009). Quando excitado, o anel indol pode doar elétrons para as moléculas vizinhas, resultando em supressão da fluorescência do triptofano, geralmente por pequenas moléculas(SCHRÖDER et al., 2017).

Diferentes métodos têm sido utilizados para acessar a ligação de ligantes a proteínas incluindo equilíbrio de partição, ultrafiltração, turbidimetria, calorimetria de titulação isotérmica, espectrometria de massa, cromatografia de afinidade, fluorescência, ressonância magnética nuclear, etc. (MOCZ & ROSS, 2013). Os métodos

espectroscópicos estão entre as técnicas mais fáceis de usar, rápidas e específicas utilizadas para investigar a interação proteína-ligante.

Os ligantes podem se ligar às proteínas por meio de interação hidrofóbica, ligação de hidrogênio e interação eletrostática. Estas interações são também a força motriz para a mudança na estrutura das proteínas. Esses ligantes podem, portanto, afetar a estrutura nativa de proteínas (WANG et al., 2007; HEMAR et al., 2011).

Cada subunidade de BLG contém dois resíduos de triptofano, Trp 19 e Trp 61, estando este último numa posição mais exposta ao solvente e muito próximo da ligação dissulfureto formada entre Cis 66 e Cis 160. Os triptofanos expostos a solventes exibem um rendimento quântico menor, que pode ser extinto pela proximidade com ligações dissulfeto. O Trp 19, por outro lado, encontra-se enterrado no cálice e, devido à sua inacessibilidade relativa a solventes espera-se que seja o único Trp fluorescente (CROGUENNEC et al., 2004b).

Além da cavidade interna do β-barril, que é um sítio ideal para ligantes hidrofóbicos, três outras regiões da BLG são descritas como locais para a ligação de ligantes: a superfície hidrofóbica em forma de bolsa entre a α-hélice a e o β-barril, a superfície exterior perto dos resíduos Trp19-Arg124 e a interface monômero/monômero (LIANG & SUBIRADE, 2010; MENSI et al., 2013).

A BLG pode se ligar a moléculas hidrofílicas, hidrofóbicas ou anfipáticas. As constantes de ligação para diferentes compostos com BLG variam amplamente de $1,5X10^{2}$ $mol.L^{-1}$ para 2-heptanona, $6,8X10^{5}$ $mol.L^{-1}$ para palmitato até $5,0X10^{7}$ $mol{\cdot}L^{-1}$ para retinol (LIANG et al., 2008). Compostos aromáticos polares, tais como fosfato de p-nitrofenil, 5-fluorocitosina, elipticina e protoporfirina, ligam-se ao sítio localizado na superfície externa da proteína. Um estudo de fluorescência dinâmica sobre a interação de BLG com 1-anilinonaftaleno-8-sulfonato indicou dois locais de ligação incluindo os locais de ligação internos e externos (COLLINI et al., 2000). Foi relatado ainda, que a BLG pode se ligar a íons metálicos, ácidos graxos, vitaminas, fármacos e polifenóis (SNEHARANI et al., 2010; DIARRASSOUBA et al., 2013; LE MAUX et al., 2014; GHALANDARI et al., 2015).

Compostos hidrofóbicos tais como a curcumina ligam-se a BLG predominantemente por interações hidrofóbicas dentro do cálice, enquanto que os

compostos hidrofílicos podem ligar-se a sítios externos (SNEHARANI et al., 2010). O BC, que tem elevada hidrofobicidade, apresenta uma afinidade importante para BLG. O retinol e o ácido palmítico se ligam na mesma cavidade interior, ao contrário dos carotenoides, que possuem diferentes locais de ligação (MENSI et al., 2013).

A ligação ao BLG também impacta nas propriedades físicas e químicas e na atividade biológica dos compostos bioativos. BLG pode formar complexos solúveis em água com retinol e BC e poderia assim proteger estes compostos lipofílicos da degradação por calor, oxidação e irradiação (HATTORI et al., 1995). O uso de BLG é pelo menos tão eficiente quanto a formação de emulsão/micelas para promover a absorção intestinal de β-caroteno (MENSI et al., 2014). A complexação com a proteína proporciona um aumento significativo na hidrosolubilidade do composto anfipático trans-resveratrol e um ligeiro aumento na sua fotoestabilidade (LIANG et al., 2008).

Alguns estudos relataram anteriormente a interação entre BC e BLG, com constantes de dissociação aparentes menores do que as encontradas para outros ligantes tais como, retinol, retinal e ácido palmítico, indicando capacidade de ligação elevada (DUFOUR & HAERTLÉ, 1991; MENSI et al., 2013; HOSSEINI et al., 2015). No entanto, existe uma falta de informações relativas à influência do pH e da desnaturação pelo calor na ligação do BC a BLG.

Além de suas muitas funções fisiológicas e farmacêuticas, a LIS também tem a capacidade de transportar fármacos. Os estudos sobre as interações entre fármacos e LIS têm grande importância para elucidação do processo de transporte e metabolismo de fármacos, relação estrutura/função da LIS e na essência química da interação entre a biomacromolécula e pequenas moléculas (WANG et al., 2009).

Vários estudos de ligação entre LIS e moléculas pequenas, como a vitamina C e B12, a baicaleína, a benzocaína, o ácido retinóico e o cloranfenicol foram investigados nos últimos anos (DING et al., 2009; LI et al., 2013; YANG et al., 2015).

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CAPÍTULO 2 - EFFECT OF pH AND NaCl ADDITION ON EMULSIFYING PROPERTIES OF LYSOZYME AND β-LACTOGLOBULIN MIXTURES

ABSTRACT

β-lactoglobulin (BLG) is an abundant whey protein with emulsifier, nutritional and transport properties, largely used as an ingredient. It has a low isoelectric point (IEP) that allows its electrostatic interaction with high IEP proteins such as lysozyme (LYS), an egg white protein with IEP in pH~10.7. Emulsions may be used to incorporate lipophilic compounds, dissolving and releasing them in aqueous mediums. In this study, we investigated the interaction between LYS and BLG in O/W emulsions in order to explore the possibility of improving emulsion stability using these two proteins. The effect of pH and salt addition on its emulsifying properties (interfacial protein concentration and composition, oil droplet size and charge, flocculation) and kinetic stability of emulsion was evaluated. The pH influenced mainly the surface charge of emulsions affecting the interaction of proteins, final size and stability of emulsions. The increase of ionic strength influenced negatively the stability of emulsions, mainly due charge screening, what increased droplets sizes and led to phase separation.

Keywords: protein mixtures, emulsion stability, interfacial tension, interfacial concentration

1. Introduction

Different nutrient encapsulation systems for food application, based on emulsified systems have been developed using proteins such as whey proteins(CHEN et al., 2006; EGAN et al., 2013; WANG et al., 2016; CHEN et al., 2017), soy and wheat (CHEN & SUBIRADE, 2009), caseins(LESMES et al., 2010; ZIMET et al., 2011); or polysaccharides, such as alginate, carrageen, pectin and chitosan (GUZEY & MCCLEMENTS, 2006; BENGOECHEA et al., 2011; BENJAMIN et al., 2012; WEI & GAO, 2016). However, there are several challenges for the development and production of food grade delivery systems, such as avoiding product toxicity, ensuring chemical stabilization of bioactive ingredients and sensorial characteristics, digestibility and release of bioactives (TOKLE et al., 2010).

Emulsions may be defined as a mixture of two liquids with low solubility in each other, wherein one liquid, the dispersed phase, is dispersed in the other, the continuous phase (WALSTRA, 2003). The interfacial tension, generated by asymmetry of intermolecular interactions between the molecules of the two liquid phases, can be significantly reduced by the addition of amphiphilic molecules, or surfactants (MASON et al., 2006).

The ability of milk proteins to adsorb at the oil/water interface and stabilize emulsions has been explored by the food industry in the preparation of nutritional products, food formulations, cream liqueurs and dairy desserts (SINGH, 2011). Milk proteins are known to be excellent emulsifiers due their amphiphilic nature (WALSTRA et al., 2010), reducing the surface tension at the oil/water interface and forming interfacial films with different rheological properties, making it possible to obtain stable emulsions at relatively low proportions of protein/oil (about 1:60). In such emulsions, the interfacial concentration of protein increases with increasing protein concentration, reaching a maximum value of about 2.0-3.0 $mg.m^{-2}$ (WALSTRA, 2003; SINGH, 2011).

β-Lactoglobulin (BLG) is naturally found in milk whey, has a molar mass of 18.3 $kg.mol^{-1}$ and isoelectric point at pH≈5 (CHEFTEL et al., 1985). It belongs to the family of Lipocalins (proteins with transport function) whose main characteristic is its ability to bind small lipophilic molecules in its central hydrophobic cavity in order to minimize the contact of these molecules with polar solvents (FLOWER, 1996). Among these

molecules, can be mentioned retinol, fatty acids, fat-soluble vitamins and cholesterol (FLOWER et al., 2000; KONTOPIDIS et al., 2002).

Egg white proteins are also used as ingredient in the food industry due their functional properties, which include gelling ability, foaming and emulsifying properties (HANDA et al., 1998). Lysozyme (LYS) is a protein extracted from egg white with a molar mass around 14 $kg.mol^{-1}$, a molecule radius of 15 Å and isoelectric point at pH≈10.7 (TYN & GUSEK, 1990). Given its basic nature, it affects the properties of other proteins by interacting with them (LI-CHAN et al., 1995). LYS is a more compact protein, whereas BLG has a more flexible structure, which implies BLG to have higher affinity to adsorb at the oil/water interface (ZHU et al., 2007; SCHRÖDER et al., 2017).

Technological properties of proteins are determined by their molecular characteristics such as molar mass, conformation, flexibility, polarity and hydrophobicity (MCCLEMENTS et al., 2009). During adsorption at an oil/water interface, the protein can unfold and orient the hydrophobic parts towards the oil phase and the polar ones towards the aqueous phase (SCHRÖDER et al., 2017). The properties of the solution, such as pH and ionic strength, as well as the interaction of these biopolymers with other components present in foods, also affect its functionality (DICKINSON, 2003; SINGH & SARKAR, 2011).

In this study, intending to overcome the instability of single protein emulsions, we produced O/W emulsions using BLG and LYS (single and mixed) as interfacial agents. Emulsifying properties (oil droplet size, flocculation, interfacial protein concentration and composition) and kinetic stability of such emulsions were determined.

2. Materials and Methods

2.1 Materials

Chicken egg white Lysozyme (> 90%) was purchased from Sigma-Aldrich Chemical (St. Louis, MO, EUA), bovine β-lactoglobulin (> 90%) was purchased from Davisco Food International Inc. (La Sueur, MN, EUA). Sunflower oil (Liza, Cargill Foods, SP, Brazil) was purchased at a local market. All others chemicals were of analytical grade and deionized water made by Milli-Q system (Millipore, USA) with sodium azide 0.02% was used throughout the experiments.

2.2 Solution preparation

Protein (1.4 mmol.L^{-1}) stock solutions were prepared by dispersing 1.12 g of LYS, 1.43 g of BLG in 50 ml of deionized water containing 0.02% w/v sodium azide (anti-microbial agent) and kept under refrigeration overnight for complete solubilization. The pH of the solutions was adjusted to 4.0 or 7.0 using HCl or NaOH (0.1 M).

Mixtures with final protein concentration of 0.7 mmol.L^{-1} and different LYS:BLG molar ratios (1:0, 3:1, 1:1, 1:3 and 0:1) were prepared from stock solutions at different pH (4.0 and 7.0) and with NaCl concentration of 0 or 100 mmol.L^{-1}.

2.3 Dynamic surface tension determination

The dynamic surface tension of the solutions prepared accord to item 2.2 was assessed by a pendant drop analysis using a contact angle and surface tension measurer (Easy drop, DSA 25, Krüss, Germany), attached to a volume control system for constant injection flow during drop formation. The variation of the Laplacian shape of a pendant drop with a constant volume of 15 µL in the protein solution was measured as a function of adsorption time (15 min) and was used to determine the time dependence of the drop surface tension. The drops were formed on the tip of a stainless steel needle (diameter=1.8 mm) and the drop image was monitored by the equipment software where the interfacial tension between phases was determined by the Laplace equation. The measurements were performed at ambient temperature (25 °C).

The equilibrium surface tension (γ_{eq}) was determined by adjusting an exponential equation (1) to the experimental data of surface tension decay.

$$\gamma = \gamma_{eq} + Aexp(-Bt) \quad (1)$$

where $\gamma(mNm^{-1})$ is the surface tension at time t (s), γ_{eq} is the equilibrium surface tension and $A(mNm^{-1})$ and B (s^{-1}) are constants.

2.4 Emulsion preparation

Oil/water emulsions were prepared using as continuous phase water solutions of lysozyme or β-lactoglobulin as well as mixtures of the two proteins (LYS:BLG molar ratios of 3:1; 1:1 and 1:3, at a total protein concentration of 0.7 mmol.L^{-1}). The continuous phases at fixed pH and NaCl concentration were mixed with the oil phase to obtain 10% sunflower oil and 90% aqueous phase (w/w). The emulsions were prepared by mixing the phases in a high speed blender (Omni Macro ES, USA) equipped with a 25 mm diameter probe at 7500 rpm for 3 min, followed by four passes through a high-pressure homogenizer (EmulsiFlex, Avestin, Canada) at 10,000 psi, at room temperature.

2.5 Emulsion characterization

2.5.1 Determination of interfacial protein concentration and composition

To estimate the protein concentration in the continuous phase, the emulsions were centrifuged at 15,000 x g for 40 min at 20 °C (5804R, Eppendorf, Germany) for phase separation. The bottom aqueous phase, that contained the non-adsorbed proteins, was carefully extracted with the aid of a syringe, dispersed in ultrapure water and centrifuged again at the same condition. The two bottom-phase were collected and filtered through PVDF syringe filters with a pore size of 0.45 μm.

The quantification of the non-adsorbed protein was performed by high-performance liquid chromatography (HPLC) (LC-10AD VP, SHIMADZU, Japan). The Chromatographic separation was performed with a reverse phase column apHera™ C18 Polymer (25 cm × 4.6 mm; 5 μm, Supelco). Elution was carried out at 40 °C and flow rate of 0.5 mL.min^{-1}, using as mobile phase a mixture of deionized water, pH 2.5 (solvent A) and acetonitrile (solvent B) with the following gradient: 0-8 min; 30-60% B; 8-10 min: 60-80% B; 10-20 min: 80-30% B; 20-30 min: 30% B. The injection volume was 50 μL and the detector wavelength was set at 215 nm. Quantification was based on peak areas.

The interfacial protein concentration (Γ, mg.m^{-2}) was estimated according to Eq. 2, where $(C_i - C_b)$ is the difference between the amount of protein used to prepare the emulsion and the protein present in the continuous phase (mg.mL^{-1}), and S_o (m^2.mL^{-1} of

emulsion) is the surface area of the oil droplets determined from the Eq. *3* (YE & SINGH, 2006).

$$\Gamma = \frac{(C_i - C_b)}{S_o} \quad 2$$

$$S_o = \frac{6\phi}{d_{32}} \quad 3$$

where ϕ is the droplet volume fraction, d_{32} is the volume-surface mean droplet diameter. The composition of proteins present on the surface was calculated as the percentage of total protein content.

2.5.2 ζ-potential

ζ-potential measurements were performed in a Laser Doppler Micro-electrophoresis instrument (Zetasizer Nano-ZS, Malvern Instruments Ltd, Southborough, UK). The ζ-potential was calculated from the electrophoretic mobility, determined by measuring the direction and speed of movement of a droplet in a well-defined electric field.

The emulsions were subjected to the analysis immediately after preparation and after 7 days of storage, at 25.0±0.5 °C. Samples were appropriately diluted to a droplet concentration of about 0.05% (w/w) with ultrapure water in the corresponding pH and NaCl concentration. Then, the emulsions were poured in capillary cell with gold electrodes and analyzed to a 173° scattering angle at 25.0±0.1 °C.

The ζ-potential (mV) was calculated from the electrophoretic mobility (μ, $V.m^2.s^{-1}$) using the Henry equation (Eq. 8).

$$\mu = \frac{2\varepsilon\zeta}{3\eta} f(ka) \quad 4$$

where ε is the electrical permeability ($F.m^{-1}$), η is the solvent viscosity (Pa.s) and $f(ka)$ was 1.5 which is the Smoluchoski approximation. ζ potential was automatically determined from the Zetasizer Software. Each individual result was automatically

calculated as the average of 5 measurements and the results were expressed as mean and standard deviation of at least two repetitions.

2.5.3 Droplet size measurements

The droplet size distributions were determined by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd, Southborough, UK). This technique measures the diffusion of particles moving under Brownian motion, and converts this to size and size distribution using the Stokes-Einstein relationship.

The emulsions were subjected to analysis immediately after preparation and after 7 days of storage, at 25 °C. The particle diameter range and the number of photon counts per second (kilo count per second -kcps) were measured at 25.0±0.1 °C. To avoid multiple scattering effects, freshly prepared emulsions were diluted to a concentration of 0.05% w/w using deionized water containing 0.02% of sodium azide, at the corresponding pH and NaCl concentration.

The particle size was reported as z-average diameter and the polidispersity index. The data from size measurement was submitted to cumulant analysis from where the z-average diameter and PDI values were extracted. Each individual result was automatically calculated as the average of 5 measurements with 20 sub-runs each. The results were expressed as mean and standard deviation of at least two repetitions.

2.5.4 Physical stability

The physical stability of the emulsions was assessed by the creaming stability test, where aliquots of emulsion (5 g) were transferred to sealed graduated glass tubes and stored for 7 days at 25.0±0.5 °C, protected from light. Sudam III (a red dye) was previously added to sunflower oil before the homogenization process to facilitate the visualization of phase separation. Aliquots of 2 mL were also stored in sealed tubes for further evaluation of the droplet size distribution and the ζ-potential. Photographic images were taken using a digital camera after storage.

The extent of creaming was characterized by the creaming index (CI) that features the serum layer formed at the bottom of the tubes. The total height of the emulsion (H_t, cm) and the height of the aqueous layer (H_a, cm) resulting from creaming of the oil

droplets during storage were measured. The creaming index (CI) was calculated from the Eq. Eq. 9 (GU et al., 2004).

$$IC = \frac{H_a}{H_t} \times 100\% \qquad 5$$

Results were expressed as mean and standard deviation of at least two repetitions.

2.6 Experimental design and statistical analysis

The emulsifying properties of LYS/BLG stabilized emulsions were compared within different pH (4.0 and 7.0), NaCl addition (0 and 100 mmol.L^{-1}) and LYS:BLG mixtures molar ratios (1:0, 3:1, 1:1, 1:3 and 0:1). The chosen pH represent two different condition were the charge are opposes and in presence or absence of NaCl. The emulsion was characterized by droplet size, surface electrical charge (ζ-potential), protein surface concentration and composition and creaming index (CI). A 2x2x5 factorial was performed to evaluate the effect of these factors on emulsion characteristics.

The experiments were conducted in a random order to minimize the effect of external variables. The data collected from the experiments were subjected to analysis of variance ($p<0.05$). Means comparisons were performed by Tukey test at 5% error probability. Mean comparisons through time were performed by a paired t-test ($p<0.05$).

The adequacy of fitted models for surface tension decay was evaluated by the statistical significance of the model ($p<0.05$) and the coefficient of determination (R^2). The significance of the model parameters was assessed by Student's t test ($p <0.05$). All statistical analyzes were performed using SAS software (version 9.0, SAS Institute Inc., Cary, NC, USA), available for the Universidade Federal de Viçosa (UFV).

3. Results and discussion

3.1 Protein characterization

Initially, the ζ-potential of individual proteins and protein mixtures were determined (Figure 2.1) in order to support further insights into their behavior in

emulsions. The solutions were prepared in different pH (4.0 and 7.0) and NaCl addition (0 and 100 mmol.L^{-1}).

The ζ -potential of LYS solution without NaCl varied from +21 mV at pH 4.0 to +12 mV at pH 7.0. This finding is in agreement with the fact that as the pH is increased approaching to the IEP point of LYS (≈10.7) when less NH^{3+} radicals are ionized decreasing the value of ζ-potential. On the other hand, the ζ-potential of BLG solution changed from +14 mV to –12 mV as the pH changed from 4.0 to 7.0, as the IEP of BLG is between those pH (≈ 5.0).

The ζ-potential decreased, in modulus, with salt addition for both proteins due to the reduction of the electrical double layer (BENGOECHEA et al., 2011). This effect seems to be more pronounced for LYS, which showed a higher decrease in ζ-potential. This may be related with either the conformation of individual protein molecules or the structure of the protein in the solution that change upon addition of salt.

When the proteins were mixed at pH 7.0, the net electrical charge approaches zero for 1:1 LYS:BLG molar ratio, indicating charge neutralization once the proteins exhibited opposite charges. In mixtures with higher molar ratios (1:3 and 3:1) the ζ-potential was closer to the one of the protein in higher proportion. These results are in agreement with previous studies that have reported similar behavior for LYS/BLG mixtures (DIARRASSOUBA et al., 2015) and for Lactoferrin/BLG mixtures (TOKLE et al., 2012).

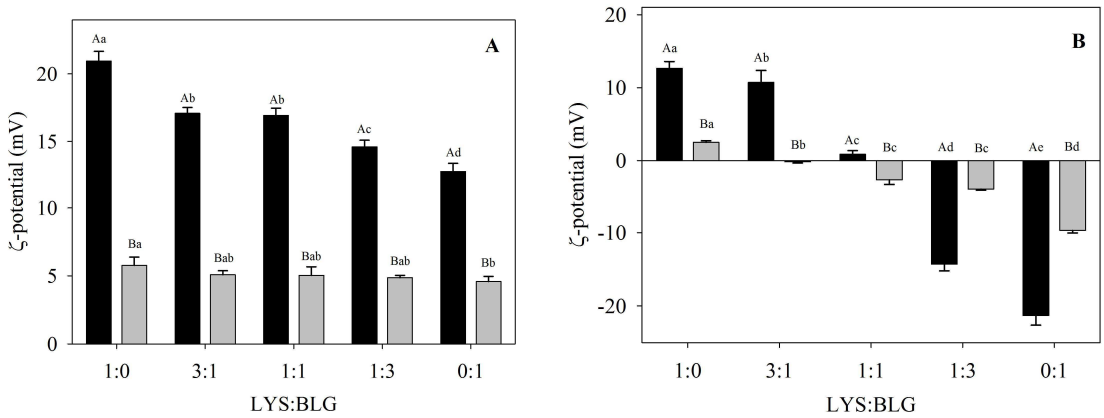

Figure 2.1 – Dependence of the particle electrical charge (ζ-potential) on LYS:BLG mixtures composition at NaCl concentration of 0 (black) and 100 mmol.L^{-1} (grey) at pH 4.0 (A) and 7.0 (B).

Means followed by the same letters, lowercase for same NaCl concentration and uppercase in same protein ratios, do not differ among themselves ($p>0.05$) by Tukey's test.

3.2 Dynamic surface tension

In order to evaluate the behavior of the proteins at the air/water (A/W), the surface tension reduction was measured by a drop shape analysis using a pendant drop tensiometer. Figure 2.2 and Figure 2.3 present the dynamic behavior of the superficial tension for 900 s, for pure proteins and LYS:BLG mixtures of 3:1, 1:1 and 1:3 molar ratios, at pH 4.0 and 7.0.

The curves indicate the dynamics of surface adsorption. BLG presented faster rates of adsorption to the surface than LYS at both pH as shown by a rapid decrease in the surface tension. It may influence in the final composition at the interface.

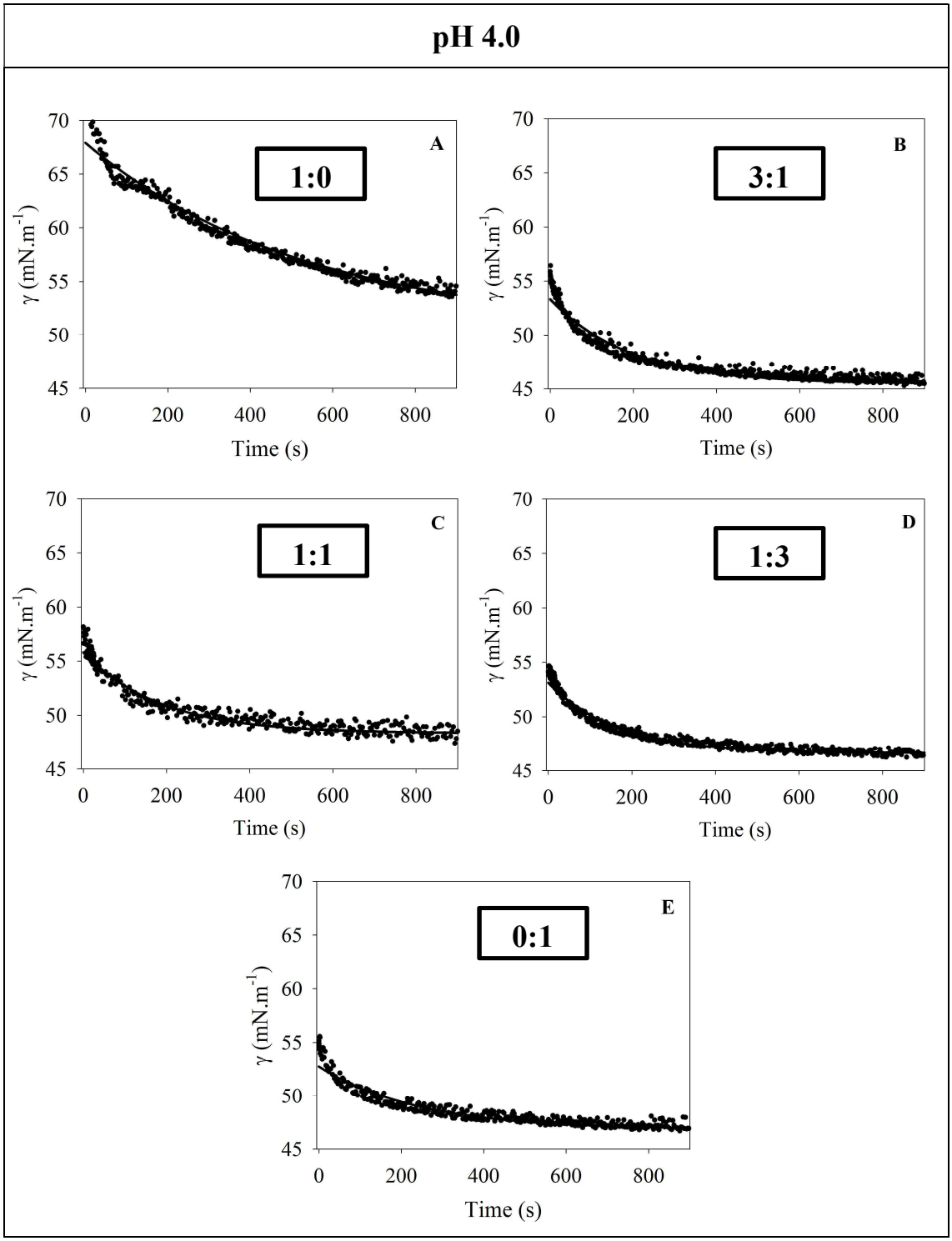

Figure 2.2 – Dynamic behavior of surface tension for Lysozyme (A), mixture of Lysozyme and β-lactoglobulin with molar ratios of 3:1 (B); 1:1 (C) and 1:3 (D) and β-lactoglobulin (E), at pH 4.0.

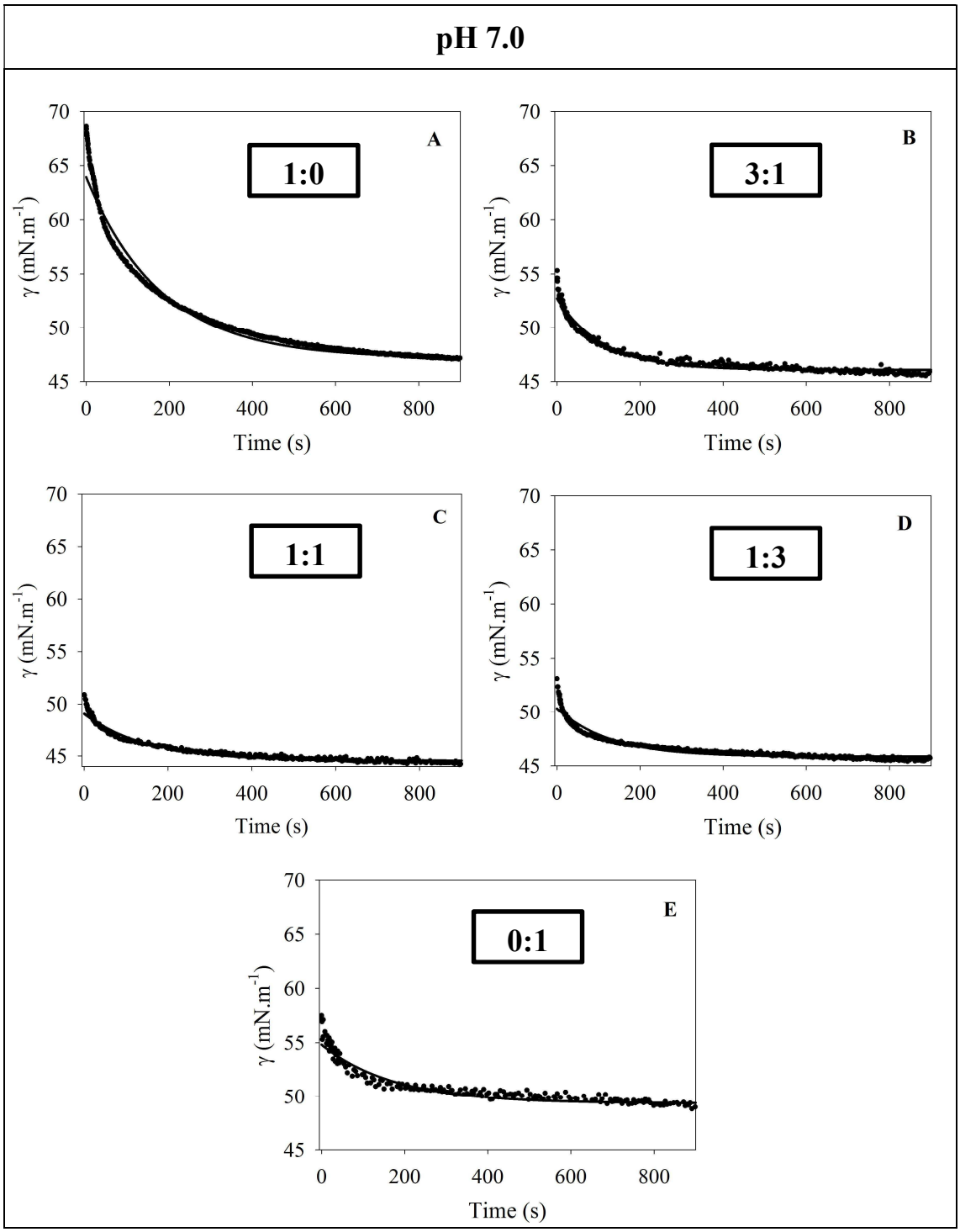

Figure 2.3 – Dynamic behavior of surface tension for Lysozyme (A), mixture of Lysozyme and β-lactoglobulin with molar ratios of 3:1 (B); 1:1 (C) and 1:3 (D) and β-lactoglobulin (E), at pH 7.0.

Once LYS is less flexible it takes more time to organize at the surface and reach equilibrium (BEVERUNG et al., 1999). LYS presented lower superficial tension reduction, in this concentration, probably due to its highly hydrophobic and stable character, with surface hydrophobicity being responsible for the superficial tension dynamic of this protein at initial times. According to BEVERUNG et al. (1999), LYS adsorbs strongly, but retains a more compact structure and this greater stability seems to promote the long time dynamics of surface tension decrease. The rigid globular structure of LYS hinders its surface unfolding and intermolecular contacts, which explains the slower tension reduction observed for both pH.

The dynamic of adsorption of LYS is indeed peculiar once it may have an initial plateau depending on the concentration (ALAHVERDJIEVA et al., 2008). At the concentration used this phenomena was not determinant and the exponential equation could still be adjusted satisfactorily, but it explain slower tension reduction of LYS. At later times, surface hydrophobicity becomes less important, as conformational changes promoting further aggregation determine the interfacial tension (SCHRÖDER et al., 2017). The ability of molecules adsorbing to the surface is related to its stronger surface activity and flexibility (DAMODARAN, 2005; SCHRÖDER et al., 2017).

When BLG and LYS were mixed, the superficial tension was reduced in higher extension than the proteins alone. This phenomenon was also observed for LYS and ovalbumin mixtures (LE FLOCH-FOUÉRÉ et al., 2010). However, it seems that BLG influenced the dynamic more strongly, as shown by the sharper decrease at initial times, even for the mixtures.

At pH 7.0, where the proteins exhibited opposite charges, it was observed a rapid decrease in the surface tension of the mixtures, probably due to a faster interfacial layer formation. On the other hand, at pH 4.0 the mixtures exhibited a slower decrease in the surface tension because of rearrangements and exchange of molecules at the surface.

The equilibrium surface tension values were obtained by the adjustment of Eq. 1 to the interfacial tension data and are shown in Table 2.1. All adjustments exhibit R^2>0.95. As LYS at pH 4.0 did not reach equilibrium at the time window of 900 s, data was collected for another 600 s and the total data was used for the adjustment (data not shown).

Table 2.1 – Equilibrium surface tension (γ_{eq}, $mN.m^{-1}$) for Lysozyme and β-lactoglobulin mixtures in different molar ratios and pH obtained by adjustment of the equation 1.

pH	LYS:BLG ratio				
	1:0	3:1	1:1	1:3	0:1
4.0	50.3 ± 0.6^{Aa}	44.8 ± 0.7^{Ca}	44.9 ± 0.2^{Ca}	46.2 ± 0.8^{Ba}	47.0 ± 0.2^{Bb}
7.0	47.6 ± 0.6^{ABb}	46.9 ± 1.2^{BCa}	45.0 ± 0.6^{Da}	45.5 ± 0.6^{CDa}	49.2 ± 0.0^{Aa}

Means followed by the same letters, uppercase in lines and lowercase in columns, do not differ among themselves ($p>0.05$) by Tukey's test.

Both protein showed different surface activity at pH 4.0 and 7.0 though with different trend. LYS presented lower equilibrium surface tension value than BLG at pH 7.0 and BLG was more active at pH 4.0. As can be seen from the results of ζ-potential (Figure 2.1) the charge density of BLG is greater at pH 7.0 than at pH 4.0, so the adsorption was expected to be greater at pH 7.0, leading to a more effective surface tension reduction. Furthermore, at pH 4.0 a greater extension of dimers/tetramers are formed comparing to pH 7.0, and these bigger structures are less flexible resulting in lower superficial activity (TAULIER & CHALIKIAN, 2001). An increase in the pH, decreased the surface activity for BLG, in a similar behavior to that found by ENGELHARDT et al. (2013), whose surface tensions changed from 47.6 $mN.m^{-1}$ at pH 3.0 to 51.5 $mN.m^{-1}$ at pH 6.8.

The values of the equilibrium surface tension of LYS obtained in this work were slight smaller than 53 $mN.m^{-1}$, obtained by LE FLOCH-FOUÉRÉ et al. (2010) at pH 7.0 and prepared with phosphate buffer, what may explain the difference. LYS is indeed less recognized by its interfacial properties by itself, the main benefit of studying it is the possibility of complexing with other proteins of smaller IEP.

As observed for the dynamic of surface tension reduction, the proteins interact at the surface resulting in additive effect for surface tension reduction. The lack of protein net charge implies in weaker repulsive interactions between the protein molecules, increasing protein adsorption at the interface leading to the lower surface tension values (MERCADANTE et al., 2012). Surface activity of proteins mixtures was higher than the proteins alone, apparently due high adsorption at the interface in charge nulling conditions.

3.3 Emulsion characterization

Protein-coated emulsions are charged, which leads to electrostatic repulsion between neighboring protein molecules at the interface, as well as between the two neighboring emulsion droplets. The electrostatic repulsion keeps the adsorbed protein molecules at a certain distance from each other and hampers the formation of both non-covalent and covalent bonds within the adsorption layer (TCHOLAKOVA et al., 2006).

Therefore, the stability of such emulsions is governed mainly by long-ranged electrostatic and van der Waals forces. NaCl addition and pH are important factors governing the electrostatic repulsion, the structure of the adsorption layers and the emulsion stability.

Emulsions of LYS, BLG and LYS:BLG mixtures (3:1, 1:1 and 1:3 molar ratio) were prepared by high pressure homogenization at the pH 4.0 and 7.0 and with or without NaCl addition. The emulsions were characterized in terms of interface composition, size distribution, surface charge, and physical stability.

3.3.1 Interface composition

The composition of the O/W interface was determined by the difference of protein composition and concentration at the bulk and the total protein used in the emulsion preparation (Figure 2.4). The importance of this data is to show the disposal of protein molecules at the interface. The amount of adsorbed protein and the structure of the adsorption layer play a very significant role in protein emulsion stability, that may occur either by a viscoelastic adsorption layer or a significant surface force barrier against coalescence (TCHOLAKOVA et al., 2005).

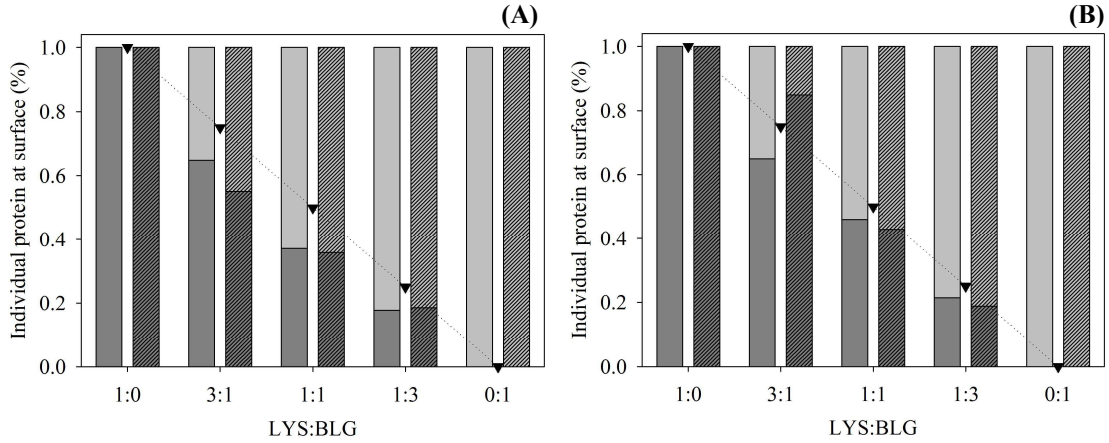

Figure 2.4 – Relative proportions of lysozyme (darker grey) and β-lactoglobulin (lighter grey) at the droplet surface of emulsions made of different LYS:BLG mixtures composition at NaCl concentration of 0 (solid) and 100 mmol.L^{-1} (crosshatched) at pH 4.0 (A) and 7.0 (B).
The dotted line represents the proportion of the proteins used to prepare the emulsion.

For almost all the conditions, BLG exhibited higher proportion at the interface than LYS. Generally, the protein that diffuses faster will prevail at the interface at initial times and its molecular flexibility and surface hydrophobicity will dictate their maintenance at the interface (RAIKOS, 2010). Probably, the BLG molecules displace LYS from the interface during the emulsification, as they are more flexible and adsorb more strongly at the interface as observed for ζ-potential values close to BLG ones (PUPPO et al., 2005). This behavior is less marked at pH 7.0 at which a complex occurs leading to accumulation of both proteins at the interface.

The NaCl addition was expected to increase protein adsorption to the interface, but did not change the proportion. The only significant difference was found for 3:1 LYS:BLG ratio, where the proportion of LYS at the interface overcome the bulk proportion, probably due adsorption of more than one layer of proteins. As seen above, at pH 7.0, especially after NaCl addition a higher adsorption occur, mainly for LYS.

Interfacial concentration (Γ, mg.m^{-2}) values were obtained from the ratio of the concentration at the interface by the total surface area and the results are shown in Table 2.2 and Table 2.3.

Table 2.2 – Interfacial concentration of LYS ($mg.m^{-2}$) for different molar ratio of LYS:BLG mixtures, NaCl concentration and pHs.

pH	NaCl ($mmol.L^{-1}$)	LYS:BLG molar ratio				
		1:0	3:1	1:1	1:3	0:1
7.0	0	2.1 ± 0.3^{Bb}	4.4 ± 0.2^{Aab}	7.8 ± 0.1^{Ba}	1.6 ± 0.1^{Ab}	-
	100	7.8 ± 0.4^{Ab}	8.1 ± 0.1^{Aa}	13.0 ± 0.7^{Aa}	3.4 ± 0.1^{Ab}	-
4.0	0	4.5 ± 0.9^{Aba}	3.5 ± 0.04^{Ca}	1.9 ± 0.2^{Ca}	1.6 ± 0.2^{Aa}	-
	100	2.3 ± 0.2^{Ba}	3.5 ± 0.5^{Ca}	3.5 ± 0.05^{Ca}	1.9 ± 0.1^{Aa}	-

Means followed by the same letters, lowercase in lines and uppercase in columns, do not differ among themselves ($p>0.05$) by Tukey's test.

Table 2.3 – Interfacial concentration of BLG ($mg.m^{-2}$) for different molar ratio of LYS:BLG mixtures, NaCl concentration and pHs.

pH	NaCl ($mmol.L^{-1}$)	LYS:BLG molar ratio				
		1:0	3:1	1:1	1:3	0:1
7.0	0	-	2.4 ± 0.2^{Ac}	9.2 ± 1.4^{Ba}	6.0 ± 0.6^{Bbc}	6.7 ± 1.4^{Bb}
	100	-	1.4 ± 0.2^{Ac}	17.3 ± 1.0^{Aa}	14.7 ± 1.0^{Aa}	8.8 ± 0.7^{Bb}
4.0	0	-	1.9 ± 0.04^{Ab}	3.3 ± 0.7^{Cb}	7.5 ± 0.2^{Ba}	8.0 ± 1.5^{Ba}
	100	-	2.9 ± 0.1^{Ac}	6.4 ± 0.4^{BCbc}	8.6 ± 0.5^{Bb}	13.6 ± 0.9^{Aa}

Means followed by the same letters, lowercase in lines and uppercase in columns, do not differ among themselves ($p>0.05$) by Tukey's test.

At pH 7.0 the proteins BLG and LYS are oppositely charged, which resulted in the formation of complexes by electrostatic interactions when they are mixed. Protein complexation enhanced the amount of protein available at the interface. Conversely, at pH 4.0 an independent adsorption would occur at the interface and the final composition would be defined by competition during adsorption phase.

Theoretical results for Γ corresponding to a single layer of BLG or LYS deposited at the interface were 2-3 $mg.m^{-2}$ and 1.9 $mg.m^{-2}$, respectively (ACTON et al., 1990; ERICKSON et al., 2000; TCHOLAKOVA et al., 2005). According to MARTINET et al. (2005), larger Γ can be resulted of aggregation of molecules on the surface and occurrence

of more than one layer. The results found in this work (> 5 $mg.m^{-2}$) indicated the occurrence of more than one layer at the interface of emulsions.

Interface concentration is highly influenced by droplet size. Therefore, aggregation may overestimate values of interfacial concentration, which have to be analyzed with parsimony. It happens because large diameters will result in small area compared to small diameters, and it is a square proportion, i.e., a droplet diameter 10 times bigger than other will result in a superficial area 100 times greater (MCCLEMENTS, 2007).

In general, proteins exhibits a maximum adsorption density near or at the IEP (IDENYI et al., 2006). However, there were no differences ($p>0.05$) in Γ for LYS nor BLG at pH 4.0 and 7.0. Only emulsions with 1:1 molar ratios showed difference in Γ for pH 4.0 and 7.0. At pH 7.0 it was observed higher values for Γ.

When both proteins are in a higher proportion (3:1 or 1:3 molar ratios), Γ approached the values for pure proteins ($p>0.05$). LYS/BLG electrostatic complexes may be enhanced by NaCl addition, what facilitated LYS adsorption when BLG was presented (DAMODARAN et al., 1998). Indeed, Γ_{LYS} was the same for 3:1 and 1:1 molar ratios than for pure LYS, at pH 4.0, even starting from a smaller concentration of LYS. For pH 7.0, Γ_{LYS} was the same for 1:3 molar ratio and bigger for 1:1 comparing to pure LYS.

At pH 4.0, NaCl addition increased Γ_{BLG} for 1:1 and 1:3 molar ratio and for pure protein. The same behavior was observed at pH 7.0, however, the increase was more prominent, probably due reduction in electrostatic repulsion (SRINIVASAN et al., 2000).

3.3.2 Surface charge

When measuring the ζ-potential, the important focus is on the magnitude of the ζ-potential and not whether it is positive or negative. The larger the magnitude of the ζ-potential, more repulsion will occur, decreasing aggregation leading to increasing dispersion stability. If the ζ-potential is low, then there are no enough forces to repel the particles from one another, and the dispersion instability will give way to aggregation. A typical line that divides stability and instability is at +30 mV or -30 mV (MCCLEMENTS, 2005). The higher the magnitudes beyond those values, the more stable the colloidal system.

The ζ-potential of the emulsion droplets was determined by measuring the electrophoretic mobility of oil droplets dispersed in aqueous solution of desired pH and salt concentration (Table 2.4).

Table 2.4 – Droplet electrical charge (ζ-potential, mV) of emulsions with different LYS:BLG mixtures composition, NaCl concentration and pHs.

pH	NaCl ($mmol.L^{-1}$)	LYS:BLG molar ratio				
		1:0	3:1	1:1	1:3	0:1
7.0	0	38.9 ± 4.1^{Ba}	6.8 ± 1.1^{Bb}	-1.5 ± 0.2^{Cb}	-17.2 ± 3.9^{Cc}	-48.8 ± 2.6^{Dd}
	100	20.9 ± 1.6^{Ca}	8.6 ± 2.1^{Bb}	-13.7 ± 4.3^{Dc}	-16.0 ± 1.0^{Cc}	-24.8 ± 1.2^{Cc}
4.0	0	58.6 ± 4.0^{Aa}	23.3 ± 4.6^{Ab}	24.6 ± 0.0^{Ab}	25.0 ± 1.4^{Ab}	27.0 ± 0.1^{Ab}
	100	18.7 ± 2.0^{Da}	13.3 ± 2.5^{ABa}	11.2 ± 1.0^{Ba}	11.6 ± 1.3^{Ba}	16.2 ± 1.0^{Ba}

Means followed by the same letters, lowercase in lines and uppercase in columns, do not differ among themselves ($p>0.05$) by Tukey's test.

Droplet electrical charges in emulsions are different when compared to pure proteins in solution. Adsorption may expose/hinder charged patches presented at the protein surface modifying the net electrical charge. Also, once the new interface formed is bigger, counter ions may interact with some patches of the molecules, modifying the double electric layer and consequently the ζ-potential. The effect of pH on protein stabilized emulsions is generally related to pH-induced conformational changes in proteins (DAMODARAN et al., 2010; CHEN et al., 2017).

ζ-potential of emulsions formed by protein mixtures were lower than |30| mV, indicating poor stability due electrostatic repulsion. LYS formed cationic emulsion droplets at both pH, with the ζ-potential of the emulsions at pH 4.0 being higher than that of the emulsion at pH 7.0, which is closer to its IEP. Differently, BLG formed anionic droplets at neutral pH and cationic droplets at pH 4.0.

At pH 4.0, the ζ-potential was influenced by BLG, even for high proportion of LYS (3:1). The BLG predominated at the interface for both pH as can be seen by a rapid trend of ζ-potential values of mixtures toward the ζ-potential of emulsions containing only BLG. LYS molecules located at the interface may have been dislocated by BLG molecules promoted by electrical repulsion.

At pH 7.0, on the other hand, the proteins present opposite charge with the same magnitude, resulting in ζ-potential close to zero on same molar ratio. The fact that the droplets became neutrally charged at 1:1 molar ratios, at pH 7.0, suggests that there was an electrostatic attraction between LYS and BLG at the interface (TOKLE et al., 2012).

NaCl addition decreased the absolute value of ζ-potential once it reduces the double layer. The NaCl addition also approximate the molecules of the complex formed by decreasing screening effects, leading to increase of the protein accumulation at the interface. For 1:1 Ratios at pH 7.0 the charge was not just neutralized in absence of NaCl. Nevertheless a significant reduction occurred. BLG at pH 7.0 was affected by NaCl addition, but not at the same extension as LYS and therefore dominated interfacial charge behavior. NaCl is reported to strongly influence the net electrical charge of proteins with high IEP (MELA et al., 2010).

3.3.3 Size distribution

The mean diameter (Table 2.5) and PDI were obtained for emulsions with different proportion of LYS and BLG, at pH 4.0 and 7.0.

Table 2.5 – Mean droplet diameter (nm) of emulsions with different LYS:BLG mixtures composition, NaCl concentration and pHs

pH	NaCl (mmol.L^{-1})	LYS:BLG molar ratio				
		1:0	3:1	1:1	1:3	0:1
7.0	0	249.4 ± 1.5^{Bc}	788.7 ± 131.5^{Bab}	954.1 ± 127.9^{Ba}	598.9 ± 108.2^{Bb}	294.1 ± 39.4^{Cc}
	100	574.6 ± 137.0^{Ac}	1531.2 ± 219.6^{Aa}	1704 ± 131.5^{Aa}	935.2 ± 49.4^{Ab}	435.5 ± 19.9^{BCc}
4.0	0	286.5 ± 28.8^{Bb}	344.2 ± 23.8^{Cab}	357.9 ± 40.5^{Cab}	550.5 ± 39.8^{Bab}	583.2 ± 19.4^{Aba}
	100	286.1 ± 36.4^{Bb}	586.6 ± 37.3^{Ba}	659.7 ± 90.4^{Ba}	644.9 ± 58.1^{ABa}	784.4 ± 81.0^{Aa}

Means followed by the same letters, lowercase in lines and uppercase in columns, do not differ among themselves ($p>0.05$) by Tukey's test.

The PDI is calculated as the dispersion of the correlation function graphic. A high PDI may indicate high propensity to droplet aggregation and Ostwald ripening (MCCLEMENTS, 2007). All the emulsions exhibited PDI below 0.6, being suitable to size distribution analysis. Emulsions prepared in the presence of NaCl presented PDI

around 10% bigger than emulsions without NaCl. Emulsions produced from mixtures showed higher PDI values, indicating the occurrence of flocculation, consistent with lower stability results observed.

LYS stabilized emulsions exhibited diameters smaller than 300 nm for both pH. They were smaller than BLG stabilized emulsions at pH 4.0, but statistically similar for pH 7.0 ($p>0.05$). At pH 4.0, BLG emulsions presented higher sizes, what can be explained by its smaller ζ-potential.

At pH 7.0 the proteins have opposite charge, which led to strong attraction and complexes formation. These complexes exhibited large size and low surface activity and resulted in larger diameters with higher PDI, probably by accumulation at the interface and droplet aggregation, even more intense than BLG at pH 4.0. At this pH, when the proteins charges are positive, it seems to occur a competition in the interface, when BLG displace LYS molecule present at the interface.

As observed for ζ-potential and surface values results, BLG seems to dominate the behavior of the interface for BLG/LYS mixtures, causing an increase in size towards BLG-coated emulsion.

The only situation when a difference in size upon NaCl did not occur was for LYS at pH 4.0, where, besides the decrease of ζ-potential the size remains small. The NaCl addition had a particular strong effect in BLG stabilized emulsions at pH 4.0, probably favoring dimers formation. At pH 7.0, bigger emulsions were formed presenting flocculation at small times, detected by high size diameters.

The main effect of NaCl addition was effects of charge screening. It may diminishes repulsion among molecules at the interface, favoring molecules adsorption, but it will also impact on droplets stabilization by repulsion due the same electrostatic forces, increasing flocculation rates (CHEN et al., 2017).

3.3.4 Physical stability

There was significant difference between emulsions after 7 days of storage as shown in Figure 2.5.

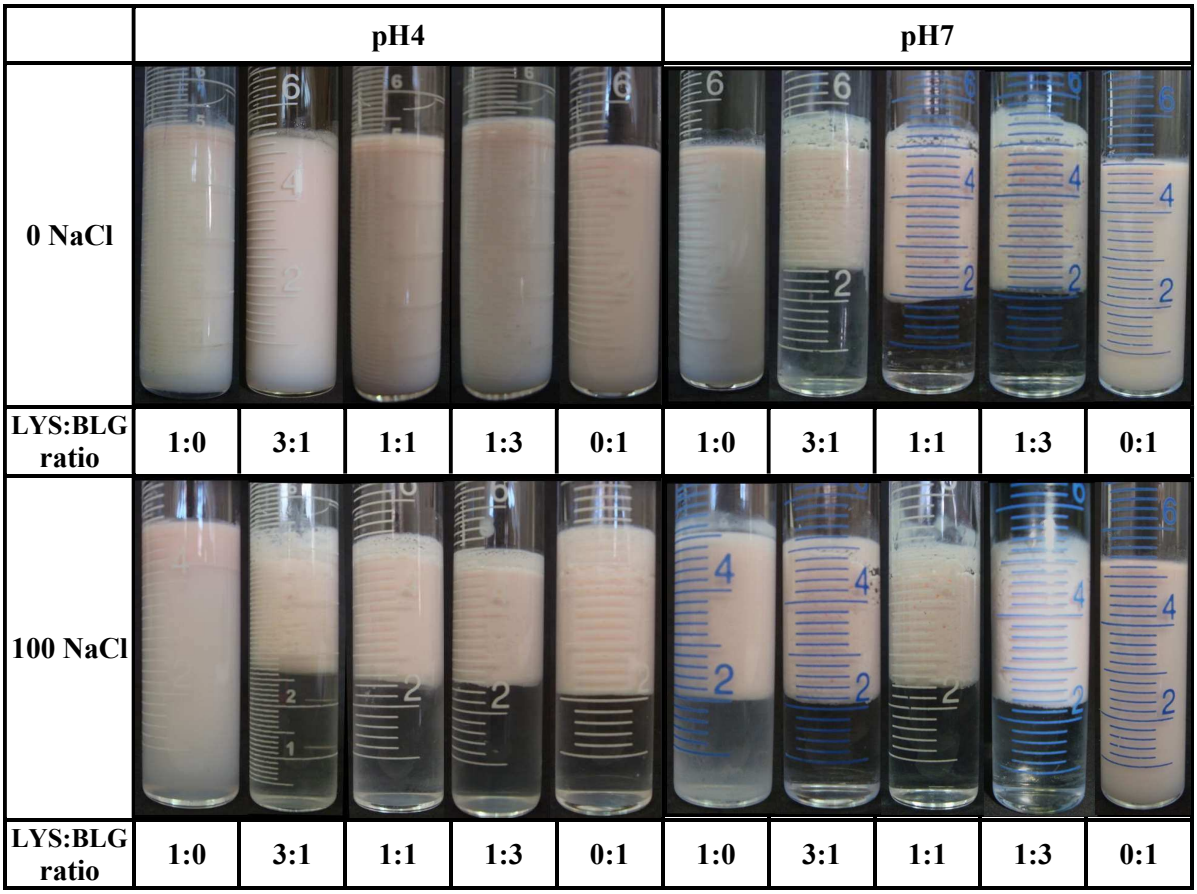

Figure 2.5 – Visual images and creaming index (CI) of emulsions made of different LYS:BLG mixtures composition at NaCl concentration of 0 (black) and 100 mmol.L^{-1} (grey) at pH 4.0 (A) and 7.0 (B).

Emulsions prepared with pure proteins or its mixtures at pH 4.0, without NaCl, showed similar behavior, being stable during experimental times. In those, no transparent aqueous phase was noted, only a droplet gradient caused by difference in droplets creaming velocity due to size difference. At pH 7.0, electrostatic interaction between BLG and LYS molecules caused the charges to be cancelled out leading to droplet aggregation by flocculation. High flocculation rates produced bigger droplets that were observed immediately after emulsion preparation. The increasing in size augmented the creaming velocity leading to phase separation, as seen by a visible serum layer.

The creaming index (CI) provides indirect information about the extent of aggregation of the droplets in the emulsion. High creaming index indicates droplets aggregation that occurred in most cases with a formation of a clear phase below a dense cream phase. All the emulsions that were stable presented CI values smaller than 10% (Table 2.6). On the other hand, high values for CI were detected for mixtures at pH 7.0,

when the lack of electrostatic repulsion allowed aggregation, resulting in a macroscopic phase separation.

Table 2.6 – Creaming index (CI) of emulsions with different LYS:BLG mixtures composition, NaCl concentration and pHs

pH	NaCl ($mmol.L^{-1}$)	LYS:BLG molar ratio				
		1:0	3:1	1:1	1:3	0:1
7.0	0	7.8 ± 2.6^{Bb}	45.6 ± 3.5^{Aa}	39.0 ± 4.4^{Aa}	38.1 ± 4.3^{Ba}	3.0 ± 1.4^{Bb}
	100	44.2 ± 1.6^{Aa}	44.2 ± 2.6^{Aa}	42.5 ± 3.5^{Aa}	46.0 ± 2.8^{Aba}	8.1 ± 0.8^{Bb}
4.0	0	$2.9 \pm 1.\ 5^{Ba}$	3.8 ± 0.2^{Ba}	7.2 ± 3.1^{Ba}	3.9 ± 0.1^{Ca}	7.2 ± 1.1^{Ba}
	100	13.0 ± 1.4^{Bb}	53.6 ± 0.8^{Aa}	45.9 ± 0.6^{Aa}	49.9 ± 0.1^{Aa}	50.8 ± 2.4^{Aa}

Means followed by the same letters, lowercase in lines and uppercase in columns, do not differ among themselves ($p>0.05$) by Tukey's test.

The stability index indicate creaming aggregation and occurred in most cases with a formation of a clear phase under a dense cream phase. For emulsion with CI values smaller than 10% it was observed the presence of a lighter region, separated from the other region which presents a gradual increase of color intensity (what may be attributed to droplet size increase) probably due to different rates of flocculation due to difference in sizes distribution (high PDI). High values for CI were detected only for mixtures at pH 7.0, when attractive electrostatic forces are enhanced.

Phase separation occurs when the charge of the complex formed between the proteins reached neutrality and the lack of electrostatic repulsion allowed aggregation, resulting in a macroscopic phase separation (SOUZA et al., 2012).

The flocculation strongly increases at NaCl addition, due to suppressed electrostatic repulsion between the droplets. This effect was more pronounced at pH 7.0, for emulsion stabilized by protein mixtures. BLG-coated emulsions flocculated at pH 4.0, after NaCl addition, which is a pH close to its IEP, were the protein already has a smaller ζ-potential. Indeed, stability results corroborated with the ζ-potentials found for emulsions after NaCl addition. High values for CI indicating strong flocculation were detected for mixtures at pH 4.0 and 7.0 and also for BLG at pH 4.0.

The physical stability of the emulsions was also evaluated by change in the ζ-potential of the droplets after storage. Data obtained after storage are shown in Table 2.7.

Table 2.7 – ζ-potential of LYS:BLG emulsions with different mixture composition after 7 days of storage at 25 °C.

pH	NaCl ($mmol.L^{-1}$)	LYS:BLG molar ratio				
		1:0	3:1	1:1	1:3	0:1
7.0	0	31.2 ± 4.7	2.8 ± 2.0*	-5.7 ± 3.2	-17.7 ± 3.6	-46.4 ± 4.4
	100	19.6 ± 4.9	7.7 ± 0.9	-14.5 ± 2.7	-16.1 ± 2.5	-29.4 ± 2.1
4.0	0	53.1 ± 4.2	20.7 ± 3.6	22.8 ± 4.8	24.3 ± 3.6	19.4 ± 2.1
	100	12.9 ± 1.8*	9.4 ± 2.8	13.9 ± 2.6	10.5 ± 2.3	8.9 ± 1.6*

*Means changed with time ($p<0.05$) by a paired t-test, comparing with day 0 results.

For emulsions that present changes during storage, a reduction in ζ-potential was observed. Emulsion made at pH 7.0 and a mixture of 3:1 molar ratio, LYS and BLG emulsions at pH 4.0 and NaCl addition, exhibited a ζ-potential varying from 9.2 to 2.8, 19.2 to 12.9 and 17.1 to 8.9 mV, respectively.

4. Conclusion

Primary emulsions formed from BLG and/or LYS were prepared and emulsions compounded by a single protein were more kinetically stable than those compounded by protein mixtures. The increase of ionic strength influence negatively the stability of emulsions. BLG/LYS emulsions made at conditions of opposite charge, although with high values of superficial tension, did not presented physical stability through the storage time studied. The addition of NaCl was not enough to enhance the stability. BLG and LYS interacted even at pH they showed same charge. Although the synergy between the proteins has decreased the interfacial tension, the size of aggregates formed impaired the formation of stable emulsions.

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CAPÍTULO 3 - EMULSIFYING PROPERTIES OF LYSOZYME AND β-LACTOGLOBULIN MULTILAYERS FORMATION WITH PECTIN

ABSTRACT

Multilayers emulsions were formed by a primary emulsion of BLG or LYS, followed by the addition of a second layer of BLG, LYS or pectin, with opposite charge. The use of multilayer emulsions may overcome the kinetically instability and poor resistance to environmental adversities attributed to protein emulsions. Emulsifying properties (oil droplet mean diameter and charge, flocculation) and kinetic stability of such emulsions were determined. Emulsions stabilized by multiple layers, containing pectin, exhibited smaller diameters and greater ζ-potential, comparing to emulsion formed only by proteins. The addition of pectin, a charged and stabilizing polymer, favored the resistance of multilayer emulsions to storage and NaCl addition.

Keywords: multilayers, emulsion stability, pectin, saturation concentration, surface load

1. Introduction

Proteins are widely used to stabilize oil/water emulsions because of their ability to provide both high steric and electrostatic repulsive forces (MOREAU et al., 2003; GU et al., 2004; MCCLEMENTS, 2004; SINGH, 2011). Whey proteins, as well as those from egg white, are often used for such formulations. β-Lactoglobulin (BLG), a major constituent of whey proteins, is largely used as emulsion stabilizer and presents isoelectric point at pH≈5 and a molar mass of 18.3 $kg \cdot mol^{-1}$ (CHEFTEL et al., 1985; FRIBERG et al., 2004; GUZEY & MCCLEMENTS, 2006). Lysozyme (LYS) is a protein extracted from egg white with molar mass around 14 $kg.mol^{-1}$ and isoelectric point at pH≈10.7 (TYN & GUSEK, 1990).

Protein-based emulsions may become unstable due to modifications in spatial arrangement or size distribution of emulsion droplets, such as creaming, flocculation and coalescence, or changes in the composition of the emulsion droplets themselves, such as oxidation and hydrolysis (SINGH, 2011). New technologies have been developed aiming to improve the kinetic stability of the emulsions, which in turn depends on the type and concentration of the ingredients in the emulsion, as well as processing and storage conditions (WANG et al., 2016; CHEN et al., 2017; FAN et al., 2017).

Electrically charged biopolymers are capable of adsorbing on the surface of oppositely charged emulsion droplets (MOREAU et al., 2003; GUZEY & MCCLEMENTS, 2006; LORENZIS et al., 2008; BENGOECHEA et al., 2011; WEI & GAO, 2016). These droplets coated by multilayer interfacial membranes exhibited superior stability to environmental factors (such as heat treatments, drying, freezing and mechanical agitation) than those stabilized by single membranes due to the increase in interface thickness and changes in their rheological characteristics (MCCLEMENTS, 2004; LESMES et al., 2010; SCHMELZ et al., 2011; AZARIKIA et al., 2017).

Protein-coated emulsions are charged, which leads to electrostatic repulsion between neighboring protein molecules at the interface, as well as between the two neighboring emulsion droplets. The electrostatic repulsion keeps the adsorbed protein molecules at a certain distance from each other and hampers the formation of both non-covalent and covalent bonds within the adsorption layer (TCHOLAKOVA et al., 2005;

BURGOS-DÍAZ et al., 2016). NaCl addition and pH are important factors governing the electrostatic repulsion, the structure of the adsorption layers and the emulsion stability.

The stability of the primary emulsion as well as the correct concentration of the components of the subsequent layers (saturation concentration) are essentials for obtaining kinetically stable and reproducible multilayer emulsions (MCCLEMENTS, 2005). Multilayer emulsions can be formed in two main ways: by blending all ingredients together or by adding one component at a time. In the second method, called layer-by-layer (LBL) deposition, a primary emulsion is formed by the homogenization of oil, water and surfactant, resulting in small droplets coated by an emulsifier layer. Thereafter, a secondary emulsion is formed by mixing, to the primary emulsion, an oppositely charged polyelectrolyte thereby promoting charge reversion (GUZEY & MCCLEMENTS, 2007). This method allows better control of the relative concentration of the added reagents, especially using saturation concentrations (LORENZIS et al., 2008).

This technique allows the stepwise adsorption of several components (polyelectrolytes, nanoparticles, proteins, enzymes, etc.). The growth layer is regulated by electrostatic attraction, leading to the formation of multilayer with thickness finely controlled (CARPENTER et al., 2018). In addition to electrostatic attraction, other interactions such as hydrogen bonds or hydrophobic interactions may be involved in the layer by layer assembly.

In this study, we investigated the interaction between LYS, BLG and pectin in O/W emulsions, exploring the possibility of making multilayered emulsions using these two proteins and this polysaccharide. Multilayered emulsions stabilized by subsequent layers of BLG, LYS and/or pectin in different pHs were produced. Emulsifying properties (oil droplet mean diameter and charge, flocculation) and kinetic stability of such emulsions were determined.

2. Materials and Methods

2.1 Materials

Chicken egg white Lysozyme (> 90%) was purchased from Sigma-Aldrich Chemical (St. Louis, MO, EUA), bovine β-lactoglobulin (> 90%) was purchased from Davisco Food International Inc. (La Sueur, MN, EUA), citric fruit peel pectin (GENU pectin type 102 AS, LM, DE=30%) was donated by CPKelco (São Paulo, Brazil) and used without further purification. Sunflower oil (Liza, Cargill Foods, SP, Brasil) was purchased at a local market. All others chemicals were of analytical grade and deionized water made by Milli-Q system (Millipore, USA) with sodium azide 0.02% was used throughout the experiments.

2.2 Multilayer emulsion preparation

Protein (1.4 $mmol.L^{-1}$) or pectin (25 $mg.mL^{-1}$) stock solutions were prepared by dispersing 1.12 g of LYS, 1.43 g of BLG or 1.25 g of pectin in 50 ml of deionized water containing 0.02% w/v sodium azide (anti-microbial agent) and kept under refrigeration overnight for complete solubilization and hydration. The pH of the solutions was adjusted to 4.0 or 7.0 using HCl or NaOH (0.1 M).

Oil/water emulsions were prepared using as continuous phase pure lysozyme or β-lactoglobulin (total protein concentration=0.7 $mmol.L^{-1}$). Aqueous solutions of proteins at the desired pH and NaCl concentration were mixed with the oil phase to obtain 10% sunflower oil and 90% aqueous phase (w/w).

Primary emulsion (P-O/W) was prepared homogenizing the mixture with a high speed blender (Omni Macro ES, USA) equipped with a 25 mm diameter probe at 7500 rpm for 3 min, followed by four passes through a high-pressure homogenizer (EmulsiFlex, Avestin, Canada) at 10,000 psi, at room temperature.

The component of the second layer was added to P-O/W emulsion, as shown in Table 3.1, and mixed on a magnetic stirrer for 30 min at room temperature. The same procedure was repeated for the third layer. The final multiple emulsion (M-O/W) consisted of 1.5% (m/m) oil, 0.105 $mmol.L^{-1}$ proteins and 1 $mg.mL^{-1}$ pectin for secondary

emulsion and 0.75% (m/m) oil, 0.0525 mmol.L^{-1} proteins and 0.5 mg.mL^{-1} pectin for tertiary.

Table 3.1 – Combinations of LYS and BLG proteins and the polysaccharide pectin.

Code	pH	1st layer	2nd layer	3rd layer
1	4.0	LYS	Pectin	-
2	4.0	LYS	Pectin	BLG
3	4.0	BLG	Pectin	-
4	4.0	BLG	Pectin	LYS
5	7.0	LYS	BLG	-
6	7.0	LYS	Pectin	-
7	7.0	BLG	LYS	-
8	7.0	BLG	LYS	Pectin

Light microscopy images were taken with an axial-mounted Zeiss Axio Scope A1 microscope (Carl Zeiss, Germany). A drop of emulsions (20 μL) was poured on an objective slide, covered with a cover slip and observed using the 100x objective lenses. Photographic images were taken using a digital camera after storage.

Multilayered emulsions were evaluated for droplet size distribution, electrical charge (ζ-potential) and creaming stability (CI), immediately after preparation and after storage at 25.0±0.5 °C for 24 h, as described in item 2.3. The effect of the addition of NaCl (0 or 100 mmol.L^{-1}) after preparation of emulsions was assessed diluting the emulsions at a ratio of 1:1 with ultrapure water at the same pH and a 1 mmol.L^{-1} NaCl stock solution.

2.2.1 Saturation concentration and surface load at saturation

In order to determine the lowest possible concentrations at which the stabilization of the multilayer occurs, the minimum amount of protein or pectin required to cover the surface of the emulsion droplets (Γ_{sat}) was defined (YE & SINGH, 2007).

The ζ-potential of P-O/W was measured through consecutive and increasing addition of the second layer component according to Table 3.1. This addition was done automatically by an autotitrator (MPT-2, Malvern Instruments Ltd, Southborough, UK) coupled to Zetasizer NanoZS.

The critical concentration where the droplet surfaces became saturated was established by modeling curves of ζ-potential dependence on component concentration. So, values for C_{sat} were obtained by fitting the Eq. 6 to the experimental data (GUZEY & MCCLEMENTS, 2007). This empirical equation assumes that the droplet surfaces are saturated when the change in ζ-potential [Δζ(c)] is approximately 95% of the total change [Δζ(Sat)].

$$\frac{\Delta\zeta(c)}{\Delta\zeta(sat)} = \frac{\zeta_C - \zeta_{sat}}{\zeta_0 - \zeta_{sat}} \approx e^{\left(-\frac{C}{3.C_{sat}}\right)} \qquad 6$$

where ζ_0, ζ_C and ζ_{sat} are the measured ζ-potential (mV) for the primary emulsion, the multilayered emulsion at concentration of the second component (C, mmol.L^{-1}) and when saturated on the oil droplet surface (C_{sat}, mmol.L^{-1}), respectively.

From the saturation concentration, the surface load at saturation was determined (Γ_{sat}) (Eq. 7), which is a variable more useful to compare once it is not dependent on the size nor the concentration of emulsion droplets and specifies the amount of adsorbed component.

$$\Gamma_{sat} = \frac{c_{sat} d_{32}}{6\phi} \qquad 7$$

where, c_{sat} is the mass of material adsorbed to the surface of the droplets per unit volume of emulsion (kg.m^{-3}), d_{32} is the volume-surface mean droplet diameter (nm), and ϕ is the droplet volume fraction.

2.3 Emulsion characterization

2.3.1 ζ-potential

ζ-potential measurements were performed in a Laser Doppler Micro-electrophoresis instrument (Zetasizer Nano-ZS, Malvern Instruments Ltd, Southborough, UK). The ζ-potential was calculated from the electrophoretic mobility, determined by measuring the direction and speed of movement of a droplet in a well-defined electric field.

The emulsions were subjected to analysis immediately after preparation and after storage at 25.0 °C. Samples were appropriately diluted to a droplet concentration of about 0.05% (w/w) with ultrapure water in the corresponding pH and NaCl concentration. Then, the emulsions were poured in capillary cell with gold electrodes and analyzed to a 173° scattering angle at 25.0±0.1 °C.

The ζ-potential (mV) was calculated from the electrophoretic mobility (μ, $V.m^2.s^{-1}$) using the Henry equation (Eq. 8).

$$\mu = \frac{2\varepsilon\zeta}{3\eta} f(ka) \qquad 8$$

where ε is the electrical permeability ($F.m^{-1}$), η is the solvent viscosity (Pa.s) and $f(ka)$ was 1.5 which is the Smoluchoski approximation. ζ potential was automatically determined from the Zetasizer Software. Each individual result was automatically calculated as the average of 5 measurements and the results were expressed as mean and standard deviation of at least two repetitions.

2.3.2 Droplet mean diameter measurements

The droplet size distributions were determined by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments Ltd, Southborough, UK). This technique measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship.

The emulsions were subjected to analysis immediately after preparation and after storage at 25 °C. The particle diameter range and the number of photon counts per second (kilo count per second (kcps)) were measured at 25.0±0.1 °C. To avoid multiple scattering effects, freshly prepared emulsions were diluted to a concentration of 0.05% w/w using deionized water containing 0.02% of sodium azide, at the corresponding pH and NaCl concentration.

The particle size was reported as z-average diameter and the polidispersity index. The data from size measurement was submitted to cumulant analysis from where the z-average diameter and PDI values could be extracted. Each individual result was

automatically calculated as the average of 5 measurements with 20 sub-runs each. The results were expressed as mean and standard deviation of at least two repetitions.

2.3.3 Physical stability

The physical stability of the emulsions was assessed by the creaming stability test, where aliquots of emulsion (5 g) were transferred to sealed graduated glass tubes and stored for at 25.0±0.5 °C), protected from light. Sudam III (a red dye) was previously added to sunflower oil before the homogenization process to facilitate the visualization of phase separation. Aliquots of 2 mL were also stored in sealed tubes for further evaluation of the droplet size distribution and the ζ-potential and photographic images were taken using a digital camera after storage.

The extent of creaming was characterized by the creaming index (CI) that features the serum layer formed at the bottom of the tubes expressed as a percentage of the total volume of emulsion in the tube. The total height of the emulsion (H_t, cm) and the height of the aqueous layer (H_a, cm) resulting from creaming of the oil droplets during storage were measured. The creaming index (CI) was calculated by the Eq. 9 (CARPENTER et al., 2018).

$$IC = \frac{H_a}{H_t} \times 100\% \qquad \textit{Eq. 9}$$

Results were expressed as mean and standard deviation of at least two repetitions.

2.4 Experimental design and statistical analysis

The experiments were conducted in completely randomized design to minimize the effect of external variables. The data collected from the experiments were subjected to analysis of variance. When significant for F test ($p< 0.05$), means were compared by a Tukey test at 5% error probability. Mean comparisons through time were performed by a paired t-test ($p<0.05$).

The adequacy of fitted models for C_{sat} was evaluated by the statistical significance of the model ($p<0.05$) and the coefficient of determination (R^2). The significance of the model parameters was assessed by Student's t test ($p <0.05$). All statistical analyzes were

performed using SAS software (version 9.0, SAS Institute Inc., Cary, NC, USA), available for the Universidade Federal de Viçosa (UFV).

3. Results and discussion

3.1 Addition of BLG or pectin to LYS-stabilized emulsions

The behavior of LYS and BLG emulsion upon the addition of increasing concentration of the second layer component was assessed and the optimum concentrations were calculated. Primary emulsions of LYS were titrated with increasing and subsequent pectin or BLG concentrations. Emulsion droplets electrical charge was measured (Figure 3.1 and Figure 3.2). These experiments would give an idea of optimum concentration of components of second and tertiary layers as well as to provide information about the adsorbed layer.

ζ-potential values of pectin at pH 4.0 and 7.0 were -37.3 and -54.0 mV, respectively, in conformity with results reported by SOUZA et al. (2012), who found 34.4 e -50.3 mV for pH 4.0 and 7.0, respectively. ζ-potential for LYS emulsions were 56 and 26 mV, for pH 4.0 and 7.0, respectively, and for BLG 26 and -50, for pH 4.0 and 7.0 respectively. Based on charges, the secondary layers were chosen from opposite charge components.

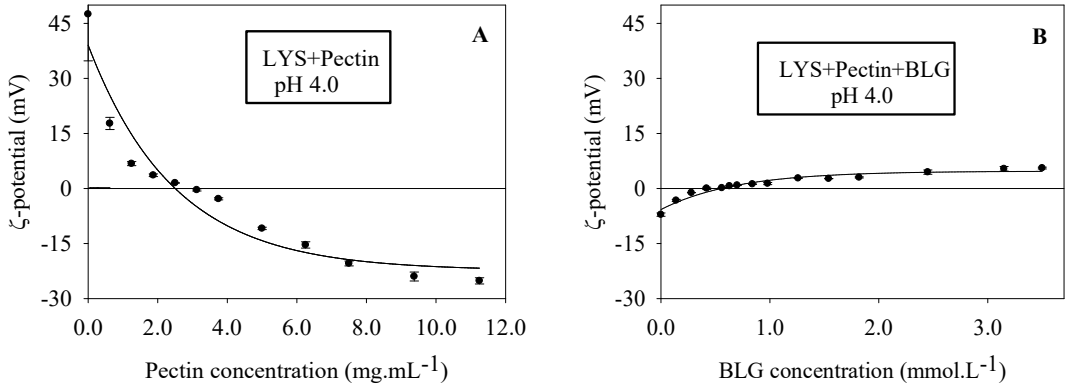

Figure 3.1 – Changes in ζ-potential of P-O/W emulsion droplets (10% oil and 0.7 mmol.L^{-1} LYS) as a function of added pectin (A) and M-O/W emulsion droplets (5% oil, 0.375 mmol.L^{-1} LYS and 3.1 mg.mL^{-1} pectin) as a function of added β-lactoglobulin (B) at pH 4.0.

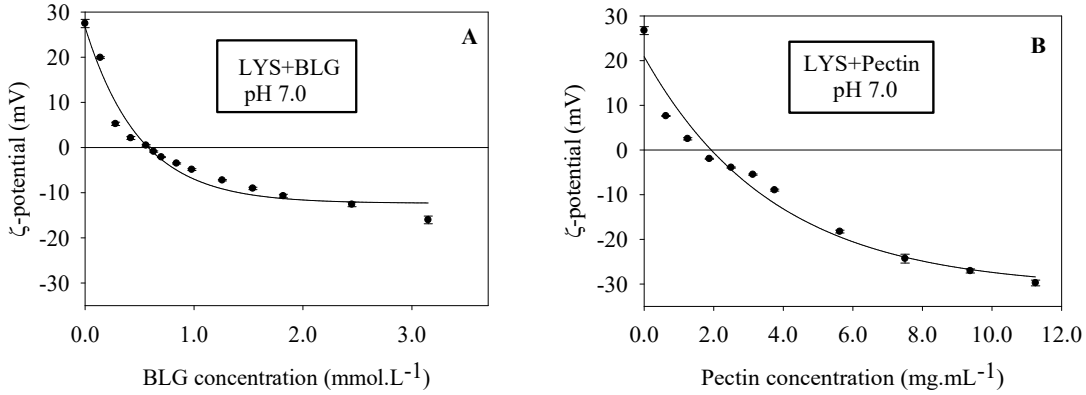

Figure 3.2 – Changes in ζ-potential of P-O/W emulsion droplets (10% oil and 0.7 mmol.L^{-1} LYS) as a function of added of added β-lactoglobulin (A) and pectin (B) at pH 7.0.

The ζ-potential of LYS-coated emulsions changed from positive to negative values as pectin or BLG concentration was increased, indicating that those anionic molecules adsorbed to the surface of cationic protein-coated droplets until a critical concentration was reached, after which the droplet surfaces were presumably covered with pectin/BLG molecules and the ζ-potential reached a plateau. The same behavior was observed when secondary emulsions were titrated with pectin.

The occurrence of this plateau in all conditions may be related to the absence of available cationic binding sites on the protein-coated droplets that were saturated with anionic groups from the pectin/BLG molecules or an electrostatic repulsion between adsorbed and non adsorbed pectin/BLG molecules.

3.2 Addition of LYS or pectin to BLG-stabilized emulsions

Primary emulsions of BLG were titrated with increasing and subsequent pectin or LYS concentrations. Emulsion droplets electrical charge was measured (Figure 3.3 and Figure 3.4).

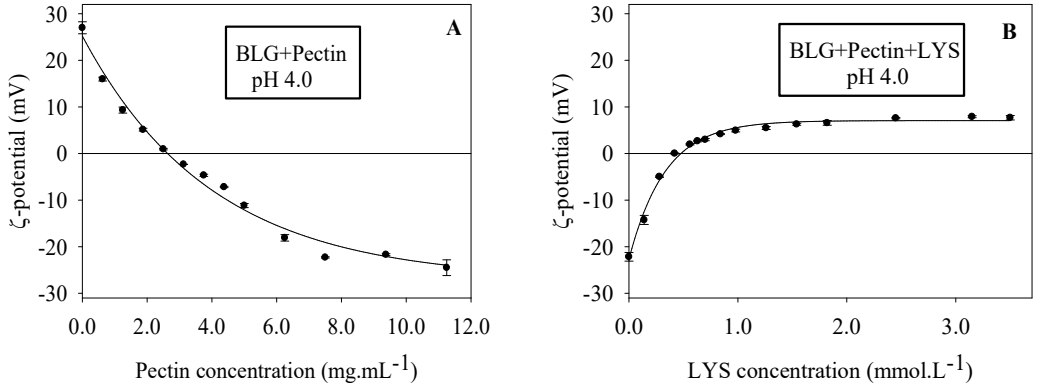

Figure 3.3 – Changes in ζ-potential of P-O/W emulsion droplets (10% oil and 0.7 mmol.L^{-1} BLG) as a function of added pectin (A) and M-O/W emulsion droplets (5% oil, 0.375 mmol.L^{-1} BLG and 3.1 mg.mL^{-1} pectin) as a function of added lysozyme (B) at pH 4.0.

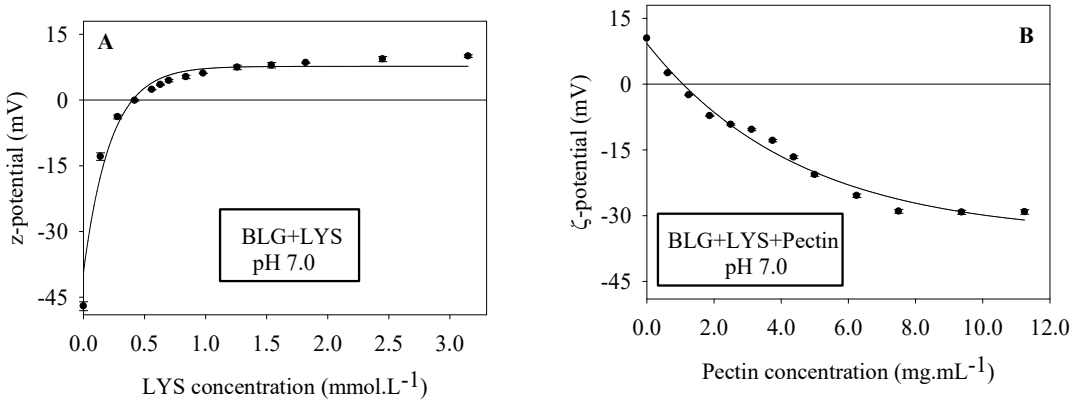

Figure 3.4 – Changes in ζ-potential of P-O/W emulsion droplets (10% oil and 0.7 mmol.L^{-1} BLG) as a function of added lysozyme (A) and M-O/W emulsion droplets (5% oil, 0.375 mmol.L^{-1} BLG and 0.375 mmol.L^{-1} LYS) as a function of added pectin (B) at pH 7.0.

The same behavior for titration of LYS-coated emulsions was observed for BLG-coated emulsions, with charge reversal indicating adsorption of the secondary or tertiary component to the surface of emulsion. A sharper change in ζ-potential was observed when adding LYS, what may be related to the stability of this molecule that adsorb at the interface forming a thick layer and due to its low flexibility do not change over time. The

evaluation of the ζ-potentials indicated that electrostatic interaction could occur between pectin and LYS at both pH.

Emulsions titrated with pectin reached higher values of ζ-potential, indicating that those emulsions would be more stable than protein-coated secondary emulsions.

3.3 Saturation concentration and surface load at saturation

The minimum amount of protein or pectin required to cover the surface of the emulsion droplets (c_{sat}) were obtained (Table 3.2) and represents the lowest possible concentrations at which the stabilization of the multilayer occurs.

From the saturation concentration, the surface load at saturation was determined (Γ_{sat}) which is a variable more useful to compare once it is not dependent on the size nor the concentration of emulsion droplets and specifies the amount of adsorbed component.

Table 3.2 – Saturation concentration (c_{sat}) and surface load at saturation (Γ_{sat}) of primary, secondary or tertiary emulsions made of LYS, BLG or pectin at different pH.

pH	1st layer	2nd layer	3rd layer	c_{sat} (mg.mL^{-1})	Γ_{sat} (mg.m^{-2})
4.0	LYS	Pectin	-	7.4	6.0
	LYS	Pectin	BLG	38.2	- *
	BLG	Pectin	-	10.9	14.7
	BLG	Pectin	LYS	14.3	- *
7.0	LYS	Pectin	-	11.1	12.8
	LYS	BLG	-	27.6	27.6
	BLG	LYS	-	9.5	10.4
	BLG	LYS	Pectin	13.6	16.1

* Tertiary emulsion exhibited diameter out of equipment range and Γ_{sat} could not be calculated.

Greater quantities of BLG are necessary for interface stabilization, evidenced by high values of c_{sat}. Although both proteins present same molecular size, LYS is more rigid, what indicates that, once it is adsorbed low rearrangement occurs after adsorption.

BLG, on the other range, may settle more molecules at the interface (GIRARD et al., 2002). The results indicate that electrostatic interactions are also involved in BLG/pectin and LYS/pectin systems.

3.4 Multilayer emulsion formation and characterization

The layer-by-layer (LBL) deposition technique offers a promising way to prepare emulsions using electrostatic attraction of charged biopolymers to oppositely charged droplets (CARPENTER et al., 2018). In this method, an interfacial membrane that consists of multiple layers of biopolymers is prepared by adsorbing consecutive layers of oppositely charged biopolymers onto a primary emulsion prepared using a conventional homogenization technique.

Multilayers emulsion were prepared by adding a second component to a primary emulsion previously prepared. The concentration of the subsequent layer component was determined by analyzing the results obtained as in item 3.3, but the final concentration was fixed for both proteins.

Interactions of pectin with proteins are sensitive to various factors, including aqueous solution conditions (pH, ionic strength, calcium ion content), as well as the molecular considerations (p.e. degree of esterification) (LEROUX et al., 2003; BAYARRI et al., 2014).

The emulsions were assessed by size distribution, ζ-potential and physical stability to time and NaCl addition. There was significant difference ($p<0.05$) in the ζ-potentials of the primary, secondary and tertiary emulsions (Table 3.3), which suggests that the overall composition of interfacial layers was affected by the following molecule layer.

Table 3.3 – ζ-potential (mV) of primary, secondary or tertiary emulsions made of LYS, BLG or pectin at different pH, immediately after formation and after 24 h storage.

pH	1st layer	2nd layer	3rd layer	ζ-potential (mV)	
				Day 0	Day 1
4.0	LYS	-		47.5 ± 0.7[a]	48.3 ± 0.9
	LYS	Pectin	-	-9.9 ± 1.1	-7.5 ± 0.2
	LYS	Pectin	BLG	-2.0 ± 0.1	-2.0 ± 0.1
	BLG	-		25.5 ± 0.4	25.1 ± 0.4
	BLG	Pectin	-	-17.3 ± 0.4	-19.3 ± 1.3
	BLG	Pectin	LYS	6.3 ± 0.4	5.5 ± 0.1
7.0	LYS	-		30.6 ± 0.6[a]	19.6 ± 0.3
	LYS	Pectin	-	-32.1 ± 0.5[a]	-35.5 ± 0.7
	LYS	BLG	-	-13.0 ± 0.2[a]	-5.8 ± 1.1
	BLG	-		-52.8 ± 0.8	-51.0 ± 1.1
	BLG	LYS	-	9.3 ± 0.0[a]	6.2 ± 0.3
	BLG	LYS	Pectin	-29.0 ± 1.0	-27.7 ± 1.0

Values followed by [a] indicates significant difference between means ($p<0.05$) by a paired t-test.

The evaluation of ζ-potential indicate that the amount of the component was enough to cover the interface and modulate the final ζ-potential, except for the third layer formed by BLG addition to pectin and LYS M-O/W, at pH 4.0. As expected, emulsions added of pectin had a negative ζ-potential for both pH, the electronegativity increased with increasing pH. The same occurred when proteins were added, the emulsion had a ζ-potential equivalent to that of proteins in solution.

Electrostatic attraction takes place between the positively charged patches on protein molecules and negatively charged carboxyl groups of pectin. Unfolding of protein structure during emulsification can expose extra charged groups and generate more linkages with the polysaccharide, like hydrophobic interactions and hydrogen bonds (LEROUX et al., 2003).

LYS/BLG M-O/W emulsions changed their ζ-potential due to molecular arrangements during storage. As observed for primary emulsions, LYS was more suitable to ζ-potential changes through storage. Emulsions with pectin in the last layer were less suitable to ζ-potential changes.

The mean diameter and PDI were obtained for M-O/W emulsions of BLG, LYS and/or pectin (Table 3.4).

Table 3.4 – Mean droplet diameter (nm) and polidispersity index of primary, secondary and tertiary emulsions made of LYS, BLG or pectin at different pH, immediately after formation and after 24 h storage.

pH	1st layer	2nd layer	3rd layer	Diameter		PDI	
				Day 0	Day 1	Day 0	Day 1
4.0	LYS	-		296.7 ± 59.0[a]	350.7 ± 46.3	0.317 ± 0.016[a]	0.341 ± 0.012
	LYS	Pectin	-	481.6 ± 33.0[a]	447.6 ± 29.4	0.455 ± 0.067	0.444 ± 0.040
	LYS	Pectin	BLG	> 2000	> 2000	-	-
	BLG	-		474.1 ± 34.3	509.7 ± 134.2	0.318 ± 0.004	0.355 ± 0.017
	BLG	Pectin	-	807.9 ± 40.9	753.5 ± 117.4	0.589 ± 0.013	0.552 ± 0.017
	BLG	Pectin	LYS	> 2000	> 2000	-	-
7.0	LYS	-		324.6 ±29.3	335.3 ± 18.5	0.342 ± 0.040[a]	0.356 ±0.022
	LYS	Pectin	-	690.7 ± 59.4	629.9 ± 37.5	0.540 ± 0.057	0.559 ± 0.008
	LYS	BLG	-	598.8 ± 11.9[a]	> 2000	0.401 ± 0.056	-
	BLG	-		483.6 ± 44.3	497.6 ± 11.2	0.344 ± 0.030[a]	0.425 ± 0.029
	BLG	LYS	-	655.9 ± 249.5	556.3 ± 215.0	0.369 ± 0.055	0.412 ± 0.105
	BLG	LYS	Pectin	709.2 ± 77.0	943.1 ± 182.3	0.387 ± 0.082[a]	0.552 ± 0.032

Different letters indicate significant difference between means ($p<0.05$) by a paired t-test.

The diameter of tertiary emulsions could not be measured by the equipment so they were indicated as bigger than 2000 nm. Emulsion diameters increased as the

subsequent layers were added, in all the multilayer emulsions ($p<0.05$). Tertiary emulsions with proteins as the last layer were not stable and presented bigger diameters, probably due flocculation.

The stabilization mechanism of polysaccharide-protein is different from that of pure proteins emulsion. The formation of protein-polysaccharide complexes at the interface created a viscous gel-like structure around the oil droplets, providing steric hindrance to droplet aggregation (GHARSALLAOUI et al., 2010; BENJAMIN et al., 2012).

Pectin forms a thicker layer leading the increasing diameters for all M-O/W emulsions, that remained stable due steric repulsion. Over the period of storage (24 h at 25 ºC) some M-O/W emulsions decreased in size what may be attributed to molecular rearrangements of protein-pectin complexes at the interface and also dehydration of pectin molecules (LEROUX et al., 2003).

The emulsions were availed at the final of storage time using a microscope and a digital camera (Figure 3.5 and Figure 3.6).

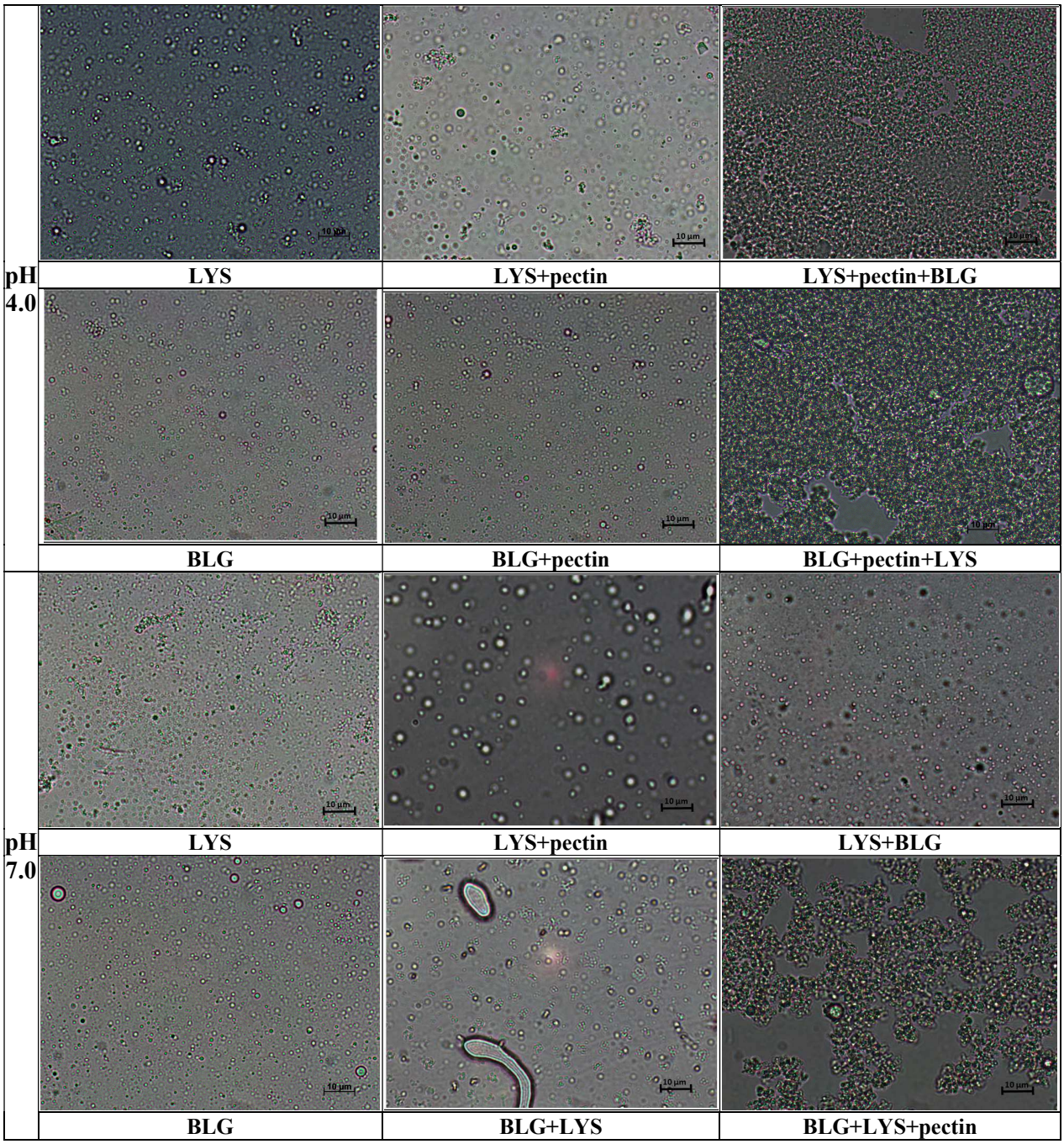

Figure 3.5 – Microstructures of primary, secondary and tertiary emulsions at different pH after 24 h storage at 25 °C.

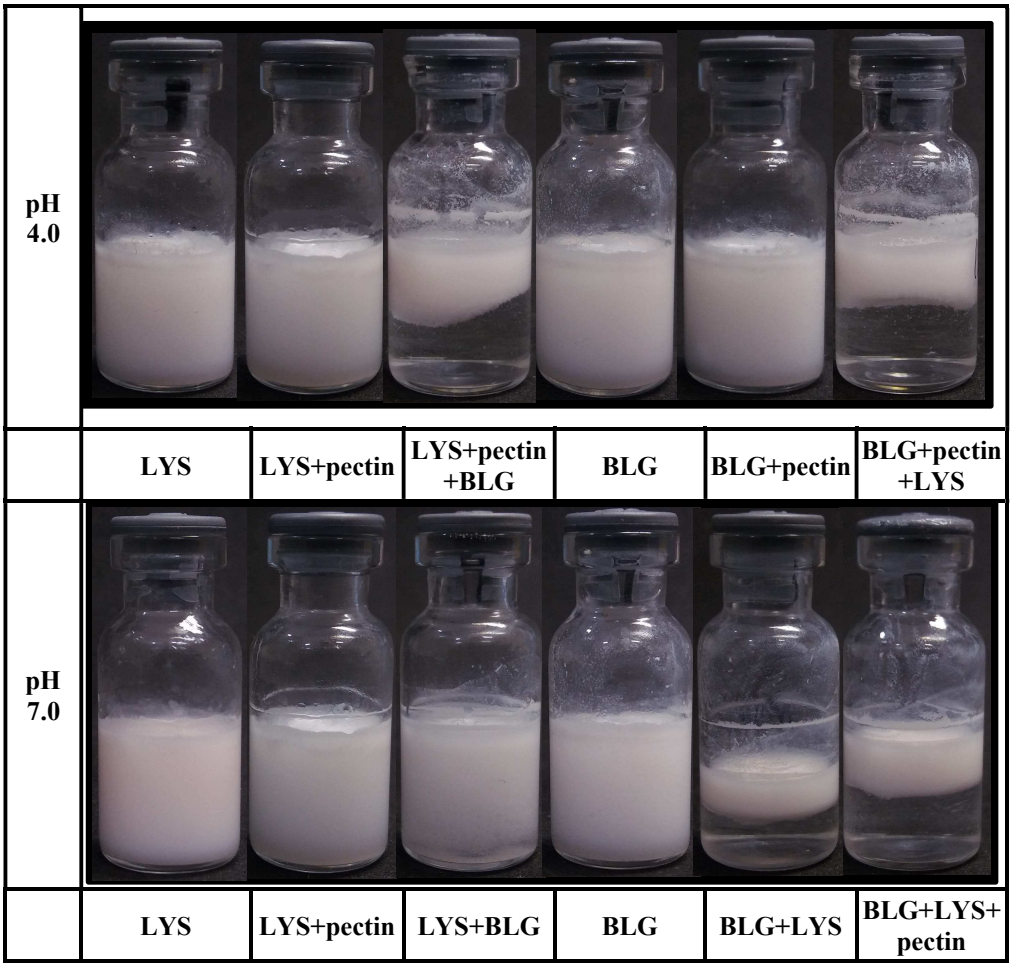

Figure 3.6 – Visual images of primary, secondary and tertiary emulsions at different pH after 24 h storage at 25 °C).

M-O/W emulsions prepared with the addition of pectin had higher surface charge and had good stability against creaming during stability test. This indicated that pectin has deposited at the interface, increasing electrostatic and steric repulsion within oil droplets.

As can be seen in Figure 3.6, tertiary emulsions and secondary emulsion formed by LYS added of BLG at pH 7.0 were not stable to storage for 24 h. Microscopies showed agglomerates in tertiary emulsions, indicating the occurrence of flocculation (MCCLEMENTS et al., 2007). Emulsions with high flocculation rates also presented bigger diameters and low ζ-potential. The lack of charge diminished electrostatic repulsion between droplets, what led to droplet agglomeration and destabilization of resulting emulsions.

3.5 Multilayer stability through NaCl addition

One of the main advantages of the use of multilayers emulsion is the possibility of improve the resistance of the emulsion to environmental stresses (AZARIKIA et al., 2017). In this experiment, the emulsions were submitted to the presence of NaCl and stored for 24 h at 25±0.5 °C (Figure 3.7). Once tertiary emulsions were not stable to storage, their stability to NaCl was not assessed.

Code	A1	A2	B1	B2	C1	C2
CI	0%	58%	13%	64%	22%	60%

Figure 3.7 – Visual images and creaming index of emulsions after NaCl addition, and with (1) and without (2) a secondary layer of pectin. Lysozyme, pH 4.0 (A), β-lactoglobulin, pH 4.0 (B) and Lysozyme (C), pH 7.0 after 24 h storage at 25 °C.

The addition of a secondary layer of pectin improved emulsion stability to salt addition when compared to primary emulsions, in both pHs. The creaming index was more pronounced for emulsion with visible aggregation.

Adsorption of either Na^+ or Cl^- at the interface can neutralize some of the ionic groups of pectin or the protein, resulting in reduced interfacial charge and poor protein-pectin interaction (MAO et al., 2014). However, after salt addition, droplet aggregation occurred and phase separation took place mainly for primary emulsions. The stability to NaCl addition was also evaluated by ζ-potential, diameter and PDI measurements (Table 3.5 and Table 3.6).

Table 3.5 – ζ-potential (mV) of primary and secondary emulsions made of LYS, BLG and/or pectin at pH 4.0 and 7.0, after NaCl addition.

pH	1st layer	2nd layer	ζ-potential (mV)	
			Day 0	Day 1
4.0	LYS	-	$3.2 \pm 0.3^{A*}$	6.7 ± 1.8
	LYS	Pectin	-15.4 ± 0.8^{A}	-17.3 ± 0.4
	BLG	-	$5.4 \pm 0.4^{A*}$	6.2 ± 0.6
	BLG	Pectin	-15.0 ± 0.6^{A}	-15.4 ± 0.7
7.0	LYS	-	13.8 ± 0.4^{A}	10.4 ± 1.4
	LYS	Pectin	-19.8 ± 1.9^{A}	-20.3 ± 0.2

Different letters indicate significant difference between means ($p<0.05$) in emulsions after NaCl addition.
* shows difference in emulsions by a paired t-test, in day 0 and 1.

Table 3.6 – Mean diameter (nm) and polydispersity index of primary and secondary emulsions made of LYS, BLG or pectin at different pH, after NaCl addition.

pH	1st layer	2nd layer	Diameter		PDI	
			Day 0	Day 1	Day 0	Day 1
4.0	LYS	-	804.7 ± 69.4^{A}	716.8 ± 106.6	$0.576 \pm 0.039^{*}$	0.863 ± 0.112
	LYS	Pectin	1004.2 ± 53.5^{A}	1154.0 ± 145.1	$0.705 \pm 0.035^{*}$	0.467 ± 0.026
	BLG	-	$1020.3 \pm 52.3^{A*}$	1379.7 ± 124.4	0.457 ± 0.053	0.461 ± 0.185
	BLG	Pectin	842.3 ± 74.7^{a}	1280.3 ± 31.1	0.504 ± 0.011	0.448 ± 0.044
7.0	LYS	-	1020.7 ± 118.3^{A}	1096.7 ± 60.5	0.451 ± 0.096	0.487 ± 0.022
	LYS	Pectin	$687.1 \pm 90.8^{*}$	961.1 ± 103.0	$0.479 \pm 0.031^{*}$	0.356 ± 0.023

Different letters indicate significant difference between means ($p<0.05$) in emulsions after NaCl addition.
* shows difference in emulsions by a paired t-test, in day 0 and 1.

ζ-potential values changed after NaCl addition, for P-O/W and M-O/W emulsions. However, P-O/W were more susceptible to charge changes, even the ones with initial high ζ-potential values. The main effect was observed for LYS-coated emulsions because it affected more significantly its ζ-potential, destabilizing the forming emulsions. Charge and emulsion diameter varied significantly, resulting in an unstable emulsion.

A significant increase in size occurred for P-O/W emulsions, as well as for M-O/W emulsion formed by LYS and pectin at pH 4, condition with the lowest ζ-potential among M-O/W emulsions. The other M-O/W emulsions exhibited good stability to NaCl addition, exhibiting little change in mean droplet diameter. BENJAMIN et al. (2013) also observed an increase in emulsion diameter for P-O/W BLG emulsions and an improvement in emulsion stability after pectin addition. Pectin increases the thickness of emulsion layers as long as provides steric repulsion, overcoming opposite effect of charge screening that may have occurred due ζ-potential reduction (MOREAU et al., 2003).

4. Conclusion

Multilayer emulsion could be formed using BLG, LYS and pectin in consecutive layers of opposite ζ-potential. Tertiary emulsions were not stable to storage, showing intense agglomeration. On the other hand, multilayer emulsions formed by the addition of pectin, a charged and stabilizing polymer, were resistant to storage and NaCl addition.LYS/BGL have been previously studied for complex formation but multilayer emulsions could be formed, especially using pectin as a complementary layer, expanding application.

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CAPÍTULO 4 - BINDING OF β-CAROTENE TO β-LACTOGLOBULIN AND LYSOZYME - A SPECTROSCOPIC STUDY

ABSTRACT

The interaction between β-carotene (BC) and β-lactoglobulin (BLG) or Lysozyme (LYS) in aqueous solution was investigated by fluorescence spectroscopic at pH 4.0 and 7.0. The effect of BC on the conformation of proteins was analyzed by UV-absorption and intrinsic fluorescence. The quenching rate constants and binding constants calculated indicated the static quenching mechanism and medium binding force. The binding constants for BLG were 1.64 and $0.67x10^4$ $L.mol^{-1}$, at pH 7.0 and 4.0, respectively and for LYS 0.98 and $1.47x10^4$ $L.mol^{-1}$, at pH 7.0 and 4.0, respectively. The binding ability of the BC to both proteins decreased significantly with denaturation, indicating partial inactivation of the binding sites. The results indicated that BC binding to proteins occurs with high probability.

Keywords: whey protein, egg protein, β-carotene, binding constants, fluorescence spectroscopic

1. Introduction

Different methods have been used to access binding of ligands to proteins including partition equilibrium, ultrafiltration, turbidimetry, isothermal titration calorimetry, mass spectrometry, affinity chromatography, fluorescence, nuclear magnetic resonance, etc. (MOCZ & ROSS, 2013). Spectroscopic methods are among the most user-friendly, rapid and accurate techniques used to investigate the protein-ligand interaction.

Fluorescence quenching can be defined as the decrease in the quantum yield of fluorescence from a fluorophore molecule induced by molecular interactions with quencher molecules. During a collisional quenching, the quencher diffuses to the fluorophore during the lifetime of the excited state and upon contact, the fluorophore returns to the ground state, without emission of a photon. In the static quenching situation a nonfluorescent complex is formed between the fluorophore and the quencher (LAKOWICZ, 2007).

The protein intrinsic fluorescence is due aromatic amino acids: tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) (DAMODARAN et al., 2010). Protein intrinsic fluorescence has been widely used to obtain information on the binding equilibrium between protein and ligands (BI et al., 2004; WANG et al., 2009; LI et al., 2013; LI et al., 2017), to evaluate the effect of adverse conditions (i.e. heating, denaturation, ion addition) on the structure of proteins(MANDERSON et al., 1999; BUSTI et al., 2005; SCHRÖDER et al., 2017) and to determine binding sites (LANGE et al., 1998; COLLINI et al., 2000).

β-lactoglobulin (BLG), a protein of molar mass of 18.3 $kg \cdot mol^{-1}$ and isoelectric point around pH~5.2, is the main protein among whey proteins. Each subunit of BLG contains two tryptophan residues, Trp 19 and Trp 61, the latter being in a substantially solvent-exposed position and in close proximity to the disulfide bond formed between Cys 66 and Cys 160. Solvent-exposed tryptophans exhibit smaller quantum yield and can be quenched by the proximity with disulfide bonds. Trp 19, on the other hand, is buried in the calyx, and from its relative inaccessibility to the solvent is expected to be the sole fluorescent Trp (CROGUENNEC et al., 2004).

BLG has been reported to bind metal ions, fatty acids, vitamins, pharmaceuticals and polyphenols (SNEHARANI et al., 2010; DIARRASSOUBA et al., 2013; LE MAUX et al., 2014; GHALANDARI et al., 2015). BLG can bind either hydrophilic, hydrophobic

or amphiphilic molecules. The binding constants for different compounds with BLG may vary widely from 1.5 10^2 L.mol^{-1} for 2-heptanone and 6.8 10^5 L.mol^{-1} for palmitate to as high as 5.0 10^7 L.mol^{-1} for retinol (LIANG et al., 2008).

Lysozyme (LYS) is a 14.3 Kg·mol^{-1} basic protein exceptionally abundant in egg white, with a very high isoelectric point at pH~11 (SATO et al., 1998). It consists of a high portion of aromatic amino acid residues including six tryptophan (Trp) and three tyrosine (Tyr) residues. Three of Trp residues are located at the substrate binding sites, two in the hydrophobic matrix box, while one is separated from the others. Trp62 and Trp108 are the most dominant fluorophores (>80%) and they are located at the substrate binding sites (DING et al., 2009).

Various binding studies between LYS and several small molecules, such as vitamin C and B12, baicalein, benzocaine, retinoic acid and chloramphenicol have been investigated in recent years (DING et al., 2009; LI et al., 2013; YANG et al., 2015). However, only few studies of LYS and BC have been performed (LI et al., 2017), but not focusing on implications of protein structure changes.

β-carotene (BC) is an important member of the carotenoid family and is an orange-colored pigment abundant in plants and fruits. BC is a precursor of vitamin A and has strong antioxidant activity (VAN LOO-BOUWMAN et al., 2014).

BC possesses poor heat stability, low solubility *in vivo* and is highly susceptible to oxidation, limiting its bioavailability and its use as a functional ingredient in various foods and drug formulations (MENSI et al., 2014). Hence, new technologies increasing the stability of BC in foods were investigated and many carrier particles have been developed in order to improve the stability and dispersibility of these unstable bioactive lipophilic molecules (LOVEDAY & SINGH, 2008).

Some studies have previously reported the binding affinity of BC and BLG, with smaller apparent dissociation constants than the one found for other ligands such as, retinol, retinal and palmitic acid, indicating high binding properties (DUFOUR & HAERTLÉ, 1991; MENSI et al., 2013; HOSSEINI et al., 2015). However, there is a lack of information regarding influence of pH and heat denaturation on BC binding to BLG, as well as for LYS.

Both proteins and pigments are subjected to temperature and pH changes during food processing and it is important verify how those changes impact on protein/BC interaction. In the present work, the interaction of β-carotene with two globular proteins, namely β-lactoglobulin and lysozyme, was investigated in pH 4.0 and 7.0 and after heat-denaturation. The binding parameters were calculated from fluorescence quenching measurements.

2. Experimental section

2.1 Materials

Chicken egg white Lysozyme (>90%), β-carotene (>97%) and spectrophotometric grade ethanol were purchased from Sigma-Aldrich Chemical (St. Louis, MO, EUA); bovine β-lactoglobulin (>90%) was purchased from Davisco Food International Inc. (La Sueur, MN, EUA) and used without further purification. All others chemicals were of analytical grade and deionized water made by Milli-Q system (Millipore, USA) with sodium azide 0.02% was used throughout.

2.2 Apparatus

A Cary Eclipse Fluorescence spectrophotometer (Varian Inc., USA) was applied for the fluorescence measurements, coupled with a Varian Temperature Controller (Varian Inc., USA). A Biomate 3 UV–Vis spectrophotometer (Thermo Electron, USA) was employed for acquiring the absorption spectra. Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano-ZS, Malvern Instruments (Southborough, UK). A PH-201 digital pH meter (Hanna instruments, USA) was used for pH measurements, calibrated with pH 4.01 and 7.01 buffers. The temperature of the solutions was controlled (T = 25.0±0.5 °C) by a refrigerate chamber with photoperiod control (model EL202, EletroLab, Brasil; with three lamps GE Universal Duramax 20 W and intensity of 1.800-2.000 lx).

2.3 Procedures

BLG and LYS stock solutions with final concentration of 0.4 $mmol.L^{-1}$ (0.82 e 0.63% m/v BLG e LYS, respectively) were prepared and maintained for 12 h at 4 °C for complete hydration of proteins. To obtain denatured proteins the stock solutions were heated to 85 °C for 20 min followed by cooling in an ice bath for 10 min. Stock solution of BC was prepared at a concentration of 5 $mmol.L^{-1}$ (2.68% m/v) by dissolving in ethanol. This solution was then diluted to 0.05 $\mu mol.L^{-1}$ in sodium azide 0.02 % before mixtures preparation.

The BC/BLG or BC/LYS mixtures were prepared by combining appropriate amounts of the β-carotene solution and protein solutions to the desired concentrations and a final concentration of phosphate buffer of 10 $mmol.L^{-1}$. These solutions were subsequently left in the dark at 25.0±0.5 °C overnight prior to measurements.

All samples were prepared in test tubes covered with aluminum foil. Sodium azide solution and buffers were filtered in a 0.22 um nylon filter before use. Sodium azide 0.02% was used as preservative. The amount of ethanol added never exceed 4% v/v which has been proved not affect the protein fluorescence properties (ROUFIK et al., 2006; LI & LI, 2011).

The intrinsic fluorescence of the proteins in solution (10 $\mu mol.L^{-1}$) was measured from 305 to 450 nm at an excitation wavelength of 295 nm at 25 °C. The spectral resolution of both excitation and emission was 5 nm and the scan rate was 120 $nm.min^{-1}$. The fluorescence of BC/BLG or BC/LYS mixtures with different BC concentrations was also measured. BC light stability was evaluated exposing protein/BC solutions to light for 7 days and recording the absorbance at 450 nm at day 0, 1 and 7.

The protein stock solutions were diluted 20 times with appropriate buffer solution at the same pH, and size distribution of proteins was determined by DLS. The particle size diameter was reported as a surface-weighted mean diameter ($d_{3,2}$). Each individual result was automatically calculated as the average of 5 measurements with 20 sub-runs each. The particle electric charge (ζ-potential) was calculated from electrophoretic mobility obtained by a laser doppler velocimetry instrument. ζ-potential values were calculated as the average of 5 measurements.

2.4 Statistical analysis

Measurements were performed at least two or three times and analyzed by ANOVA using the SAS software (version 9.0, SAS Institute Inc., Cary, NC, USA). Results were reported as means and standard deviations. Comparison of means was carried out using Tukey or a paired t-test for comparison in time, both at a confidence level of 0.05.

2.5 Calculation

2.5.1 Analysis of fluorescence quenching mechanism

The Stern-Volmer equation (Eq. 1) can be derived considering the fluorescence intensities observed in the absence and presence of quencher, considering the fluorescence intensity (F) observed for a fluorophore is proportional to its concentration in the excited state ([F]).

$$\frac{F_0}{F} = \frac{\gamma + K_q[Q]}{\gamma} = 1 + K_q\tau_0[Q] \qquad 1$$

where $\gamma = \tau^{-1}$ is the decay rate of the fluorophore in the absence of quencher and τ is the lifetime of the fluorophore. In the absence of quenching the excited-state population decays with a rate γ, which is the sum of radiative and non-radiative decay rates. In the presence of quencher, there is an additional decay rate $K_q[Q]$. which is the Stern-Volmer equation.

The quenching data were analyzed according to Stern-Volmer equation (Eq. 2).

$$\frac{F_0}{F} = 1 + K_{SV}[BC] = 1 + K_q\tau_0[BC] \qquad 2$$

where F_0 and F are the fluorescence intensities in the absence and presence of BC, $[BC]$ is β-carotene concentration, K_{SV} is the Stern–Volmer quenching constant, K_q is the biomolecular quenching rate constant and τ_0 is the average lifetime of fluorophores without quencher.

The maximum scatter collision quenching constant, K_q, of various kinds of quenchers with biopolymers is 2×10^{10} $L.mol^{-1}.s^{-1}$ (WARE, 1962). Smaller diffusion-limited quenching constants are expected for larger molecules once they have smaller diffusion coefficients. Smaller values are found for larger molecules which present smaller diffusion coefficients and larger K_q indicates some kind of binding interaction (LAKOWICZ, 2007). The duration of the static of collisional static quenching is represented by the quenching rate constants obtained by K_{SV}.

2.5.2 Binding constants and number of binding sites

Whenever determined that the binding of a pair of molecules is static and a complex is formed, it is desirable to obtain information regarding binding equilibrium.

Binding constants are derived assuming that there are the same and independent binding sites n in the protein, that is, at each binding site there is the same capacity for the protein binding with the ligand. Equation 3 describes the reaction between the ligand (L) and the protein (P).

$$P + nL = L_nP \qquad 3$$

where L_nP is the new complex formed between the protein (P) and the ligand (L) whose binding constant is K_b. Based on the reaction (Eq. 3) different methods to determine K_b and n can be applied.

The main approach used to calculate the binding constant (K_b) and the number of binding sites in the protein (n) (BI et al., 2004; LIANG et al., 2008; WANG et al., 2009) can be derived from the definition of equilibrium constant (Eq. 4).

$$K_b = \frac{[L_nP]}{[L_f]^n[P_f]} \qquad 4$$

Here, $[L_nP]$ is the concentration of the new complex, $[L_f]$ is the free concentration of the ligand, n is the number of the binding sites and $[P_f]$ is the free concentration of the protein.

Assuming that the fully complexed protein is non-fluorescent we obtain the Eq. 5.

$$F = F_0 - F_0\frac{[L_nP]}{[P_t]} \qquad 5$$

Similarly, as in the static quenching process, the fluorescence intensity for the fluorescent system is directly proportional to the free concentration (also considering that the complex formed is nonfluorescent) (Eq. 6).

$$\frac{[P_f]}{[P_t]} = \frac{F}{F_0} \qquad 6$$

Introducing $[L_nP]$ and $[P_f]$ from equations 5 and 6 in equation 4 yields to the static quenching equation (Eq. 7).

$$log\left(\frac{F_0 - F}{F}\right) = log\, K_b + n\, log[L_f] \qquad 7$$

This is the reciprocal log equation, widely used in many experiments. However, because $[L_f]$ is not easy to quantify it is commonly replaced by $[L_t]$. This assumption is only plausible for $[L_f] \gg [P_t]$. As it was not the case for the current study and because increasing ligand concentration could result in increasing inner-effects, a different relation was used in order to obtain the binding constants.

In equation 7, the other assumption is that the complex formed is non-fluorescent, i.e., the protein fluorescence is totally quenched in presence of the additive (WEI et al., 2010). In a different approach BI et al. (2004) adopted an alternative method to calculate K_b and n, considering a step interaction to ligand to different binding sites and under the hypothesis that there are the same and independent binding sites n in the protein, obtaining eq. 8.

$$log\left[\frac{F_0 - F}{F}\right] = nlogK_b - n\, log\left(\frac{1}{[L_t] - \frac{(F_0 - F)[P_t]}{F_0}}\right) \qquad 8$$

Regarding equation 8, no assumption needs to be made, once $[L_t]$ is known. It is then a more appropriate method to estimate K_b and n, as in some studies (BI et al., 2005; WANG et al., 2007; PANG et al., 2012).

After the calculation of the binding constant (K_b), standard Gibbs free energy change ($\Delta G°$) could be estimated according to Eq. 9.

$$\Delta G° = -\,RT \ln (K_b) \qquad 9$$

where T is the temperature (K) and R is the universal gas constant (8.3145 $J.mol^{-1}.K^{-1}$).

3. Results and Discussion

3.1 Size distribution and electrical charge of proteins

Proteins were characterized in terms of size and electrical charge. The measurements were carried in protein solutions with the same concentration used for fluorescence analysis (10 µmol.L^{-1}) in 10 mmol.L^{-1} phosphate buffer in 0.02% (m/v) sodium azide.

Dynamic light scattering (DLS) experiments permitted to obtain the size distribution (expressed as frequency of particle volume) of native and denatured proteins at pH 4.0 and 7.0 (Figure 4.1).

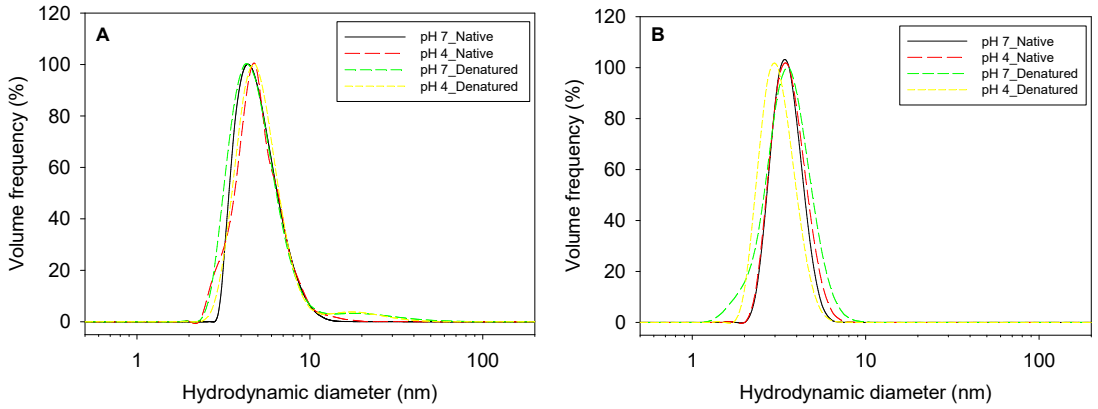

Figure 4.1 – Particle size distribution curves of 10 µmol.L-1 (A) BLG and (B) LYS in 10 mmol.L-1 phosphate buffer at different pH.

BLG exhibited an average diameter of 5.3 nm, equivalent to more than 99% of particle in native state and corresponding to dimers size (ZÚÑIGA et al., 2010). The particle size evidenced dimer association even for pH 4.0 that is the in frontier pH for larger aggregates formation. After denaturation, a 22.9 nm peak appeared, being equivalent to about 5% of particle volume for BLG in both pH. It may be associated with aggregation after structure destabilization, which expose charged and hydrophobic groups.

Indeed, protein concentration, pH, temperature and ionic strength affect monomer-dimer equilibrium and consequently the proportion of monomers and non-covalent dimers in solution (MERCADANTE et al., 2012). Apparently, the low protein

concentration prevents further aggregation and size increasing, even for denatured proteins (ZIMMERMAN et al., 1970; SNEHARANI et al., 2010).

For LYS a single peak of about 3.5 nm was found for all conditions, corresponding to the monomer size (NEMZER et al., 2013). It has been reported that LYS molecules exist as monomers at pH <5 and dimers from pH 5 to 10 (SATO et al., 1998). Despite that, the occurrence of monomers even at pH 7.0 may be also attributed to highly diluted protein solution.

As expected from the isoelectric point of LYS (pH~10.7), it exhibited a positive charge at both pH, more intense for pH 4.0, which is further from IEP than at pH 7.0 (Figure 4.2). As the IEP for BLG (pH~5.2) is between the pH values studied there was a charge inversion, from negative at pH 7.0 to positive at pH 4.0.

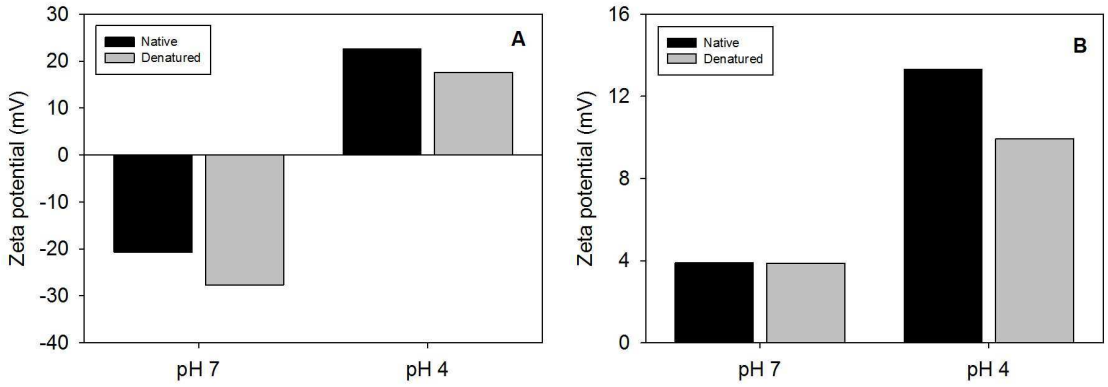

Figure 4.2 – ζ-potential of 10 μmol.L^{-1} (A) BLG and (B) LYS in 10 mmol.L^{-1} phosphate buffer at different pH.

Denaturation modified ζ-potential of proteins, except for LYS at pH 7.0, presumably because the rigidity of this protein (DAMODARAN et al., 2010). Also, ζ-potential measurements of native protein in aqueous solution with pH adjusted with 0.1 mol.L^{-1} NaOH or HCl were -38.4 and 34.6 mV for BLG at pH 7.0 and 4.0, respectively, and 26.4 and 42.8 mV for LYS at pH 7.0 and 4.0, respectively. These differences exemplify the interference of medium components, such as phosphate buffer in electrical charge of proteins.

Although the presence of phosphate ions affected the electrophoretic mobility of both proteins (CUGIA et al., 2013), reducing then the ζ- potential value, the proteins does

not show any binding sites for phosphate (CROGUENNEC et al., 2000). So the presence of phosphate was not expected to interfere in binding properties of the proteins.

3.2 β-carotene light stability

Light is one of the most potent factors affecting BC degradation during storage. Thus, it is important to determine the efficiency of the proteins to protect BC when exposed to light. The light stability at 25 °C was assessed by measuring the absorbance of protein/BC complexes, pure proteins and BC, at 450 nm after the mixture and after 1 and 7 days. The absorbance of pure proteins was subtracted from the absorbance of the complex and the percentage of reduction of initial absorbance is showed in Figure 4.3.

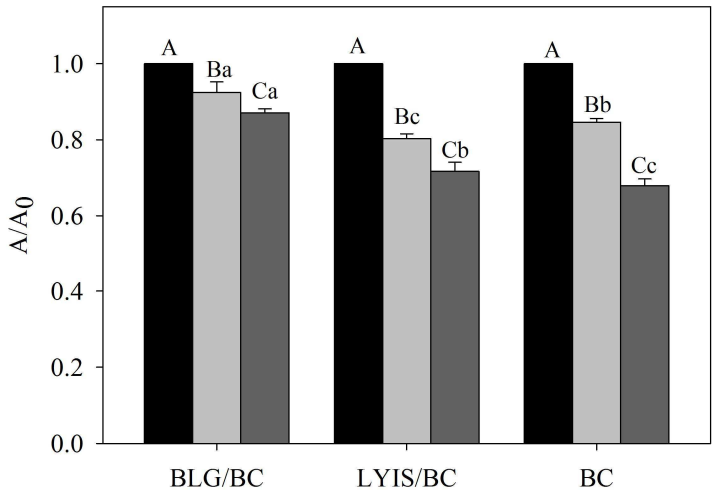

Figure 4.3 - Percentage of absorbance reduction for β-carotene in solution (BC) and β-lactoglobulin (BLG-BC) and Lysozyme (LYS-BC) complexes, at the day 0 (■), 1 (■) and 7 (■). Means followed by the same letters, uppercase for same composition and lowercase in same days, do not differ among themselves ($p>0.05$) by Tukey's test.

The absorbance of BC solution reduced for all conditions with a significant difference on the first day and even at the seventh ($p<0.05$). However, when BC was added to a solution contained BLG the reduction was less pronounced ($p<0.05$), indicating a stronger protection of BC. The absorbance of BC when in a solution containing LYS decayed marked on day 0, but at the end of the experiment, it showed a smaller reduction of absorbance when compared to BC alone.

Those results corroborate with others studies (HATTORI et al., 1995; MENSI et al., 2014) attesting BLG capacity on protecting compounds from light degradation but also indicates that LYS interaction may offer some protection as well, corresponding to its secondary function of carrying molecules.

3.3 Fluorescence spectra of BLG and LYS

The intrinsic fluorescence emission of proteins can typically be either quenched or enhanced and shift to a shorter (blue shift) or a longer wavelength (red shift). Enhancements of the fluorescence emission result from a reduction of intra molecular quenching of Trp residues of the protein. Conversely, quenching occurs when the fluorescence emission of the Trp residue is hindered by increased intra molecular interactions or upon binding of a ligand. A red shift on the maximum wavelength indicates that the Trp residue has moved from a nonpolar environment to a more polar region and when surrounded by a more hydrophobic environment the peaks are blue shifted (DING et al., 2009; LIANG & SUBIRADE, 2012).

The fluorescence emission spectra of BLG and LYS obtained by excitation at 295 nm (Figure 4.4) allowed the confirmation of structural changes on the protein upon pH change and denaturation. Changes in the emission spectra of Trp residues often occur in response to conformational transitions, subunit association, substrate binding or denaturation.

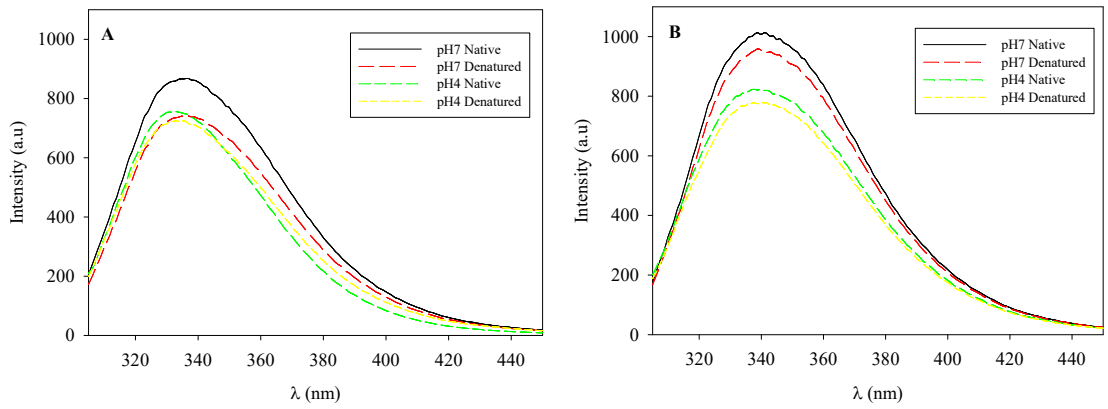

Figure 4.4 - Fluorescence emission spectra of (A) BLG and (B) LYS at different pH and structure. [LYS] = [BLG] = 10 μmol.L^{-1} and λ_{exc}=295 nm.

BLG and LYS exhibit a blue-shift when moving from pH 7.0 to 4.0, which is a λ_{max} decreasing, indicating a decreasing in the polarity on the microenvironment of the fluorophore due to variations on protein conformation (LIANG & SUBIRADE, 2012).

For LYS, this difference may be attributed to increasing in electrical charge at pH 4.0 what may have led to hindering of tryptophan residues. At pH 7.0, BLG molecules exhibited an opening of the calyx site and an increase in sulfhydryl group accessibility for reaction with sulfhydryl reagents (DOMÍNGUEZ-RAMÍREZ et al., 2013). Then, emission maximum was sensitive to the state of association of BLG and the protein conformation close to the sulfhydryl group Cys121.

The fluorescence emission maximum wavelengths for both proteins, as observed in the fluorescence spectra, are characteristic of tryptophan residues placed in a relatively hydrophobic environment, which may be hindered in the interior of the proteins. The hydrophobic character of tryptophan neighborhood is additionally suggested by the comparison with the maximum fluorescence of free D,L-tryptophan in aqueous solution is around 355 nm, evidencing a more hydrophilic environment what confirms the hydrophobic character of tryptophan neighborhood in the proteins (DUFOUR et al., 1994).

3.4 Influence of BC on proteins fluorescence spectra

The fluorescence spectra corrected for F_0 are shown in Figure 4.5 and Figure 4.6. The fluorescence intensity of both BLG and LYS decreased gradually with increasing [BC] for all the conditions studied. No shifts in λ_{max} were observed resulting from the association of the proteins with BC, indicating that albeit BC quenched the fluorescence of proteins it did not changed their structure.

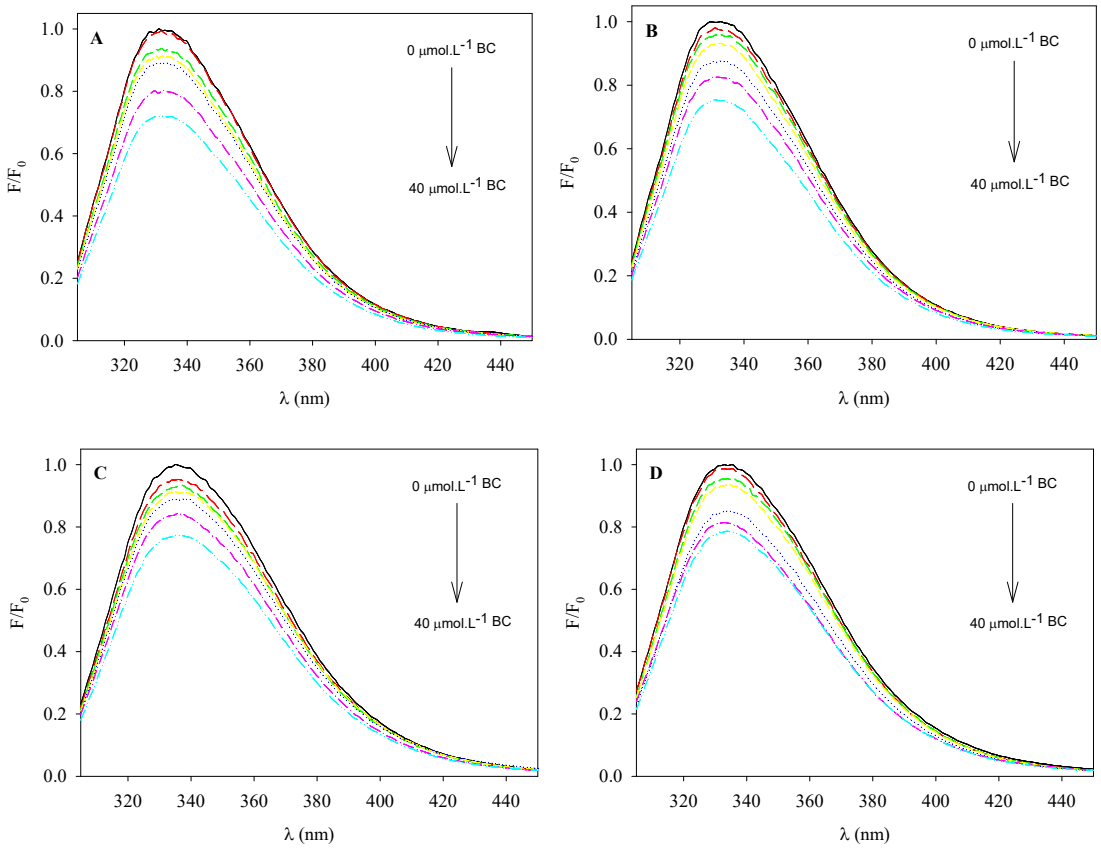

Figure 4.5 – Effect of BC on native (A and B) and denatured (C and D) BLG fluorescence (λ_{ex}= 295 nm) at pH 7.0 (A and C) and 4.0 (B and D). Arrow indicates an increase in BC concentration (from 0 to 40 µmol.L^{-1}).

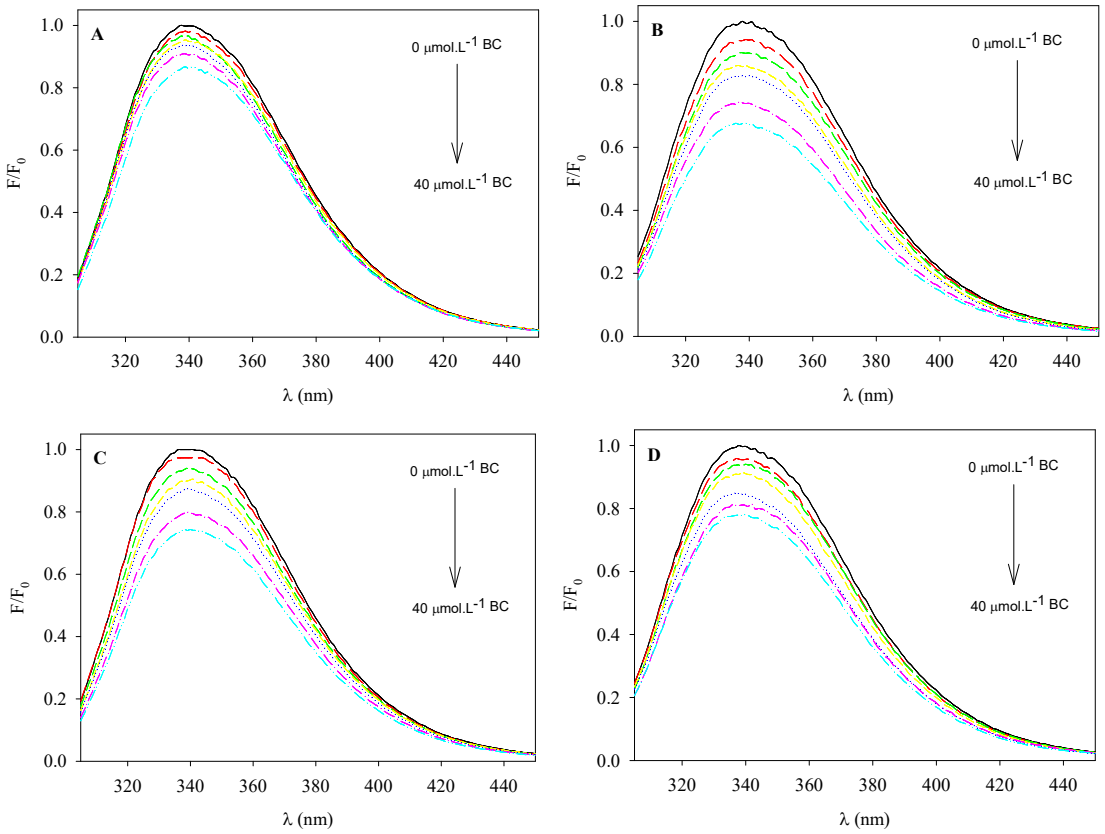

Figure 4.6 – Effect of BC on native (A and B) and denatured (C and D) LYS fluorescence (λ_{ex}= 295 nm) at pH 7.0 (A and C) and 4.0 (B and D). Arrow indicates an increase in BC concentration (from 0 to 40 µmol.L^{-1}).

The intensity of quenching in different pH and on denatured proteins was evaluated by a Stern-Volmer plot (Figure 4.7), which relates F_0/F with the ligand concentration.

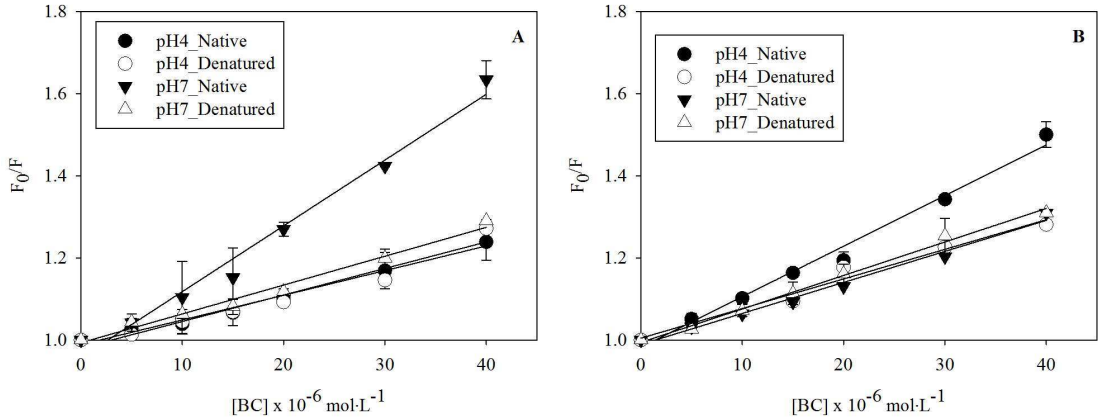

Figure 4.7 – Stern-Volmer plots (F_0/F) for (A) BLG and (B) LYS with increasing [BC] from 0 to 40 µmol.L^{-1} under different pH conditions and denatured conditions. [BLG] = [LYS] = 10 µmol.L^{-1}.

The bigger the reduction of fluorescence intensity (high F_0/F ratios), stronger is the binding between the components. Changes in the emission spectra of Trp residues occur in response to conformational transitions, subunit association, ligand binding, or denaturation. Under BC addition to protein solutions, this decrease can be attributed to quenching effects due BC binding. These results suggest that the quenching may occur by a formation of complex not only a dynamic process (HEMAR et al., 2011; LI & LI, 2011).

3.5 Analysis of fluorescence quenching mechanism

The Stern-Volmer and quenching rate constants were calculated by equation 2, using the slopes obtained by linear regression of (F_0/F) versus [BC] (Table 4.1). The values of τ_0 used in this study were 1.28×10^{-9} s and 5.0×10^{-9} s, for BLG and LYS, respectively, obtained by LIANG et al. (2008) and LI & LI (2011).

Table 4.1 – Stern-Volmer quenching constants for the interaction of BC with BLG and LYS.

		BLG			LYS		
Protein	State	K_{SV} (10^4 L.mol^{-1})	K_q (10^{12} L.mol^{-1}.s^{-1})	R^2	K_{SV} (10^4 L.mol^{-1})	K_q (10^{12} L.mol^{-1}.s^{-1})	R^2
pH 4.0	Native	0.62 ± 0.09	4.84 ± 0.73	0.979	1.29 ± 0.14	2.58 ± 0.27	0.986
	Denatured	0.66 ± 0.12	5.19 ± 0.93	0.995	0.60 ± 0.13	1.20 ± 0.26	0.933
pH 7.0	Native	1.44 ± 0.11	11.27 ± 0.82	0.988	0.78 ± 0.00	1.56 ± 0.00	0.988
	Denatured	0.69 ± 0.07	5.38 ± 0.53	0.970	0.84 ± 0.09	1.67 ± 0.17	0.996

K_q obtained in this study were at least 100 times greater than the maximum scatter collision constant, indicating the fluorescence quenching observed was mainly due static quenching than dynamic collision quenching. Further analysis were handled based on static quenching with formation of a non-fluorescent complex between the proteins and BC.

3.6 Binding parameters of BC to BLG and LYS

Once the static quenching mechanism was dominant in the interaction of the proteins and BC, it was possible to determine the binding constant for a non-fluorescence complex.

The plots of $log\left[\frac{F_0-F}{F}\right]$ versus $log\left(\frac{1}{[L_t]-\frac{(F_0-F)[P_t]}{F_0}}\right)$ (Figure 4.8) were elaborated and linear regressions were performed to obtain the slopes and intercepts, according to equations 8.

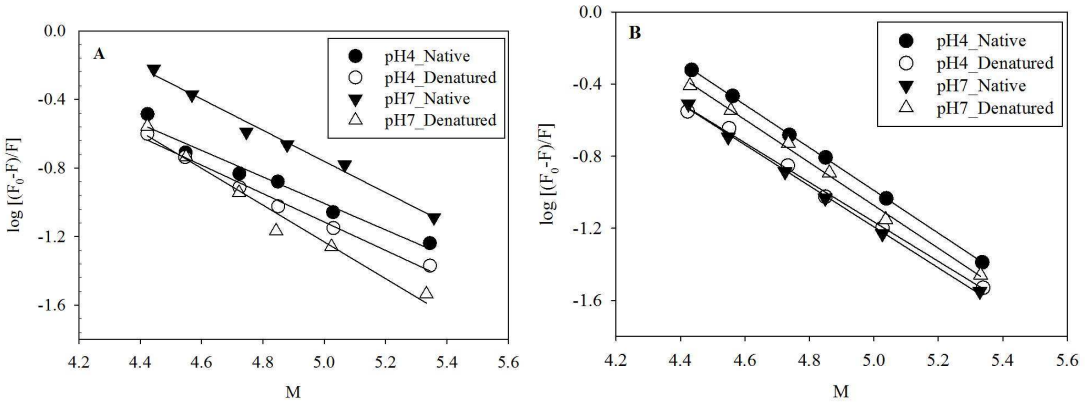

Figure 4.8 - Plots of log [(F_0-F)/F] versus M=$log\left(\frac{1}{[L_t]-\frac{(F_0-F)[P_t]}{F_0}}\right)$ for (A) BLG and (B) LYS. [BC] ranging from 5 to 40 μmol.L^{-1}; [BLG] = [LYS] = 10 μmol.L^{-1}.

The binding constants (K_b) and number of binding sites (n) were calculated by equation 8 (Table 4.2).

Table 4.2 – Binding parameters for BC/BLG and BC/LYS complex formation, at 25 ºC.

		BLG			LYS		
Protein	State	K_b (10^4 L.mol^{-1})	n	R^2	K_b (10^4 L.mol^{-1})	n	R^2
pH 4.0	Native	0.67 ± 0.14^{b}	1.01 ± 0.49^{a}	0.981	1.47 ± 0.01^{a}	1.20 ± 0.01^{a}	0.995
	Denatured	0.30 ± 0.02^{c}	0.66 ± 0.06^{b}	0.999	0.79 ± 0.18^{c}	1.08 ± 0.04^{a}	0.951
pH 7.0	Native	1.64 ± 0.11^{a}	1.09 ± 0.07^{a}	0.966	0.98 ± 0.00^{bc}	1.21 ± 0.00^{a}	0.996
	Denatured	0.25 ± 0.03^{c}	0.71 ± 0.03^{b}	0.944	1.10 ± 0.11^{b}	1.18 ± 0.05^{a}	0.993

Means with the same letter in the same column are not significantly different, by the Tukey test ($p>0.05$).

A high K_b value indicates high binding affinity. The values of K_b ranged from 10^3 to 10^4 Lmol^{-1}, suggesting that binding occurred under all conditions but varied as a function of protein, pH and upon denaturation (ALARCÓN et al., 2012; LI et al., 2017).

BLG showed greater binding capacity at pH 7.0 and in native state. The BLG possesses a loop in its secondary structure that acts as a gate to the central cavity (KONTOPIDIS et al., 2004; DOMÍNGUEZ-RAMÍREZ et al., 2013). At pH 4.0 it is in a closed conformation, limiting the access of the ligand to the most reactive site of the molecule and could then cause K_b diminishing.

Nevertheless, once it has still significant binding, even with limited access to the central calix, it may be concluded that BC molecule could bind to external sites of BLG molecule, although with less efficiency. Besides the internal cavity of β-barrel, which is an ideal site for hydrophobic ligands, three other portions of BLG have been suggested as sites for ligand binding: the surface hydrophobic pocket in a groove between the α-helix and the β-barrel, the outer surface near Trp19-Arg124 and the monomer/monomer interface (LIANG & SUBIRADE, 2010; MENSI et al., 2013).

COLLINI et al. (2000) described two main binding sites in BLG molecule, an internal (central calix) and an external one. Considering that at pH 4.0 the access to the central calix for the ligand molecule was hindered and it caused a decrease in K_b, we may say the central calix was the main binding site for BC binding to BLG and corresponds to about 60% of the binding, considering the diminishing in K_b from pH 7.0 to 4.0.

The same consideration may be made regarding denaturation. After denaturation, the protein loses its tertiary structure what, for BLG implicates in losing the central calix structure (DOMÍNGUEZ-RAMÍREZ et al., 2013). Even more, the denaturation caused a reduction in K_b even for the protein at pH 4.0, indicating the destruction of binding sites. Protein denaturation also may expose functional groups buried inside the protein structure, affecting interfacial and binding properties (APENTEN et al., 2002).

Denaturation caused a significant decreased in K_b for BLG in both pHs and LYS at pH 4.0. BLG exhibited the major decrease probably due its more organized binding site, the central calix, which was completely unstructured upon heating. According to BUSTI et al. (2005), denaturation may expose binding sites of protein structure and increase binding of small hydrophilic molecules, such as acrylamide. However, when the binding occurs with hydrophobic molecules the solvent exposition has a reverse effect, diminishing binding.

The BC/BLG molar binding ratio at pH 7.0 was one, with denaturation causing a decreasing of n, to around 0.5, which is the situation when one molecule of BC binds to two molecules of BLG. DUFOUR & HAERTLÉ (1991) found a 1:2 stoichiometry to BC/BLG binding and attributed it to the presence of two β-ionone rings in BC, which may interact with two BLG molecules. BC could also bind at the dimer interface, what would result in $n = 0.5$. MENSI et al. (2013) related that BC did not compete for the same site of retinol, which is known to bind to the central calix.

LYS showed greater K_b values at pH 4.0 and was affected by denaturation in this pH. As seen for the maintenance of ζ-potential values in pH 7.0, k_b was significantly similar for denatured and native proteins, corroborating with the high stability of the protein in this condition where no changes in the number of BC binding site were observed. The BC/LYS molar binding ratio were the same for all conditions ($p>0.05$) and similar to those found by LI et al. (2017), suggesting that one molecule of BC binds to one molecule of LYS.

Standard Gibbs free energy change ($\Delta G°$) values, directly calculated from K_b values, indicate a driving force for the association of proteins and BC. The results are shown in Table 4.3.

Table 4.3 – Standard Gibbs free energy change ($\Delta G°$) values for BC/BLG and BC/LYS complex formation at 25 ºC for different pH and protein states.

Protein	State	BLG	LYS
pH 4.0	Native	-21.8±0.5^{b}	-23.8±0.0^{a}
	Denatured	-19.8±0.2^{c}	-22.2±0.0^{c}
pH 7.0	Native	-24.0±0.2^{a}	-22.8±0.6^{bc}
	Denatured	-19.3±0.3^{c}	-23.1±0.3^{ab}

Means with the same letter in the same column are not significantly different, by the Tukey test (p>0.05).

Standard Gibbs binding free energy values ranged from –24.0 to –19.3 KJ.mol^{-1}, indicating that the binding between BC and both proteins process was favored in detriment of dissociation. More negative $\Delta G°$ values for BC binding to native BLG and LYS at pH 7.0 and 4.0, respectively, also indicated more stable complex formed at these conditions.

4. Conclusion

It was provided an approach for studying the binding of BC to LYS and BLG. The results showed that LYS and BLG fluorescence were quenched by BC through static quenching process. It was observed that at pH 4.0 conformational changes on the structure of the proteins lead to a decrease of the binding constant for BLG and an increase for LYS. In addition to changes due to pH, denaturation also influenced the protein structure and diminished BC binding.

The spectral data revealed the conformational changes of LYS and BLG upon interaction with BC, a spontaneous process as confirmed by negative standard Gibbs binding free energy values.

These results can be of fundamental importance on strategies to incorporate BC into food products through interaction with proteins. Knowing the binding behavior of BC to proteins at different pH and after denaturation can lead to optimization of current methods of hydrophobic compounds incorporation, complementing release studies.

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CONCLUSÃO GERAL

As proteínas β-lactoglobulina (BLG) e Lisozima (LIS) formaram emulsões cineticamente estáveis e com diâmetros menores que 300 nm, em pH 4,0 e 7,0. A adição de NaCl durante o preparo não melhorou a estabilidade dessas emulsões. Quando misturadas, a BLG apresentou-se com maior proporção na interface e as emulsões formadas por essas proteínas apresentaram maiores diâmetros e menor estabilidade, principalmente quando adicionadas de NaCl.

A mistura das proteínas não resultou em maior estabilidade das emulsões, o que só foi alcançado com a adição de pectina, utilizando emulsões multicamadas. Esse polímero carregado e estabilizante, favoreceu a resistência de emulsões multicamadas ao armazenamento e à adição de NaCl, podendo ser exploradas para aplicações em diferentes alimentos.

A fluorescência da LIS e BLG foi suprimida pela presença do BC por um processo de supressão estático e a constante de ligação foi afetada por mudanças na conformação das proteínas causadas pela mudança de pH e desnaturação. Resultados esses, importantes para embasar o uso dessas proteínas para incorporação do BC em alimentos, especialmente na forma de emulsões.

A partir das conclusões obtidas nesse trabalho, outros estudos posteriores podem ser sugeridos:

- Avaliar a capacidade das emulsões de LIS e BLG em proteger compostos hidrofóbicos contra luz, temperatura e oxidação;

- Avaliar o efeito de variações de pH para condições semelhantes de carga líquida das proteínas, por exemplo pH 2, 3, 4 e 5 ou 6, 7 ,8 e 9;

- Avaliar o efeito da adição de outros tipos de sais mono e bivalentes.