ELIARA ACIPRESTE HUDSON

ALBUMINA DO SORO BOVINO COMO NANOCARREADOR DE CURCUMINA

Dissertação 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 *Magister Scientiae.*

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(Coorientador)

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RESUMO

HUDSON, Eliara Acipreste, M.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Albumina do soro bovino como nanocarreador de curcumina.** Orientadora: Ana Clarissa dos Santos Pires. Coorientadores: Luis Henrique Mendes da Silva e Geany Peruch Camilloto.

A curcumina exibe diversas atividades biológicas, mas suas características de baixa solubilidade e pouca estabilidade termodinâmica limitam sua biodisponibilidade. Portanto, é indispensável estudar sua interação com a albumina do soro bovino (BSA), que é conhecida por aumentar a solubilidade e estabilidade de compostos hidrofóbicos em diversas formulações. Neste trabalho estudou-se a interação entre a curcumina e a BSA em suas conformações nativa e desnaturada, em pH 7,0, por meio de diferentes técnicas analíticas, e avaliou-se a cinética de fotodegradação da curcumina na presença da BSA. Pela análise de espectroscopia de fluorescência (EF) e de calorimetria de titulação isotérmica (ITC) os valores da constante de ligação (K_a) entre a proteína e o composto bioativo obtidos foram na ordem de 10^5 $L.mol^{-1}$, enquanto que por ressonância plasmônica de superfície (RPS) esse valor foi $3,88x10^3$ $L.mol^{-1}$. Essas diferenças são explicadas pelas limitações apresentadas por cada técnica. O experimento de competição realizado indicou que a curcumina compete com os marcadores utilizados pelos sítios I, II e III da BSA. Os valores obtidos para $\Delta H°$ por EF (-8,67 $kJ.mol^{-1}$) e por ITC (-29,11 $kJ.mol^{-1}$) indicaram que o processo de formação dos complexos foi exotérmico. Nos estudos com a BSA desnaturada houve um ligeiro aumento nos valores de K_a ($4,53x10^5$ $L.mol^{-1}$ e $2,00x10^6$ $L.mol^{-1}$, obtidos por EF e ITC, respectivamente, a 25 ℃), comparados aos valores encontrados com a BSA nativa, o que indicou que há outros locais na BSA para ligação da curcumina além dos mostrados no experimento de competição. O perfil termodinâmico foi semelhante ao obtido para a conformação nativa, mas $\Delta H°$ foi mais negativo (-16,12 $kJ.mol^{-1}$ e -42,63 $kJ.mol^{-1}$, por EF e ITC, respectivamente), indicando que a mudança de conformação da BSA proporcionou uma interação mais exotérmica. No experimento de fotodegradação, à medida que a concentração de BSA aumentou, os valores da constante de degradação (K_d) da curcumina

diminuíram exponencialmente e seu tempo de meia-vida ($t_{1/2}$) aumentou linearmente, o que indicou que a BSA protege a curcumina da fotodegradação. Esse trabalho contribui para um conhecimento mais aprofundado da interação intermolecular entre curcumina e BSA por trazer resultados obtidos por técnicas com diferentes fundamentos e parâmetros cinéticos da degradação da curcumina na presença de diferentes concentrações de BSA, o que ainda era ausente na literatura, fornecendo informações importantes para o sucesso da aplicação tecnológica da curcumina em diferentes formulações.

ABSTRACT

HUDSON, Eliara Acipreste, M.Sc., Universidade Federal de Viçosa, February, 2017. **Bovine serum albumin as nanocarrier of curcumin.** Adviser: Ana Clarissa dos Santos Pires. Co-advisors: Luis Henrique Mendes da Silva and Geany Peruch Camilloto.

Curcumin exhibits various biological activities, but its low solubility and stability characteristics limit its bioavailability. Therefore, it is indispensable to study its interaction with bovine serum albumin (BSA), which can increase the solubility and stability of several hydrophobic compounds in various formulations. Here, we studied the interaction between curcumin and BSA in its native and denatured conformations, at pH 7.0, was studied through different analytical techniques, and the kinetics of curcumin photodegradation was evaluated in the presence of BSA. By the analysis of fluorescence spectroscopy (FS) and isothermal titration calorimetry (ITC) the values of the binding constant (K_a) between the protein and the bioactive compound obtained were in the order of 10^5 $L.mol^{-1}$, whereas by surface plasmonic resonance (SPR), this value was $3.88x10^3$ $L.mol^{-1}$. These differences are explained by the limitations presented by each technique. The competition experiment performed indicated that curcumin competes with the markers used by sites I, II and III of the BSA. The values obtained for ΔH ° by FS (-8.67 $kJ.mol^{-1}$) and by ITC (-29.11 $kJ.mol^{-1}$) indicated that the process of forming the complexes was exothermic. In the studies with denatured BSA there was a slight increase in K_a values ($4.53x10^5$ $L.mol^{-1}$ and $2.00x10^6$ $L.mol^{-1}$, obtained by FS and ITC, respectively, at 298 K), compared to the values found with the native BSA, which indicated that there are other BSA sites for curcumin binding in addition to those shown in the competition experiment. The thermodynamic profile was like that obtained for the native conformation, but ΔH ° was more negative (-16.12 $kJ.mol^{-1}$ and -42.63 $kJ.mol^{-1}$, by FS and ITC, respectively), indicating that the change in conformation of the BSA provided a more exothermic interaction. In the photodegradation experiment, as the BSA concentration increased, the values of the curcumin degradative constant (K_d) decreased exponentially and its half-life ($t_{1/2}$) increased linearly, which indicated that BSA protected the curcumin

from photodegradation. This work contributes to a more deepened knowledge of the intermolecular interaction between curcumin and BSA by bringing results obtained by techniques with different fundamentals and kinetic parameters of curcumin degradation in the presence of different BSA concentrations, which was still absent in the literature, providing important information to the success of the technological application of curcumin in different formulations.

SUMÁRIO

1. INTRODUÇÃO

A curcumina é um composto que desperta muito interesse em diversas áreas de pesquisa devido a suas bem relatadas propriedades biológicas e farmacocinéticas. Entre essas propriedades biológicas pode-se citar as antioxidantes, anticarcinogênicas, antimicrobianas e anti-inflamatórias. Porém, apesar dessas propriedades, as baixas biodisponibilidade, estabilidade e solubilidade da curcumina limitam sua eficácia terapêutica e também sua utilização em alimentos. Vários compostos têm sido utilizados com a finalidade de melhorar as essas características limitantes da curcumina, incluindo diversas proteínas.

As albuminas do soro são as proteínas mais abundantes no plasma sanguíneo e são muito utilizadas como proteínas-modelo para diversos estudos. Essas proteínas são responsáveis pelo transporte de diversos compostos no organismo, o que aumenta o interesse em estudá-las.

A albumina do soro bovino (BSA) possui uma homologia estrutural de 80% com a albumina humana (HSA) e tem sido utilizada em vários estudos como carreadora de diversos compostos, incluindo a curcumina, com a finalidade de melhorar suas propriedades de solubilidade, estabilidade e controlar sua distribuição pelo organismo. No entanto, essas proteínas são passíveis a mudanças de conformação, o que muitas vezes pode afetar suas propriedades carreadoras.

A ligação de compostos às albuminas pode afetar suas funções fisiológicas, sendo relevante, portanto, estudar as propriedades de interação entre a proteína e o ligante. Além disso, a formação de complexo entre os dois pode modificar várias propriedades do composto ligado, como metabolismo e tempo de meia-vida.

Existem várias técnicas disponíveis para o estudo e caracterização de interações intermoleculares, entre as quais pode-se citar as técnicas espectroscópicas, como espectroscopia de fluorescência e espectroscopia de absorção molecular, técnicas calorimétricas, como a microcalorimetria de

titulação isotérmica e a nanocalorimetria diferencial de varredura, ressonância plasmônica de superfície e também técnicas eletrocinéticas, como medidas de potencial zeta. No entanto, como cada técnica possui suas limitações, nem sempre é possível caracterizar completamente uma interação utilizando-se apenas uma delas. Portanto, é interessante que se realize o estudo por meio do uso de várias técnicas analíticas que permitam a determinação de parâmetros fundamentais à caracterização de interações intermoleculares.

A determinação de parâmetros termodinâmicos de interação entre BSA em suas conformações nativa e desnaturada e a curcumina, obtidos por diferentes técnicas analíticas, e também o estudo do efeito da BSA e sua conformação na estabilidade fotoquímica da curcumina, obtendo-se parâmetros cinéticos de sua fotodegradação, ainda ausentes na literatura, são muito relevantes e úteis para as áreas farmacêutica e alimentícia.

2. OBJETIVOS

2.1. Objetivos gerais

Estudar a termodinâmica de interação entre a BSA e a curcumina por meio de diferentes técnicas e determinar o efeito protetor da BSA contra a fotodegradação da curcumina, nas conformações nativa e desnaturada da proteína.

2.2. Objetivos específicos

- Determinar a constante de interação, a estequiometria de formação do complexo, variação de entalpia, variação da energia livre de Gibbs e variação de entropia padrões de formação do complexo entre a curcumina e a BSA, por meio das técnicas de espectroscopia de fluorescência, ressonância plasmônica de superfície e calorimetria de titulação isotérmica;
- Avaliar o efeito da conformação da proteína sobre os parâmetros termodinâmicos de interação entre a curcumina e a BSA;
- Estudar a cinética de fotodegradação da curcumina na presença de diferentes concentrações de BSA em suas conformações nativa e desnaturada.

3. REVISÃO DE LITERATURA

3.1. Curcumina

Curcumina (1,7-bis-(4-hidroxi-3-metoxiphenil)-1,6-heptadieno-3,5-diona) (Figura 1) é um composto fenólico extraído do rizoma seco da planta *Curcuma longa.* Vogel, em 1842, foi o primeiro a isolar a curcumina, e sua estrutura foi caracterizada por Lampe e Milobedeska em 1910. É frequentemente utilizada como tempero, conservante e corante alimentício (Hatcher, Planalp, Cho, Torti, & Torti, 2008; M. Li, Ma, & Ngadi, 2013a; Naksuriya, van Steenbergen, Torano, Okonogi, & Hennink, 2016; Niu et al., 2012).

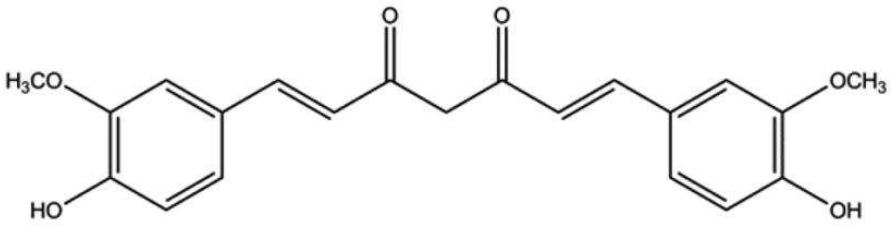

Figura 1. Estrutura molecular da curcumina. Fonte: PILETSKA et al. (2015).

Comercialmente, a curcumina é encontrada em uma mistura de curcuminóides, em que se encontram também os compostos bisdemetoxicurcumina e demetoxicurcumina. Foi relatado que algumas das atividades biológicas dessa mistura são mais fortes e evidentes que as da curcumina pura. Esses compostos são encontrados em meios não polares, bem como as formas bis-α,β-insaturada e β-dicetona da curcumina. Em soluções aquosas, solventes polares e meios biológicos, o tautômero enol da curcumina é predominantemente encontrado (Figura 2) (Naksuriya et al., 2016).

Figura 2. Formas tautoméricas enol e dicetona da curcumina. Fonte: Adaptado de NAKSURIYA et al. (2016).

A curcumina é praticamente insolúvel em água em pH ácido e neutro. Em solução tampão pH 5,0 já foi reportado que sua solubilidade é de apenas 11 ng/mL (Naksuriya et al., 2016). Já em sistemas aquosos com pH alcalino, o hidrogênio do grupo fenol é ionizado, formando o íon fenolato, o que permite a dissolução da curcumina (Kumavat et al., 2013; Pan, Zhong, & Baek, 2013). Em metanol, a curcumina é levemente solúvel; em DMSO e clorofórmio apresenta boa solubilidade (Hatcher et al., 2008).

Numerosos estudos tem reportado que a curcumina apresenta várias atividades biológicas e farmacológicas, tanto no estado sólido quanto em solução (Priyadarsini, 2009). Um papel crucial nas atividades benéficas da curcumina é exercido pelos grupos hidroxila dos anéis benzeno, pelas duplas ligações conjugadas e pela porção β-dicetona central presentes em sua estrutura (Niu et al., 2012; M. Yang, Wu, Li, Zhou, & Wang, 2013a). Os grupos hidroxila fenólicos podem doar átomos de hidrogênio, atuando como antioxidantes, e a β-dicetona age como um quelante de metais (Schneider, Gordon, Edwards, & Luis, 2015). A capacidade de eliminar radicais reativos de oxigênio e nitrogênio e sua característica de solubilidade em lipídeos são as propriedades mais importantes responsáveis pelas atividades da curcumina (Barik, Priyadarsini, & Mohan, 2003).

Atividade anti-inflamatória, anticarcinogênica, antioxidante e antimicrobiana tem sido associadas à curcumina (GB, SL, G, & ZZ, 2001; Kim, Choi, & Lee, 2003; Ruby, Kuttan, Dinesh Babu, Rajasekharan, & Kuttan, 1995). Apesar desses efeitos terapêuticos, suas características de baixa solubilidade em

água em meio ácido ou neutro dificultam sua absorção pelo organismo, além de sua baixa estabilidade em meios básicos, limitando sua biodisponibilidade. Consequentemente, seu rápido metabolismo e rápida eliminação sistêmica limitam sua eficácia terapêutica (Gupta, Patchva, & Aggarwal, 2013; M. Li et al., 2013a; Mondal, Ghosh, & Moulik, 2016). Baixos níveis séricos, distribuição limitada aos tecidos e curto tempo de meia-vida são consequências da baixa biodisponibilidade da curcumina (Anand, Kunnumakkara, Newman, & Aggarwal, 2007).

Quanto à sua estabilidade em meios ácidos, menos de 20% de curcumina se degrada em 1 hora, enquanto em meios alcalinos a degradação ocorre em 30 minutos, originando os produtos trans-6-(4'-hidroxi-3'-metoxifenil)-2,4-dioxo-5-hexanal, ácido ferúlico e vanilina (Sharma, Gescher, & Steward, 2005). Wang et al. (1997) relataram que cerca de 90% de curcumina em pH 7,2 degradou-se em 30 minutos de incubação a 37°C. Barik et al. (2003) encontraram qu e essa rápida degradação em pH 7,2 não é devido a uma decomposição química, mas sim a uma precipitação da mistura de curcuminóides. Além da instabilidade em meios básicos, a curcumina é muito sensível a luz, altas temperaturas e oxigênio (Paramera, Konteles, & Karathanos, 2011).

Devido a essas características da curcumina, várias alternativas têm sido buscadas para aumentar sua biodisponibilidade e estabilizá-la em meios aquosos. Esforços para alcançar esses objetivos incluem incorporação da curcumina em ciclodextrinas (Baglole, Boland, & Wagner, 2005; Paramera et al., 2011), micelas de surfactantes (Tønnesen, 2002), lipossomas (Isacchi et al., 2012), nanopartículas lipídicas e poliméricas (Dandekar et al., 2010; C. Li et al., 2012; Yallapu, Jaggi, & Chauhan, 2012), e também em proteínas (Barik et al., 2003; Esmaili et al., 2011; M. Li, Ma, & Ngadi, 2013b; Mitra, 2007; Mohammadi & Moeeni, 2015; Sahu, Kasoju, & Bora, 2008; Sneharani, Karakkat, Singh, & Rao, 2010; Sneharani, Singh, & Appu Rao, 2009; Tapal & Tiku, 2012; M. Yang et al., 2013a).

A utilização de proteínas para incorporação e melhora das características de solubilidade, estabilidade e biodisponibilidade da curcumina apresenta

algumas vantagens, pois a grande maioria das proteínas são naturais, não tóxicas e disponíveis (M. Yang et al., 2013a).

Esmaili et al. (2011) estudaram a interação da curcumina com micelas de β-caseína de leite de camelo por meio das técnicas de espectroscopia de fluorescência e de absorção molecular (UV-vis), e avaliaram os efeitos dessa interação sobre a solubilidade, citotoxicidade e atividade antioxidante da curcumina. Os autores encontraram que as micelas de β-caseína interagem com a curcumina principalmente por interações hidrofóbicas e que a solubilidade da curcumina foi aumentada pelo menos 2500 vezes. Sua atividade antioxidante também foi maior comparada à curcumina livre e sua citotoxicidade para a linha de células de leucemia humana foi aumentada na presença das micelas, obtendo-se valores de concentração inibitória de 26,5 e 17,7 mmol/L para curcumina livre e encapsulada, respectivamente.

Um estudo sobre a interação entre curcumina e micelas de caseína bovina foi conduzido por Sahu et al. (2008), e a utilidade das micelas como veículo de entrega da curcumina para células de câncer foi avaliada. Os resultados indicaram que as moléculas de curcumina formam complexo com as micelas de caseína, com uma constante de ligação de $1{,}48 \times 10^4$ L/mol. A avaliação de citotoxicidade mostrou que a concentração inibitória da curcumina complexada foi de 12,69 μM, enquanto que a encontrada para a curcumina livre foi de 14,85 μM.

Sneharani et al. (2009) estudaram a interação da curcumina com α_{s1}-caseína. Com base nas medições espectroscópicas, concluíram que a curcumina se liga à α_{s1}-caseína, a pH 7,4, em dois diferentes locais, um com alta afinidade ($2{,}01 \times 10^6$ L/mol) e outro com uma afinidade menor ($6{,}3 \times 10^4$ L/mol). A interação entre as duas moléculas aumentou a estabilidade da curcumina a pH 7,2. Nesse pH, o tempo de meia-vida da curcumina livre em tampão foi de 8,8 minutos, enquanto que na presença da α_{s1}-caseína esse tempo foi aumentado para 340 minutos.

No trabalho de Li et al. (2013) a interação entre curcumina e a proteína β-lactoglobulina foi estudada por meio das técnicas espectroscópicas de

infravermelho e fluorescência nos valores de pH 6,0 e 7,0, obtendo-se os resultados de que a curcumina se liga a dois locais diferentes da proteína nos dois valores de pH. A ligação entre as duas moléculas proveu um aumento na atividade antioxidante da curcumina. Sneharani et al. (2010) também estudaram a interação entre curcumina e β-lactoglobulina na forma de nanopartículas. As nanopartículas preparadas foram capazes de encapsular a curcumina com uma eficiência maior que 96% e de aumentar sua estabilidade e sua solubilidade para 625 µM, contra 30 nM quando a curcumina estava livre.

Mitra et al. (2007) estudaram a ligação e a estabilidade da curcumina na presença de albumina do soro bovino (BSA) por meio de diálise e UV-vis. O autor encontrou que a curcumina se liga à BSA com uma alta afinidade ($6,3x10^6$ L/mol) e que o complexo formado oferece significativa proteção à molécula de curcumina da deterioração por irradiação UV e por exposição a meios alcalinos.

Mohammadi et al. (2009) estudaram a interação da curcumina e da diacetilcurcumina, um derivado também biologicamente ativo, com as albuminas do soro humano e bovino. Como as constantes de ligação entre a curcumina e as albuminas ($3,12x10^5$ e $3,42x10^6$ L/mol para HSA e BSA, respectivamente) foram maiores que as constantes entre diacetilcurcumina e as proteínas ($6,36x10^2$ e $2,92x10^3$ L/mol), concluiu-se que o grupo fenólico OH presente apenas na estrutura da curcumina exerce um importante papel na sua interação com as albuminas.

A estabilidade e a ligação da curcumina à BSA na presença de i-carragena, em pH 7,0, foram estudadas por Yang et al. (2013). Quando havia i-carragena no meio, um complexo por atração eletrostática entre essas moléculas e a BSA era formado, e esse complexo mostrou maior afinidade de ligação à curcumina do que a BSA sozinha. Os autores explanaram que a formação do complexo estabilizou a estrutura enovelada da BSA, provendo um ambiente mais hidrofóbico para a curcumina. Para estudar a estabilidade da curcumina a pH 7,0, as mudanças na intensidade relativa de absorção máxima da curcumina em função do tempo foram utilizadas. Como resultado, cerca de 53% de curcumina na forma livre se decompôs depois de 1000 minutos de incubação, enquanto que

cerca de 24% da curcumina ligada à BSA se decompôs. Quando havia 0,5, 1,0 e 1,5 g/L de i-carragena ligada à BSA, a degradação foi de 16, 12 e 10%, respectivamente.

3.2. Albumina do soro bovino

As albuminas do soro são as proteínas mais abundantes no plasma sanguíneo e correspondem a 60% do seu total de proteínas. São frequentemente utilizadas como proteínas-modelo para muitos estudos bioquímicos, físico-químicos e biofísicos (Gelamo, Silva, Imasato, & Tabak, 2002).

A propriedade mais importante desse grupo de proteínas é a de transporte de vários compostos de importância biológica, como ácidos graxos, hormônios e vitaminas (Bourassa, Hasni, & Tajmir-Riahi, 2011). Essas proteínas são também responsáveis por manter a pressão osmótica necessária para a distribuição adequada dos fluidos corporais entre os compartimentos intravasculares e tecidos corporais (Pacheco & Bruzzone, 2012). Essas características aumentam o interesse pelo estudos dessas moléculas, pois uma pequena alteração estrutural pode afetar suas importantes funções biológicas (Vilhena, Rubio-Pereda, Vellosillo, Serena, & Pérez, 2016).

A existência de cavidades hidrofóbicas nas albuminas do soro provê o aumento da solubilidade de substâncias hidrofóbicas no plasma sanguíneo e controlam sua distribuição pelo corpo. Alguns estudos têm mostrado que a distribuição e metabolismo no sangue de um grande número de compostos biologicamente ativos, como drogas e alguns metabólitos, são dependentes de sua afinidade com as albuminas do soro (Callister et al., 1990; Jahanban-Esfahlan & Panahi-Azar, 2016a; Sinha et al., 2016).

A BSA possui uma variedade de funções fisiológicas e importantes propriedades como estabilidade, solubilidade em meios aquosos, capacidade de ligação a vários compostos e sua homologia estrutural de 80% com a HSA (Meti, Byadagi, Nandibewoor, & Chimatadar, 2014; Shi et al., 2010). A BSA é composta por 582 resíduos de aminoácidos, dentre os quais dois são resíduos de triptofano

(Trp 134 e Trp 212). O Trp 134 localiza-se na superfície da molécula e o Trp 212 está localizado dentro de uma cavidade hidrofóbica da BSA. A HSA é composta por 585 resíduos de aminoácidos e apenas um triptofano, o Trp 214. Esses resíduos de triptofano contribuem para a característica de fluorescência intrínseca das albuminas, o que permite o estudo de interações com compostos por meio da técnica de espectroscopia de fluorescência (Cheng, 2012a).

A BSA é dividida em três domínios linearmente dispostos e estruturalmente distintos (I-III). Cada um destes domínios é composto por dois subdomínios, A e B. As cavidades de ligação da BSA localizadas nos subdomínios IIA e IIIA, também conhecidas por sítios I e II, respectivamente, são as principais regiões de ligação para compostos endógenos e exógenos. O Trp 134 encontra-se na superfície do domínio I, enquanto o Trp 212 encontra-se na cavidade hidrofóbica do domínio II (Guo et al., 2014; Shahabadi & Hadidi, 2014; Sudlow, G., Birkett, D.J., Wade, 1975). Uma representação estrutural da BSA com seus domínios e subdomínios está apresentada na Figura 3.

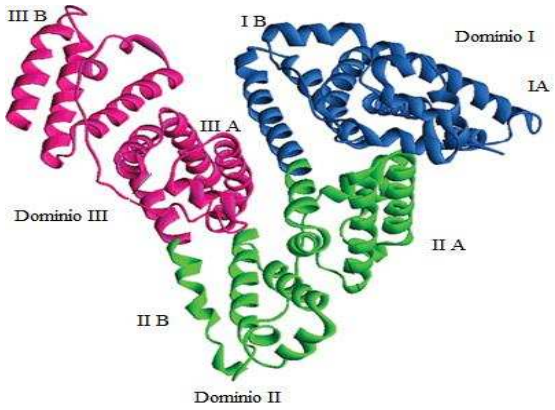

Figura 3. Representação estrutural da BSA e seus domínios. Fonte: Adaptado de METI et al. (2014).

A ligação de diversos compostos a albuminas do soro pode afetar significativamente as atividades biológicas dessas proteínas. Muitas propriedades dos compostos ligados, como metabolismo, tempo de meia-vida e penetração em membranas também podem ser afetadas. Uma ligação muito forte entre a proteína transportadora e o composto carreado pode diminuir a concentração deste no plasma sanguíneo, enquanto que uma fraca ligação pode levar a um tempo de meia-vida curto ou a uma má distribuição deste composto. Por isso, é importante conhecer e avaliar as propriedades de ligação de vários compostos de interesse a essas proteínas (Basu & Suresh Kumar, 2015; Jahanban-Esfahlan & Panahi-Azar, 2016a; Mohammadi, Bordbar, Divsalar, Mohammadi, & Saboury, 2009).

A interação entre vários compostos biologicamente ativos e a BSA tem sido estudada. Os flavonoides catequina, epicatequina, rutina e quercetina e sua interação com BSA foram estudados por Papadopoulou et al. (2005) por meio da extinção de fluorescência. Os autores encontraram que ligação de hidrogênio, ligação iônica e interação hidrofóbica dirigiram as interações entre as moléculas, e que as interações não induziram alterações conformacionais na proteína.

Cheng et al. (2012b) conduziram o estudo sobre a interação entre BSA e HSA e escopoletina, uma cumarina presente em raízes de diversas plantas e que possui atividade biológica, como antidepressiva, anti-inflamatória e antioxidante. Resultados de espectroscopia de fluorescência 3D, de absorção UV-vis e de infravermelho mostraram que as conformações das albuminas foram alteradas após a interação com a escopoletina.

Rodríguez Galdón et al. (2013) estudaram a interação entre BSA e licopeno, um carotenoide sintetizado por plantas e micro-organismos que possui potente atividade antioxidante. Os resultados de espectroscopia de fluorescência tridimensional revelaram que houve uma modificação conformacional e um aumento no diâmetro da BSA induzidos pela interação com o licopeno. A avaliação da conformação da BSA por dicroísmo circular confirmou a existência de um ligeiro desdobramento da molécula.

Li et al. (2015) estudaram em condições fisiológicas a interação da BSA e da HSA com outros dois antioxidantes, β-caroteno e astaxantina, por meio de várias técnicas espectroscópicas. Os resultados revelaram que as interações entre as moléculas foram entálpica e entropicamente dirigidas, e que forças hidrofóbicas e atração eletrostática desempenharam um importante papel sobre a interação. Adicionalmente, encontraram que as duas moléculas podem induzir mudanças conformacionais nas albuminas estudadas.

Ácido ascórbico e α-tocoferol e sua interação com BSA também foram estudados por Li et al. (2015). Estudos termodinâmicos indicaram que a interação entre as moléculas foi entalpicamente dirigida e que as interações intermoleculares envolvidas foram ligação de hidrogênio e van der Waals. Esses antioxidantes também induziram mudanças na conformação da BSA.

A interação de curcumina com BSA estudada por Mohammadi et al. (2009) indicou que não houve mudanças significativas na estrutura secundária da BSA, não afetando seu estado enovelado. As alterações induzidas pela interação da curcumina ocorreram apenas no sítio de sua ligação.

3.3. Técnicas experimentais para o estudo de interações intermoleculares

3.3.1. Espectroscopia de fluorescência

A espectroscopia de fluorescência é frequentemente utilizada em estudos de interações intermoleculares entre vários compostos e proteínas devido à sua alta sensibilidade e à variedade de parâmetros relacionados à interação que podem ser obtidos (Shahabadi & Hadidi, 2014).

Proteínas possuem fluorescência intrínseca devido à presença dos aminoácidos fenilalanina, tirosina e triptofano, sendo este último o predominante e o mais sensível a alterações em seu ambiente (Ghosh, Rathi, & Arora, 2016; Jayabharathi, Thanikachalam, & Venkatesh Perumal, 2011). As interações entre ligantes e proteínas quando ocorrem próximas aos resíduos de triptofano podem

ocasionar a extinção de sua intensidade de fluorescência (Shahabadi, Maghsudi, Kiani, & Pourfoulad, 2011).

O termo extinção ou supressão de fluorescência refere-se a qualquer processo que diminua o rendimento quântico de fluorescência de um fluoróforo. Uma variedade de causas pode induzir a extinção de fluorescência, como rearranjos moleculares, formação de complexo no estado fundamental ou colisões entre uma molécula e o fluoróforo (He et al., 2011). A Figura 4 ilustra um espectro onde a intensidade de emissão de fluorescência de uma proteína foi extinguida por uma molécula supressora.

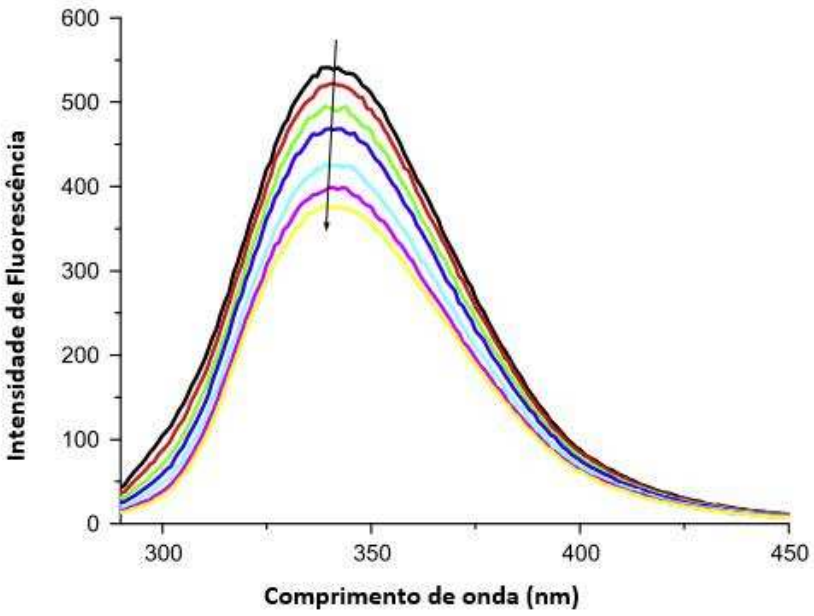

Figura 4. Efeito de concentrações crescentes de clembuterol na emissão de fluorescência de BSA em tampão Tris-HCl, pH 7,4. Fonte: Adaptado de Bi et al. (2014).

Dois mecanismos de extinção de fluorescência são conhecidos: extinção dinâmica e extinção estática. O processo dinâmico resulta de encontros difusivos entre a molécula supressora e o fluoróforo em seu estado excitado; esses encontros fazem com que o fluoróforo retorne ao seu estado fundamental sem a emissão de um fóton. O processo estático é resultado da formação de um complexo não fluorescente entre o supressor e o fluoróforo em seu estado

fundamental. Para a distinção dos mecanismos de supressão de fluorescência, pode-se examinar suas diferentes dependências com a temperatura. Como o mecanismo dinâmico é relacionado à difusão e maiores temperaturas resultam em maiores coeficientes de difusão, é esperado que a constante de supressão bimolecular aumente com o aumento da temperatura, e o inverso pode ser esperado para o mecanismo estático (Bi, Pang, Wang, Zhao, & Yu, 2014; Shahabadi & Hadidi, 2014).

A extinção de fluorescência de um fluoróforo por um supressor pode ser analisada pela equação de Stern-Volmer (Equação 1).

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \quad (1)$$

Nesta equação, K_{sv} é a constante de supressão de Stern-Volmer, F_0 e F correspondem às intensidades de fluorescência do fluoróforo na ausência e na presença do supressor, respectivamente, [Q] é a concentração da molécula supressora da fluorescência (mol/L), k_q é a constante de taxa de supressão e τ_0 é o tempo de meia-vida do fluoróforo na ausência do supressor (cerca de 10^{-8} s para proteínas).

O valor de K_q pode ser utilizado para determinar se o mecanismo de supressão é estático ou dinâmico. Quando a supressão é dinâmica, o valor máximo da constante de taxa de supressão é $2{,}0 \times 10^{10}$ $L.mol^{-1}s^{-1}$ (Shu et al., 2015).

Sendo o mecanismo estático, a constante de ligação (Ka) e o número de sítios de ligação (n) podem ser obtidos por meio da curva de regressão logarítmica dupla modificada (Equação 2) (Wei, Xiao, Wang, & Bai, 2010).

$$log\frac{F0-F}{F} = n\ logKa - n\log\frac{1}{\left([Qt]-\left(\frac{F0-F}{F0}\right)[P]\right)} \quad (2)$$

F_0 e F são as intensidades de fluorescência do fluoróforo na ausência e na presença do supressor, [Qt] e [P] são a concentração total da molécula

supressora e da proteína, respectivamente. O valor de n é obtido da inclinação da curva $log\frac{F0-F}{F}$ versus $\log\frac{1}{\left([Qt]-\left(\frac{F0-F}{F0}\right)[P]\right)}$, e o valor de Ka é o antilogaritmo da razão intercepto/inclinação.

Uma vez obtida a constante de ligação, Ka, a variação da energia livre de Gibbs padrão de formação de complexo (ΔG°) pode ser obtida por meio da equação 3.

$$\Delta G° = -RT\ lnKa \quad (3)$$

Nesta equação, R é a constante universal dos gases (8,314 J/mol.K) e T é a temperatura, em Kelvin.

O valor de ΔG°possui duas componentes, uma entálpica e uma entrópica, e a determinação destas variáveis termodinâmicas fornece informações importantes sobre as forças motrizes que regem a formação do complexo. A variação de entalpia padrão de formação de complexo (ΔH°) e a variação da entropia padrão de formação de complexo (ΔS°) podem ser obtidas a partir da aproximação de Van't Hoff (Equação 4), por meio da realização do experimento de supressão de fluorescência em várias temperaturas. Plotando um gráfico de ln Ka versus 1/T, ΔH° e ΔS°são obtidos da inclinação e do intercepto da curva, respectivamente (He et al., 2011; Shahabadi & Hadidi, 2014).

$$lnKa = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} \quad (4)$$

Na equação 4, Ka é a constante de ligação encontrada em cada temperatura, R é a constante universal dos gases e T é a temperatura estudada (K).

3.3.2. Microcalorimetria de titulação isotérmica (ITμC)

Vários eventos moleculares ocorrem quando duas moléculas interagem e passam do estado livre para o estado complexado. A técnica calorimetria de titulação isotérmica permite a obtenção direta da energia associada a esses eventos originados a partir de uma mistura de dois reagentes. Em uma análise calorimétrica típica, a adição progressiva de 10 μL de um dos reagentes é realizada em uma célula contendo o outro reagente. A cada injeção realizada, um pico é obtido em uma curva de potência versus tempo, e a integração de cada pico obtido fornece a variação da entalpia de interação associada a cada injeção. A figura 5 ilustra um experimento típico de ITμC e o termograma (curva de potência versus tempo) que é obtido (Ladbury & Chowdhry, 1996; Leavitt & Freire, 2001).

A ITμC é frequentemente utilizada para interações entre proteínas e ligantes, mas também pode ser útil no estudo de interações de quase todos os compostos bioquímicos como ácidos nucleicos, lipídios, carboidratos e outros compostos orgânicos. Uma vantagem é que ITμC é a única técnica que determina diretamente a variação de entalpia relacionada às interações intermoleculares (Linkuvienė, Krainer, Chen, & Matulis, 2016).

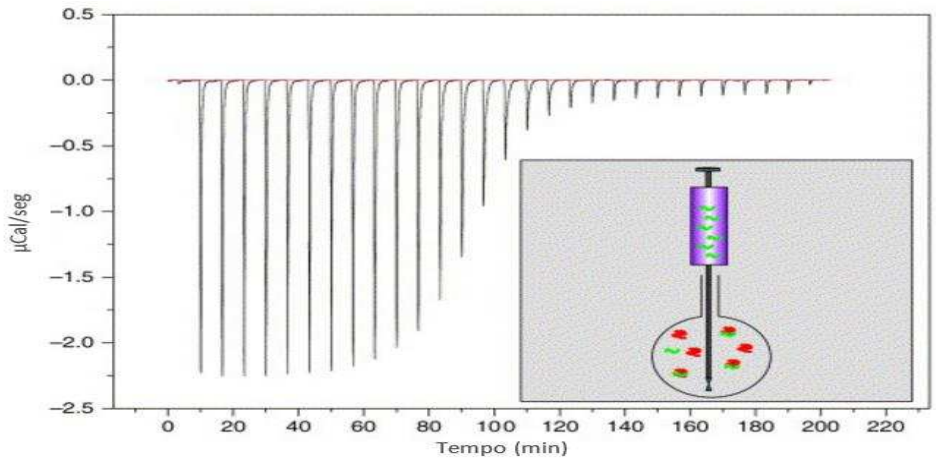

Figura 5. Exemplo de um experimento típico de iTμC. Fonte: Adaptado de Leavitt & Freire (2001).

Os parâmetros estequiometria de ligação (n), constante de ligação (Ka), variação da entalpia de interação ($\Delta_{int}H$), variação da energia livre de Gibbs padrão de formação de complexo ($\Delta G°$) e variação da entropia padrão de formação de complexo ($\Delta S°$) são parâmetros que podem ser obtidos pelo uso desta técnica calorimétrica.

A interação entre o flavonoide puerarina e BSA foi caractreizada por Xi & Fan (2010) por meio das técnicas iTμC e modelagem molecular. Os parâmetros termodinâmicos foram determinados e os autores encontraram valores de estequiometria de interação de 1,02, constante de ligação de $1,44x10^3$ L/mol, variação de entalpia de $-1,75x10^3$ J/mol e de entropia de $5,43x10^2$ J/mol, concluindo que o processo de interação entre as moléculas foi entropicamente dirigido.

Lelis et al. (2017) estudaram a termodinâmica de interação entre o corante sintético vermelho 40 com a BSA por meio das técnicas espectroscopia de fluorescência e iTμC. Os valores da constante de ligação encontrados pela técnica de fluorescência ficaram na faixa de 3,26 a $8,08x10^4$ L/mol, enquanto que pela calorimetria o valor de Ka encontrado foi de $1,03x10^6$ L/mol. Resultados diferentes também foram encontrados para a estequiometria (aproximadamente 1 pela fluorescência e 5 pela iTμC) e para os valores de $\Delta H°$ $\Delta G°$ e $T\Delta S°$ (18,59, -20,55 e 39,14 kJ/mol, a 25℃ pela fluorescência e -10,29, -28,59 e 18,30 kJ/mol pela calorimetria).

As características de rapidez na resposta e no equilíbrio térmico e a capacidade de caracterizar termodinamicamente processos de interação em um único experimento diferenciam a iTμC de outras técnicas, como cromatografia, fluorescência e eletroforese, que são técnicas menos sensíveis (Bernaczek, Mielańczyk, Grzywna, & Neugebauer, 2016).

3.3.3. Ressonância plasmônica de superfície (RPS)

Os biossensores RPS tornaram-se um método bem estabelecido para o estudo de interações intermoleculares. A RPS é uma técnica que se baseia na

alteração do índice de refração do solvente próximo à superfície do chip que ocorre durante a formação do complexo. Esses biossensores podem ser utilizados para estudar interações de qualquer sistema biológico, desde proteínas a pequenas estruturas, como fagos e células. Esta tecnologia permite monitorar interações fracas, que não são possíveis por outros sensores (Gopinath, 2010; Rich & Myszka, 2000).

O primeiro biossensor RPS foi comercialmente introduzido em 1990, desenvolvido e manufaturado por BIAcore Inc. A grande vantagem desse sistema é sua capacidade de caracterizar as interações em tempo real, sem a necessidade do uso de marcadores. Em um experimento básico de RPS, um dos reagentes, referido como ligante, está ligado à superfície do sensor, enquanto que o outro, o analito, está em solução e passa por essa superfície. Quando os dois reagentes interagem, uma resposta é gerada (Figura 6) (Myszka, 1997).

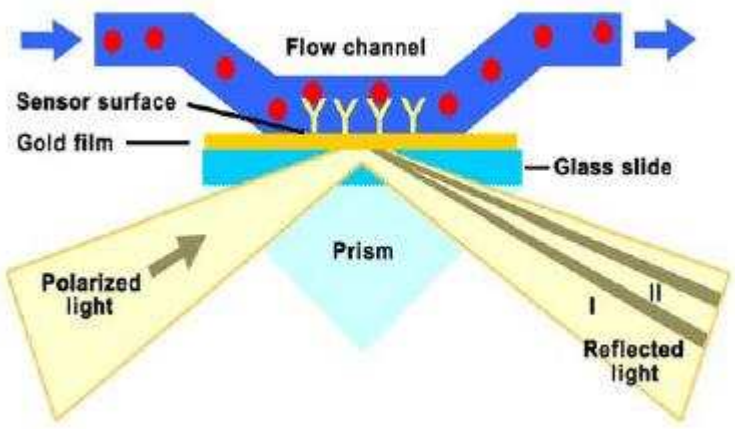

Figura 6. Representação esquemática de um biosensor Biacore.

O sinal gerado quando a interação ocorre é proporcional à massa total do analito na vizinhança da superfície do sensor; se a massa molar do analito é conhecida, a constante de ligação e a estequiometria do complexo formado podem ser determinadas (Day, Capili, Borysenko, Zafari, & Whitty, 2013).

Wear & Walkinshaw (2006) estudaram a interação entre a enzima humana ciclofilina e a droga inibidora de sua atividade, ciclosporina, utilizando as técnicas de RPS e iTµC, afim de comparar os parâmetros termodinâmicos obtidos por

cada uma. Como resultado, os autores obtiveram que os valores dos parâmetros determinados por iTμC foram praticamente os mesmos obtidos por RPS. A 25℃, foram encontrados valores de 13,4 nM para a constante de dissociação (Kd) e de -10,69, -14,4 e -3,7 kcal/mol para ΔH°, ΔG° e TΔS°, respectivamente, pela RPS, e 11,5 nM para Kd, -10,8, -14,6 e -3,8 kcal/mol para ΔH°, ΔG° e TΔS°, respectivamente, pela técnica calorimétrica.

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BOVINE SERUM ALBUMIN AS NANOCARRIER OF CURCUMIN

Abstract - Curcumin exhibits various biological activities, but its low solubility and stability characteristics limit its bioavailability. Therefore, it is indispensable to study its interaction with bovine serum albumin (BSA), which can increase the solubility and stability of several hydrophobic compounds in various formulations. Here, we studied the interaction between curcumin and BSA in its native and denatured conformations, at pH 7.0, was studied through different analytical techniques, and the kinetics of curcumin photodegradation was evaluated in the presence of BSA. By the analysis of fluorescence spectroscopy (FS) and isothermal titration calorimetry (ITC) the values of the binding constant (Ka) between the protein and the bioactive compound obtained were in the order of 105 L.mol-1, whereas by surface plasmonic resonance (SPR), this value was 3.88x103 L.mol-1. These differences are explained by the limitations presented by each technique. The competition experiment performed indicated that curcumin competes with the markers used by sites I, II and III of the BSA. The values obtained for ΔH° by FS (-8.67 kJ.mol-1) and by ITC (-29.11 kJ.mol-1) indicated that the process of forming the complexes was exothermic. In the studies with denatured BSA there was a slight increase in Ka values (4.53x105 L.mol-1 and 2.00x106 L.mol-1, obtained by FS and ITC, respectively, at 298 K), compared to the values found with the native BSA, which indicated that there are other BSA sites for curcumin binding in addition to those shown in the competition experiment. The thermodynamic profile was like that obtained for the native conformation, but ΔH° was more negative (-16.12 kJ.mol-1 and -42.63 kJ.mol-1, by FS and ITC, respectively), indicating that the change in conformation of the BSA provided a more exothermic interaction. In the photodegradation experiment, as the BSA concentration increased, the values of the curcumin degradative constant (Kd) decreased exponentially and its half-life (t1/2) increased linearly, which indicated that BSA protected the curcumin from photodegradation. This work contributes to a more deepened knowledge of the intermolecular interaction between curcumin and BSA by bringing results obtained by techniques with

different fundamentals and kinetic parameters of curcumin degradation in the presence of different BSA concentrations, which was still absent in the literature, providing important information to the success of the technological application of curcumin in different formulations.

Keywords: curcumin, intermolecular interaction, BSA conformation, photo-degradation.

1. INTRODUCTION

Curcumin, a polyphenolic compound, exhibits various biological activities, such as anti-inflammatory, anticarcinogenic, antioxidant and antimicrobial activities. However, its low solubility and low stability properties in aqueous systems make difficult its use in food formulations and its body absorption (Gupta et al., 2013; Hatcher et al., 2008; Priyadarsini, 2009).

Because of these limitations, various alternatives have been sought to increase the curcumin solubility and stability in aqueous media. Some of them is the incorporation of curcumin into cyclodextrins, liposomes and into hydrophobic protein cavities (Barik et al., 2003; Isacchi et al., 2012; Mitra, 2007; Paramera et al., 2011). Among several biomolecules, bovine serum albumin (BSA) has been extensively studied as carrier of various bioactive compounds because of its stability properties, aqueous solubility and structural homology to human serum albumin (HSA) (Meti et al., 2014; Shi et al., 2010).

Despite the studies evaluating the interaction between curcumin and BSA, all of them do not address the influence of protein conformation on the interaction properties, as well as the protection against photo-degradation provided to curcumin when the protein carries it. In addition, most of the studies only use one technique to find binding parameters. Fluorescence spectroscopy has been often used to determine thermodynamic binding parameters between curcumin and BSA (M. Yang et al., 2013a) or HSA (Mohammadi et al., 2009). However, fluorescence is restricted to binding occurring directly with fluorophore residues, such as tryptophan (Ghosh et al., 2016). In a previous work, our group found that the use of multiple techniques is strategic to determine thermodynamic binding parameters because different techniques can access interactions occurring at different sites (Lelis et al., 2017).

Here, we present a complete thermodynamic analysis of the interaction between curcumin and BSA in both native and unfolded conformation obtained by three sensitive techniques (fluorescence spectroscopy, surface plasmonic resonance and isothermal titration calorimetry). In addition, we also studied the

protection effect provided by BSA (native and unfolded) to the curcumin photo-degradation.

2. MATERIAL AND METHODS

2.1. Materials

BSA (>98%), curcumin (>80%), warfarin (reagent grade), ibuprofen (>98%), digitoxin (>92%) and dimethyl sulfoxide (analytical grade) were obtained from Sigma-Aldrich (USA). Sodium phosphate was acquired from Vetec (Brazil).

2.2. Fluorescence spectroscopy

The fluorescence measurements were carried on LS55 Fluorescence Spectrophotometer (PerkinElmer Inc, Waltham, USA), using a quartz cell having 1 cm path length. The cell was filled with 3 mL of native or denatured BSA (15 µM), at pH 7.0, containing different concentrations of curcumin (10-70 µM). The solution of denatured BSA was obtained by heating at 353 K for 10 min. To confirm the denaturation of the BSA, a differential scanning nanocalorimetry experiment was carried out. The curcumin stock solution (500 µM) was first prepared in dimethyl sulfoxide (DMSO), and then an aliquot was transferred to buffer pH 7.0, so that the final volume of DMSO had no effect on the protein structure.

The fluorescence experiments were performed out in five temperatures (293, 298, 308, 318 and 323 K). The excitation wavelength of BSA was 295 nm and slits widths for both excitation and emission were fixed at 5 nm. The emission spectra of the protein were recorded in range of 296-450 nm.

To determine the binding site of curcumin to BSA, the markers warfarin, ibuprofen and digitoxin were used. In this experiment, curcumin was added to native BSA solution with and without three probes, at same concentration of the protein, and the fluorescence quenching experiment was conducted.

2.3. Surface Plasmon Resonance (SPR)

Surface plasmon resonance analyses were conducted in a BIACORE X100 instrument (General Electric Healthcare Company, Uppsala, Sweden). BSA was immobilized on a CM5 sensor chip (GE Healthcare Company) using amine-coupling according to the recommended protocol in the Biacore X100 Handbook BR-1008-10 Edition AC. The flow cell, formed when the sensor chip was docked in the instrument, was activated for 7 min with a 1:1 mixture of 0.1 mol·L^{-1} 3-(N,N-dimethylamino)propyl-N-ethylcarbodiimide (EDC) and 0.1 mol·L^{-1} N-hydroxysuccinimide (NHS) at a flow rate of 20 µL·min^{-1}, at 298 K. Then, 30 µg·mL^{-1} BSA in 10 mmol·L^{-1} sodium acetate, at pH 7.0, was injected for 7 min, resulting in immobilized densities around 7000 Resonance Units (RU). A 7 min pulse of 1 mol·L^{-1} ethanolamine hydrochloride, pH 8.5, was then used to deactivate the excess hydroxysuccinimidyl groups on the surface that did not react with the protein. Thus, curcumin solution with concentration from 25 to 200 µM were injected to the sample cell and binding was observed as showed in the plot of resonance signal (RU) *versus* time (s).

2.4. Isothermal titration microcalorimetry (ITC)

The ITC experiments were perfomed at 298 K, using a TAM III microcalorimeter controlled by ITCRun software (TA Instruments, New Castle, DE, USA), following Lelis et al. (2017). Aqueous BSA solution (4µM) at pH 7.0 was degassed and put in the calorimetric sample cell with a total volume of 2.7 Ml. The titration solution (curcumin 100 µM) was degassed and loaded into the injection syringe. Then, 25 aliquots of curcumin solution (each 10 µL) were added into BSA solution. A dilution experiment was carried out, replacing BSA solution with a pH 7.0 buffer. Samples were constantly stirred at 300 rpm to ensure thorough mixing. Raw data obtained from a plot of power vs. injection number were transformed using the instrument software to construct a plot of enthalpy change vs. curcumin:BSA molar ratio. The values of enthalpy change of curcumin dilution (ΔH_{obs}^{dil}) were subtracted from those acquired from curcumin addition to BSA solution (ΔH_{obs}^{int}). The apparent interaction enthalpy change ($\Delta H_{app\text{-}int}$) was

calculated following the relationship $\Delta H_{app-int} = \left(\Delta \mathrm{H}_{obs}^{int} - \Delta \mathrm{H}_{obs}^{dil}\right)/n_{curc}$, where n_{curc} is the curcumin amount of substance added to the sample cell in each injection. The resulting data of $\Delta H_{app\text{-}int}$ *versus* curcumin concentration were fitted to one to one binding model (Equation 1) in order to obtain the binding constant (K_a), the binding stoichiometry (n), and the calorimetric standard enthalpy change (ΔH^{o}_{cal}) for BSA/curcumin complex formation.

$$\Delta H_{app-int} = \frac{V_c \Delta H^{o}_{lig}}{2K_a}\ 1 + K_a[Cur]_{total} + nK_a[BSA]_{total} - \left[(1 + K_a[Cur]_{total} + nK_a[BSA]_{total})^2 - 4nK_a{}^2[Cur]_{total}[BSA]_{total}\right]^{1/2} \quad (1)$$

where: V_c = Cell volume, K_a = binding constant, $[Cur]_{total}$ = total curcumin concentration in the cell, n = number of independent sites, $[BSA]_{total}$ = total BSA concentration in the cell.

2.5. Degradation kinetics of curcumin by exposure to light

Solutions containing 25 µM curcumin in the presence of different concentrations of native or denatured BSA (0-45 µM) at pH 7.0 were stored in glass bottles and placed inside a light chamber containing two fluorescent lamps corresponding to the daylight (Barros & Stringheta, 2006). Absorbance readings of samples were performed at 425 nm in a UV-vis spectrophotometer (Shimadzu UV-2550), at 298 K and at 30 min intervals, during 6 hours.

3. RESULTS AND DISCUSSION

3.1. Curcumin/BSA interaction characterized by fluorescence spectroscopy

3.1.1. Quenching mechanism and binding parameters

The intrinsic fluorescence of proteins is mainly due to its tryptophan content. BSA contains two tryptophan residues, Trp 134 and Trp 212, located on

the surface of sub-domain I B and on the hydrophobic binding core of the sub-domain II A, respectively (Bhogale et al., 2014; Guo et al., 2014).

If a ligand binds to BSA, the protein fluorescence may change. (Guo et al., 2014; Sułkowska, 2002). The conformation transition or denaturation of the biopolymer can also modify the protein fluorescence spectra (Damodaran, Parkin, & Fennema, 2008; Zhao, Liu, Chi, Teng, & Qin, 2010). In addition, protein conformation can also affect the protein-ligand binding. Therefore, fluorescence spectroscopy is a technique that can be used to access information about interactions between strategic solutes and proteins in different conformations. To know how curcumin can affect the tryptophan environment in the native BSA ($_n$BSA), at pH 7.0, the intrinsic fluorescence of this protein in presence of increasing concentrations of curcumin was studied (Figure 1). Increasing curcumin concentration, there was a decrease in the fluorescence intensity emitted by the $_n$BSA, and a wavelength shift of maximum fluorescence intensity from 345 nm to 337 nm was observed, suggesting that a BSA/curcumin complex is formed. The blue-shift in wavelenght indicates that Trp was exposed to a more hydrophobic environment, probably curcumin bound near to it (Sinha et al., 2016).

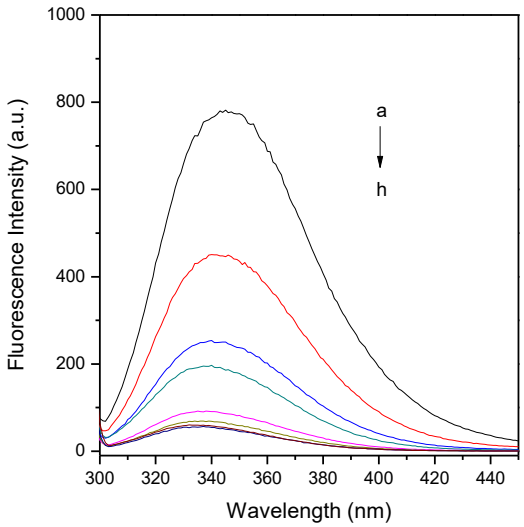

Figure 1. Fluorescence spectra of $_n$BSA (15 µM) with increasing concentration of curcumin obtained at 298 K and pH 7.0. The arrow "a → h" indicates 0, 10, 20, 30, 40, 50, 60 and 70 µM of curcumin.

The mechanism of fluorescence quenching can be dynamic, when the quenching results from collisions between the quencher and the fluorophore, or static, when a non-fluorescent complex is formed in ground state of the fluorophore (Ghosh et al., 2016; Lakowicz, 2006). To access the quenching mechanism of BSA by curcumin, the Stern-Volmer equation (Equation 2) was used.

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \quad (2)$$

where F and F_0 are the fluorescence intensities of the protein in presence and absence of quencher (curcumin), respectively; K_{SV} is the Stern-Volmer quenching constant and [Q] is the concentration of quencher; K_q is the quenching rate constant of protein fluorescence and τ_0 is the average lifetime of the protein without the quencher (10^{-8} s).

Figure 2 plots F_0/F versus [Q] curve, from which the values of K_{sv} and K_q were obtained. When the fluorescence quenching mechanism is static, the K_q is known to be greater than the maximum scatter collision quenching constant of quenchers with biopolymers ($2x10^{10}$ $L.mol^{-1}s^{-1}$) (Cheng, 2012b). Furthermore, the mechanism can be distinguished by the K_{SV} dependence on temperature: the decrease in K_{SV} values with increasing temperature indicates that the mechanism is static; otherwise, the mechanism of quenching will be dynamic (Jahanban-Esfahlan & Panahi-Azar, 2016b). The K_{SV} and K_q values obtained for curcumin interacting with $_n$BSA at different temperatures are shown in Table 1.

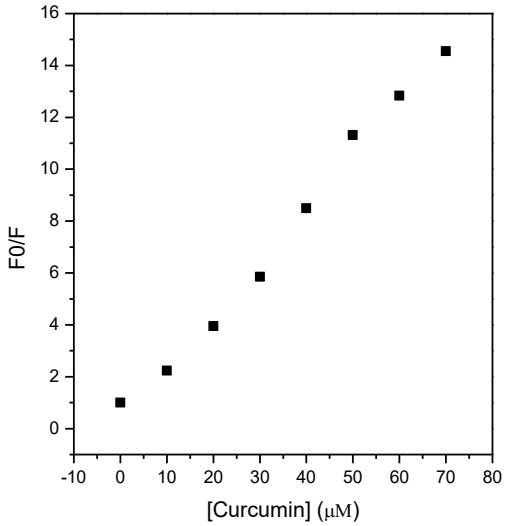

Figure 2. Stern-Volmer plot (F_0/F x curcumin concentration) for $_n$BSA interacting with increasing curcumin concentration, at 298K and pH 7.0.

Table 1. Stern-Volmer quenching constants (K_{sv}), quenching rate constants (K_q), binding constants (K_a) and number of the binding sites in the BSA interacting with curcumin, at different temperatures, and pH 7.0.

T (K)	**K_{sv} (x10^5 L.mol^{-1})**	**r^2**	**K_q (x10^{13} L.mol^{-1})**	**K_a (x10^5 L.mol^{-1})**	**r^2**	***n***
293	2.02	0.9818	2.02	2.17	0.9690	1.04
298	2.14	0.9857	2.14	1.95	0.9566	1.11
308	3.10	0.9801	3.10	1.73	0.9565	1.31
318	2.94	0.9885	2.94	1.60	0.9354	1.36
323	2.49	0.9789	2.49	1.54	0.9385	1.24

For $_n$BSA interacting with curcumin, there was no clear dependence of K_{sv} values on the temperature, but the values obtained for K_q were much higher than the maximum scatter collision quenching constant. This result, together with the observed blue-shift in the BSA emission spectrum, indicates that the fluorescence quenching mechanism for the interaction between native BSA and curcumin is static, i.e., with a complex formation between both chemical species. This is in accordance with the results obtained by Mohammadi et al. (2009), that studied the interaction between native BSA and curcumin at pH 6.4.

Assuming that BSA/curcumin complexes were formed, and that there are independent and similar binding sites for curcumin on $_n$BSA, we can determine the binding constant (K_a) and the complex stoichiometry (*n*) associated to the process described by Equation 3. There are several models used to calculate these parameters, all of them apply some approach. However, in this work, we chose the more rigorous model, without any approach in free and total concentrations of protein and curcumin, according to equation 4 (Wei et al., 2010).

$$nCurc + BSA \leftrightharpoons \mathrm{BSA} - Curc_n \tag{3}$$

$$log\frac{F0-F}{F} = n\ logKa - n\log\frac{1}{\left([Qt]-\left(\frac{F0-F}{F0}\right)[P]\right)} \tag{4}$$

where F_0 and F are the fluorescence intensities of BSA in the absence and presence of curcumin, respectively, [Qt] is the total curcumin concentration and [P] is the total BSA concentration. The *n* value can be obtained from slope of the curve $log\frac{F0-F}{F}$ versus $\log\frac{1}{\left([Qt]-\left(\frac{F0-F}{F0}\right)[P]\right)}$ (Figure 3), and the Ka value from intercept/slope antilogarithm. These values are presented in Table 1.

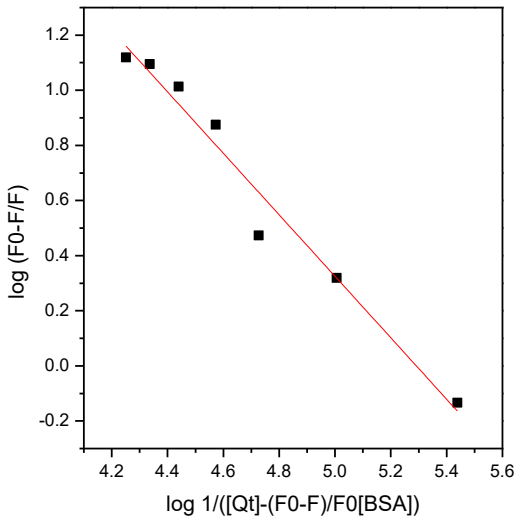

Figure 3. Plot for determination of Ka and *n* values of the complex between curcumin and native BSA, at 298 K and pH 7.0.

The K_a values were in the order of 10^5, indicating a moderated interaction between BSA and curcumin. The *n* values suggest that there is least one binding site for curcumin in the BSA molecule. Other studies have obtained Ka values with the same magnitude for curcumin interaction with BSA and other proteins (Barik et al., 2003; M. Yang et al., 2013a).

To map the binding sites for curcumin on BSA, molecular probes (warfarin, ibuprofen and digitoxin) were used. We performed a displacement competitive experiment using these molecules as markers for sites I, II and III of the BSA (Meti et al., 2014). The K_a values decreased in the presence of the markers in comparison with BSA-curcumin in its absence ($1.95x10^5$ $L.mol^{-1}$); the reduction in the presence of warfarin was 94% ($1.17x10^4$ $L.mol^{-1}$), in the presence of ibuprofen was 85% ($2.93x10^4$ L.mol-1) and of digitoxin was 71% (5.66 $x10^4$ $L.mol^{-1}$), revealing that curcumin competes with warfarin, ibuprofen or digitoxin for the sites I, II and III of BSA; or changes the protein conformation affecting the BSA binding with these three ligands. Barik et al.(2003) studied the binding kinetics of curcumin to BSA by measuring the absorbance of the ligand as a function of time and the results indicated that curcumin can bind to sites I and II with different kinetics, in

a fast and a slow processes. Ge et al. (2014) found that curcumin competes with warfarin in human serum albumin (HSA).

3.1.2. Thermodynamics analysis

To understand the driven force of interaction for the nBSA/curcumin complexation, it is important to determine the thermodynamic parameters standard Gibbs free energy change (ΔG°) , standard enthalpy change (ΔH°) and standard entropy change (ΔS°). We performed the binding studies using fluorescence in five temperatures, and the ΔG° values were obtained using the Equation 5.

$$\Delta G° = -RT\ lnKa \tag{5}$$

where R is the universal gas constant (8.3145 J.mol^{-1}.K^{-1}), T is the temperature (K), Ka is the binding constant (L.mol^{-1}).

Using the van't Hoff equation (Equation 6) and plotting *lnKa* versus *1/T* (Figure 4), we determined the ΔH° value.

$$\frac{\ln Ka_2}{\ln Ka_1} = -\frac{\Delta H°}{R}\left(\frac{1}{T_1} + \frac{1}{T_2}\right) \tag{6}$$

where Ka is the binding constant (L.mol^{-1}), ΔH° is standard enthalpy change (kJ.mol^{-1}), R is the universal gas constant (8.3145 J.mol^{-1}.K^{-1}), T is the temperature (K).

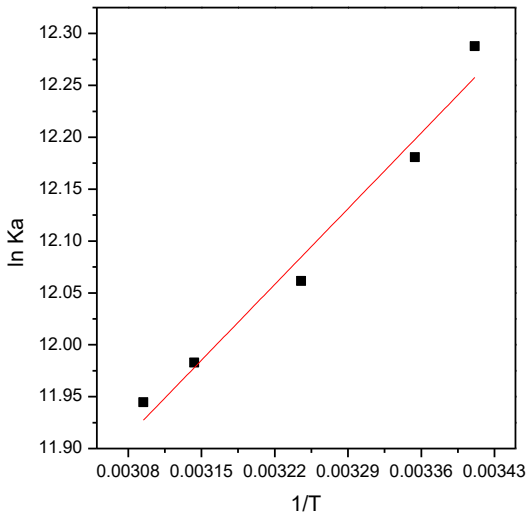

Figure 4. Plot of $\ln K_a$ versus 1/T (Van't Hoff approach) for curcumin interacting with BSA, at pH 7.0.

The ΔS° values were obtained from Equation 7. All thermodynamics parameters are shown in Table 2.

$$\Delta G° = \Delta H° - T\Delta S° \quad (7)$$

where ΔH°is standard enthalpy change (kJ.mol $^{-1}$), ΔG° is the standard Gibbs free energy change (kJ.mol^{-1}) and ΔS°is the standard entropy change (kJ.mol^{-1}K^{-1}).

Table 2. Standard enthalpy change (ΔH°), standard Gibbs free energy change (ΔG°) and standard entropy change (ΔS°) for interaction between native BSA and curcumin, obtained by fluorescence at pH 7.0.

T (K)	ΔG° (kJ.mol^{-1})	ΔH° (kJ.mol^{-1})	r^2	TΔS° (kJ.mol^{-1})
293	-29.95	-8.67	0.9550	21.28

298	-30.19	21.52
308	-30.87	22.20
318	-31.70	23.02
323	-32.09	23.42

The ΔG° values were negative, indicating that the equilibrium shown in Equation 3 favored the complex formation. Mitra (2007) found similar values, being -37.66, -38.21 and -39.42 $kJ.mol^{-1}$ at 283, 293 and 310 K for interaction between native BSA e curcumin at pH 7.4. The ΔH° values were negative while the TΔS° values were positive, indicating that thermodynamic processes of the complex formation were enthalpically and entropically driven. The entropy increasing can be explained by the hydrophobic effect, which occurs when the degree of translational freedom of water molecules released from solvation layers of both molecules interacting increases (Breiten et al., 2013; Ge et al., 2014), indicating a strong contribution of hydrophobic interactions (YANG, HU, FAN, & SHEN, 2008) and/or conformational changes of the protein. The enthalpy change associated with hydrophobic interactions is generally positive, but the negative ΔH° value indicates that specific interactions between curcumin and BSA occur in complex formation. These interactions are probably hydrogen bonds, involving the hydroxyl group of curcumin and proton acceptor groups in the BSA, along with van der Waals interactions.

In spite of the potential of fluorescence technique for studying intermolecular interaction, it is restricted to determine binding in sites containing and/or near Trp residues. Thus, to complete characterize curcumin-BSA binding, other techniques were used, namely surface plasmon resonance (SPR) and isothermal titration microcalorimetry (ITC).

3.2. Curcumin/BSA interaction characterized by SPR

After BSA immobilization on the CM5 chip, curcumin solutions with concentration from 25 to 200 µM were injected to the sample cell. Sensorgrams showing resonance signal (RU) *versus* time (s) are showed in Figure 5.

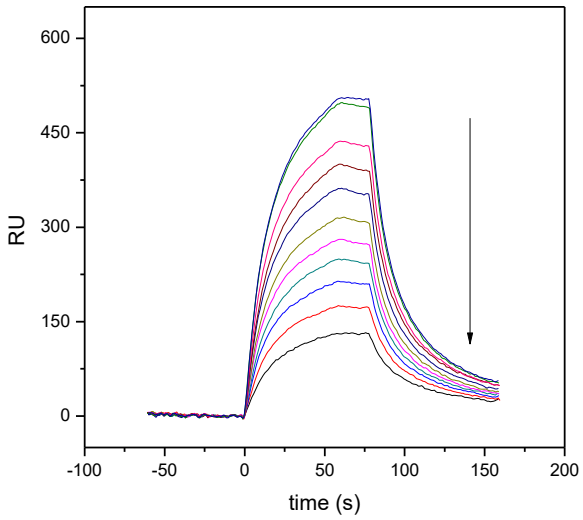

Figure 5. Sensorgrams obtained from injection of curcumin dye over BSA immobilized on a Biacore CM5 sensor chip, at 298 K. Arrow indicates the curcumin concentration increasing from 25 to 200 µM (up to down).

The RU values enhanced with time due to curcumin binding on the chip surface resulted from specific interaction between BSA and curcumin, until reach a pathamar after the saturation of binding sites of the biopolymer; in this moment, the adsorption rate is equal to the desorption rate. Then, the signal returned to baseline rapidly after dissociation induced by buffer flow. The saturation RU is denominated equilibrium response (R_{eq}), and it is dependent on curcumin concentration injected in the system. With R_{eq} data it was possible to obtain the adsorption isothermal, which is R_{eq} *versus* [curcumin] plot (Figure 6).

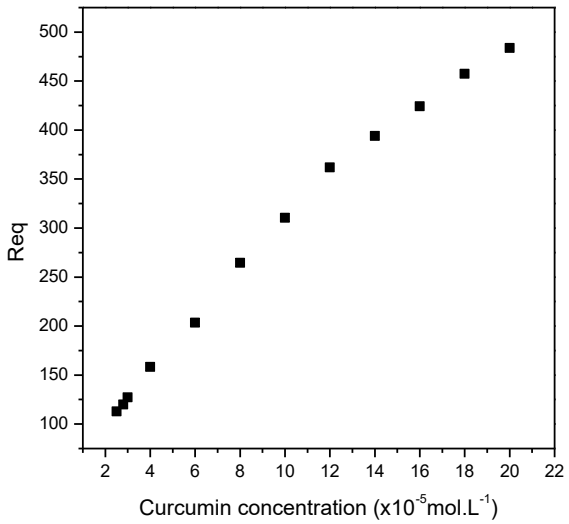

Figure 6 - Response data, obtained by SPR, at equilibrium plotted against curcumin concentration.

To determine the dissociation constant (K_D) of the BSA/curcumin interaction, the isotherm data were adjusted with a one to one binding model (Equation 8), which assumes a simple reversible interaction between curcumin and BSA, with the formation of the surface-bound protein-analyte complex. It is possible to calculate the K_a using the relationship $K_a = {}^{1}/_{K_D}$.

$$R_{eq} = \frac{CR_{max}}{K_D + C} + RI \qquad (8)$$

where RI is the contribution from the bulk refractive index of the sample, R_{max} is the maximum resonance response and C is the curcumin concentration.

Our experimental data provided K_a=3.88x10^3 L.mol^{-1} (ΔG^o = -20.47 kJ.mol^{-1}), which was 50 times smaller than K_a obtained by the fluorescence experiment. This difference could be explained considering that in SPR experiment, all BSA sites that interacted with curcumin have been detecting and not only those near to Trp residues. Thus, K_a value obtained by SPR is the product

of constant of each distinct site of BSA ($K_a = K_{a1}.K_{a2}.K_{a3}$ ... K_{an}), demonstrating that BSA/curcumin binding is more complex than that expressed by fluorimetric data.

3.3. Curcumin/BSA interaction characterized by ITC

Figure 7 plots the $\Delta H_{app\text{-}int}$ values for the curcumin interaction with $_n$BSA as a function of colorant concentration, obtained at 298 K and pH 7.0.

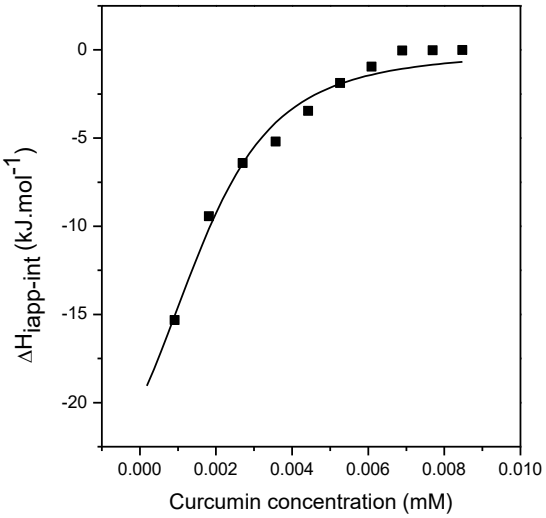

Figure 7 – $\Delta H_{app\text{-}int}$ versus curcumin concentration plot for curcumin/native BSA binding at 298 K and pH 7.0.

As curcumin concentration increased $\Delta H_{app\text{-}int}$ became less negative until that the addition of curcumin had no thermic effect. This behavior could be interpreted considering the following molecular model: at small [curcumin]/[BSA] ratio almost of all curcumin added is bond to the BSA molecule. But, when curcumin concentration increases the fraction of free binding sites on BSA decreases due to the curcumin/BSA complex formation. At [curcumin] = 0.007 mM almost all BSA molecules is complexed by curcumin. Based on this model, $\Delta H_{app\text{-}int}$ depends on curcumin concentration because modifying [curcumin]/[BSA]

ratio the fraction of ocuppied sites on BSA is altered. Fitting $\Delta H_{app\text{-}int}$ *versus* curcumin concentration to the mathematical model described by Equation 1, thermodynamic parameters of complex formation could be calculated: K_a= 9,31x10^5 L.mol^{-1}, *n*= 0.4 and ΔH^o_{cal}= -29.11 kJ.mol^{-1}.

Calorimetric K_a value was about 5 and 250 times higher than K_a values obtained by fluorescence and SPR, respectively, at 298 K. Such difference comes from different conditions and/or accessibility of available binding sites on BSA. As mentioned before, only interactions occurring at sites near to Trp are detected by fluorescence, while calorimetry finds all BSA binding sites. Regarding SPR, the protein is immobilized on a chip, which may difficult the access to some binding sites; on the other hand, both protein and curcumin are in solution, becoming interaction sites more accessible.

Another important analysis is that either for SPR or calorimetry, the K_a value results from the average of K_a values of various binding sites. However, the contribution of each constant on the K_a obtained experimentally depends on the effect of each interaction on the physicochemical property used to detect the interaction. For calorimetry, the sites whose interaction releases more enthalpic energy are those with higher contribution to calorimetric K_a. On the other hand, for SPR, the sites whose interaction causes greater variation in the refraction index on BSA/solution interface contributes more to determine K_a. Our results showed that the sites releasing more enthalpic energy were those with more intense energy (higher K_a) and they were not near Trp sites, since calorimetric K_a is higher than fluorimetric K_a. Based on this same interpretation, SPR K_a value is smaller because the same sites (with more negative ΔH^o) also contribute to higher variation in the refraction index of BSA/solution interface.

Regarding difference between calorimetric and fluorescence ΔH^o values, the explanation comes from two sources: the former is that ΔH^o calculated by fluorescence uses an approach (van't Hoff approach) while that obtained by calorimetry is a direct determination; and the second reason is that curcumin binding occurring far from Trp residues released more enthalpic energy.

The smaller n value found by ITC analysis suggests that one curcumin molecule is bond in this site, which releases more enthalpy, and at the same time in another site of another BSA molecule.

3.4. Effect of BSA tertiary structure on curcumin/BSA binding

We found by fluorescence, SPR and ITC techniques that curcumin/BSA binding occurs at different sites. Probably the change of protein conformation affects the BSA available sites to bind to curcumin and the determination of binding parameters using unfolded BSA ($_{u}$BSA) could bring important information on curcumin/BSA binding. In addition, from the technological point of view, BSA can denature due to heating, high pressure, pH change, addition of chemicals, among others, during routine of processing in the food industry (HAYAKAWA, KAJIHARA, MORIKAWA, ODA, & FUJIO, 1992). Thus, we performed the fluorescence and ITC experiments using unfolded BSA.

By fluorescence, a decrease in the protein fluorescence intensity also occurred when curcurmin bond to unfolded BSA (Figure S1, Supplementary Material), as observed for $_{n}$BSA.

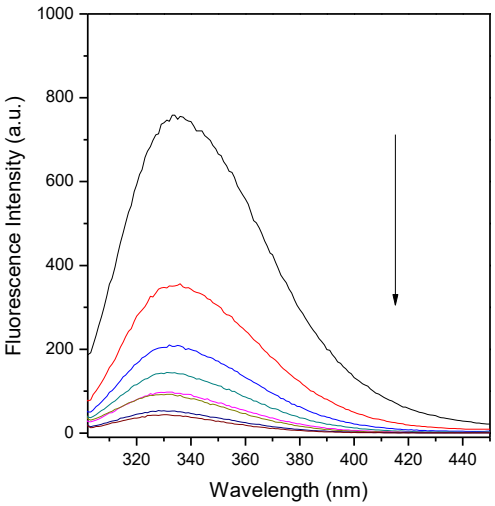

Figure S1. Fluorescence quenching spectrum of $_{u}$BSA (15 µM) by curcumin at 298 K and pH 7.0. The arrow indicates curcumin concentration ranging from 0 to 70 µM.

In the $_{u}$BSA spectra, the maximum fluorescence intensity wavelength was 334 nm, while that for the native protein was 345 nm. This difference may be due to the aggregation of $_{u}$BSA. When in the unfolded state, hydrophobic amino acids are exposed and interact by hydrophobic interactions, causing tryptophan to remain in a more hydrophobic environment (YANG, WU, LI, ZHOU, & WANG, 2013B).

Through Stern-Volmer model, curcumin/$_{u}$BSA binding also occurred by a static mechanism, i.e., due to a complex formation between both chemical species. Table S1 shows the binding parameters determined.

Table S1. Stern-Volmer quenching constants (Ksv), quenching rate constants (Kq), binding constants (Ka) and number of the binding sites (n) in the $_u$BSA interacting with curcumin, at different temperatures, pH 7.0.

T (K)	Ksv ($x10^5 L.mol^{-1}$)	r^2	Kq ($x10^{13}$ $L.mol^{-1}$)	Ka ($x10^5$ $L.mol^{-1}$)	r^2	n
293	2.58	0.9948	2.58	4.89	0.9360	0.76
298	2.35	0.9913	2.35	4.53	0.9395	0.78
308	2.24	0.9889	2.24	3.97	0.9319	0.83
318	2.27	0.9865	2.27	3.09	0.9364	0.92
323	2.47	0.9821	2.47	2.50	0.9449	0.96

For all temperatures, analyzing curcumin/$_u$BSA binding, we found a slight increase in the K_a values compared to those found for curcumin/$_n$BSA complexes, which was kept in the order of 10^5 $L.mol^{-1}$. Besides, *n* value were near 1. These results pointed to even though curcumin bind to specific sites of BSA, as showed in the experiments done with molecular probes, there are other binding sites on the protein for this molecule. Probably, after the denaturation BSA exposed their hydrophobic groups that aggregated and provided hydrophobic regions to hold curcumin (Militello, Vetri, & Leone, 2003).

The behavior of standard thermodynamic parameters for curcumin/unfolded BSA complex formation was close to that found for complex formed with the native BSA conformation, as shows the Table 3.

Table 3. Standard enthalpy change (ΔH°), standard Gibbs free energy change (ΔG°) and standard entropy change (ΔS°) obtained for interaction between unfolded BSA and curcumin, at pH 7.0.

T (K)	ΔG° (kJ.mol^{-1})	ΔH° (kJ.mol^{-1})	r^2	TΔS° (kJ.mol^{-1})
293	-31.93	-16.12	0.9472	15.81
298	-32.28			16.17
308	-33.03			16.91
318	-33.44			17.32
323	-33.49			17.37

Curcumin/$_u$BSA complex formation was enthalpically and entropically driven, however ΔH° value was more negative for unfolded BSA/curcumin complex. This result could be attributed to the higher amount of OH groups available on the protein when it was denatured, which could bind to curcumin OH groups by hydrogen bonds, and/or to a lower degree of dessolvation associated to curcumin/unfolded BSA compared to curcumin/native BSA interaction.

To confirm the fluorescence results on the conformation effect of the protein on curcumin/BSA binding, ITC experiment was conducted using unfolded BSA. Figure 8 shows the plot of $\Delta H_{app\text{-}int}$ *versus* [curcumin], obtained at 298 K and pH 7.0, for unfolded BSA.

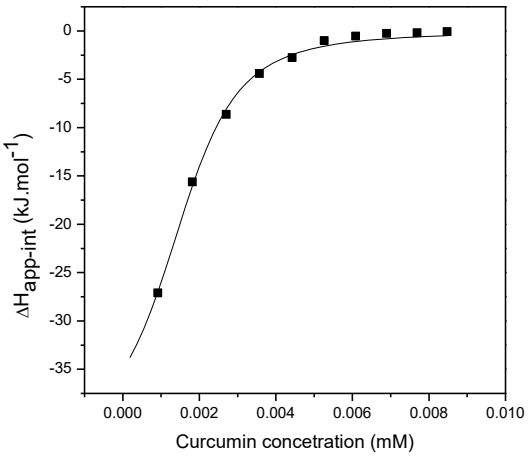

Figure 8 – $\Delta H_{app\text{-}int}$ versus curcumin concentration plot for curcumin/unfolded BSA binding at 298 K and pH 7.0

To obtain thermodynamic binding parameters, data from Figure 8 were adjusted to the model presented at Equation 1. The values were: K_a = 2.00x10^6 L.mol^{-1}, *n* = 0.4 and ΔH^o_{cal} = -42.63 kJ.mol^{-1}.

Calorimetric data followed the same profile of fluorescence data for curcumin interacting with $_u$BSA, showing that conformation change provided more intense and more enthalpically favorable interaction. As demonstrated for native BSA, calorimetric and fluorescence ΔH^o confirmed that curcumin/unfolded BSA binding also occurred at different sites.

3.5. Protective effect of BSA on curcumin photo-degradation

The fluorescence, SPR and ITC techniques showed that curcumin and BSA formed complex at pH 7.0. In addition, to determine the binding parameters between curcumin and BSA, the photo-degradation study of the complexed curcumin is relevant to a further application of this complex in different formulations. The curcumin photo-degradation may affect the consumers' health, and also may change the color and sensory properties of food products (NADI et al., 2015), originating compounds such as bicyclopentadione, vanillic acid, vanillin, ferulic aldehyde and ferulic acid (ANSARI, AHMAD, KOHLI, ALI, & KHAR, 2005; SCHNEIDER, GORDON, EDWARDS, & LUIS, 2015; TØNNESEN & KARLSEN, 1985; TØNNESEN, KARLSEN, & HENEGOUWEN, 1986).

To evaluate the protective effect of BSA on curcumin exposed to light, we determined the photo-degradation constant (K_d) of curcumin in the presence of increasing concentration of the native and unfolded protein, at pH 7.0 over time, adjusting the experimental data to Equation 8 (Abdou, Hafez, Bakir, & Abdel-Mottaleb, 2013; de Rosso & Mercadante, 2007).

$$-ln\frac{A}{A_0} = K_d t \quad (8)$$

where A is the absorbance in the end time, A_0 is the absorbance in time 0, K_d is the degradation constant, t is the storage time. The K_d values were obtained from the slope of the plot *-ln A/A₀* versus the time of light exposure (Figure S2, Supplementary Materials).

Then, the half-life time ($t_{1/2}$) was obtained by following equation:

$$t_{1/2} = \frac{ln2}{k_d} \quad (9)$$

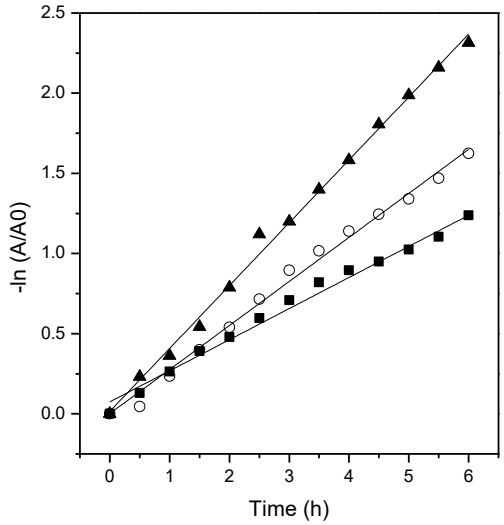

Figure S2. Plot for determination of degradations constant (k_d) of curcumin (25 μM) in buffer pH 7.0 (▲), in the presence of 35 μM of native (■) and denatured (○) BSA, pH 7.0.

Experiments were performed for different protein concentration, and the K_d and $t_{1/2}$ values as a function of BSA concentration are shown in Figure 9.

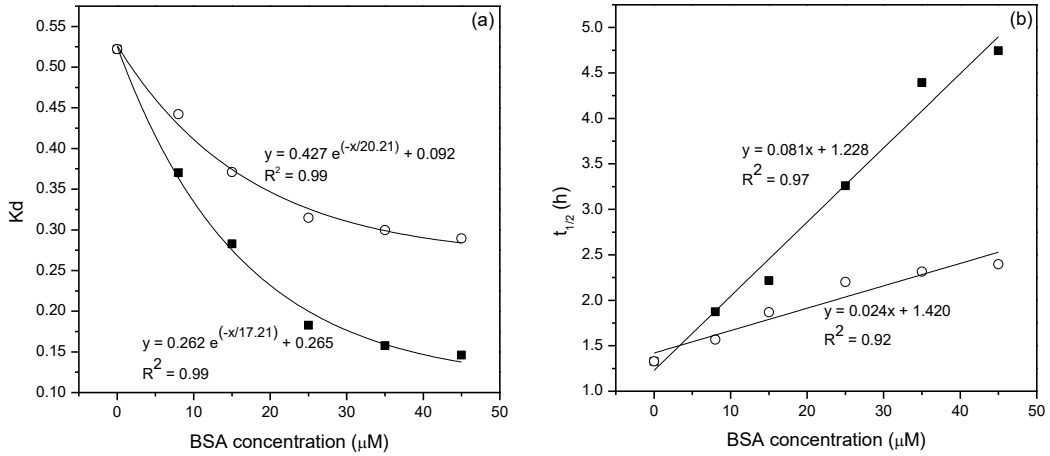

Figure 9 – Photo-degradation parameters of curcumin complexed with native (■) and unfolded (○) BSA: (a) degradation constant (K_d) and (b) half-life time ($t_{1/2}$).

As the concentration of either native or unfolded BSA increases, the curcumin K_d values decreased exponentially until achieve a pathamar, while the $t_{1/2}$ increased linearly at 0.081 h.μM^{-1} and 0.024 h.μM^{-1}, respectively. The pathamar obtained in Kd *versus* [BSA] plot indicates that above 20 μM and 30 μM for unfolded and native BSA, respectively, almost all available curcumin in the system is complexed with BSA and addition of more protein did not change the K_d value. Interestingly, at protein saturation concentration the [curcumin]/[BSA] ratios (r_S) are 0.80 and 1.20 for unfolded and native protein, respectively, with are very similar to the *n* values (0.78 and 1.10) found by fluorescence at 298 K. This similarity between *n* and r_S corroborating with the hypothesis that curcumin photo-protection occurs due to a complex formation process.

Kumavat et al. (2013) evaluated the instability of curcumin in buffer at different pH conditions and in the presence of light also by measuring absorbance over time. When curcumin in buffer pH 7.0 was incubated at 310 K, its half-life was estimated at 0.78h, or 47 minutes. Mirzaee, Mirzaee, Kooshk, Rezaei-Tavirani, & Khodarahmi (2013) studied the protective effect of β-lactoglobulin, BSA and casein on light and water-mediated degradation of curcumin at pH 7 and 310 K. The authors concluded that BSA and casein showed ability to protect

curcumin from degradation mediated by the two sources, whereas β-lactoglobulin promoted decomposition of curcumin by an unknown mechanism. However, the authors did not study the effect of BSA concentration and conformation and did not present degradation constant and half-life values.

The mechanism of photo-degradation of curcumin is still unclear, but some studies have shown that under light incidence the curcumin can act as oxygen sensitizer and may degrade (Priyadarsini, 2009; Tønnesen & Greenhill, 1992). It is well known that for molecular sensitizer mechanism occur is necessary molecular collision between curcumin and oxygen. As curcumin could bind to inner cavities of BSA, it may be suggested that within these cavities curcumin is less accessible to the oxygen present in the solvent, preventing its photo-degradation. Even though K_a values were slightly higher for curcumin/unfolded BSA, suggesting a higher amount protected curcumin than that of curcumin/native BSA complex, the denaturation process could decrease the degree of protection of curcumin collision with oxygen.

4. CONCLUSIONS

The interaction between curcumin and BSA at native and unfolded state was demonstrated. For both conformation curcumin/BSA binding was enthalpically and entropically driven. The different techniques used to study intermolecular interaction showed that curcumin/BSA binding was multi-site for native and unfolded BSA. However, BSA conformation affected the thermodynamic binding parameters, as well as the photo-degradation of curcumin.

This work showed that curcumin/BSA binding improves photochemical stability of curcumin, providing important information for successful technological application of curcumin in different formulations.

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