VIVIANNE MOLICA DE ANDRADE

SISTEMAS AQUOSOS BIFÁSICOS: DETERMINAÇÃO DE EQUILÍBRIO DE FASE E APLICAÇÃO PARA PARTIÇÃO DE ALFA-LACTOALBUMINA E BETA-LACTOGLOBULINA

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Agroquímica, para obtenção do título de *Magister Scientiae.*

VIÇOSA
MINAS GERAIS – BRASIL
2011

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APROVADA: 02 de Fevereiro de 2011.

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Dedico este trabalho aos alicerces da minha vida: meus pais, Celinho e Nazaré, meus irmãos Vivian e Vinícius, e ao meu companheiro de todas as horas, Gui.

AGRADECIMENTOS

À Deus, sempre presente em minha vida, guiando todos os meus caminhos.

Aos meus pais, Celinho e Nazaré, pelo amor, paciência, compreensão, orações, incentivo e apoio em minhas escolhas.

Aos meus irmãos, Vivian e Vinícius, pela torcida, orações e por saber que sempre posso contar com vocês.

Ao Michel, pelas conversas e desabafos.

Ao Guilherme pelo amor, companheirismo, amizade, paciência e incentivo sempre.

À toda minha família (avós, tios, tias, primos e primas) que sempre torcem por mim.

Ao Geraldo, Rozelene e Rafael, pela acolhida na família.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, à Fundação de Amparo à Pesquisa do Estado de Minas Gerais, FAPEMIG e ao Instituto Nacional de Ciências e Tecnologias Analíticas Avançadas, INCTAA, pelo apoio financeiro.

À Universidade Federal de Viçosa e ao Departamento de Química, pela oportunidade de realização deste trabalho.

Aos professores Carminha e Luis, pela confiança, orientação, ensinamentos e amizade.

Aos professores Alvaro, Gurgel e Ana Clarissa, por aceitarem participar da banca desta defesa.

Aos amigos do grupo QUIVECOM, pela amizade e convívio e em especial ao Gabriel, que me ajudou na realização dos experimentos e discussão dos resultados.

Aos amigos do Departamento de Química, pela amizade e boa convivência no trabalho.

Aos amigos Eder, Aparecida, Igor, Tiago, Maiby, Montanari, Lilian, Katalin, Leandro, pelos bons momentos no mestrado.

BIOGRAFIA

Vivianne Molica de Andrade, filha de Cirilo Antônio de Andrade e Maria Nazaré Molica de Andrade, nasceu em 24 de dezembro de 1983, na cidade de Viçosa, Minas Gerais.

Em março de 2004 ingressou no curso de Bioquímica da Universidade Federal de Viçosa, diplomando-se com o título de Bacharelado em julho de 2008.

Atualmente integra o quadro de servidores da UFV como Assistente de Laboratório do Departamento de Química. É estudante de mestrado e desenvolve pesquisa no grupo de Química Verde Coloidal e Macromolecular (QUIVECOM), sob orientação da Prof[a]. Maria do Carmo Hespanhol da Silva do Departamento de Química da UFV. Além disso, é bolsista de Apoio Técnico - Nível Superior do CNPq, no período de setembro de 2010 a agosto de 2012, desenvolvendo atividades sob orientação do Prof. Luis Henrique Mendes da Silva no grupo QUIVECOM do Departamento de Química da UFV.

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SUMÁRIO

LISTA DE SÍMBOLOS E ABREVIATURAS

Φ_i^B - Fração volumétrica do componente i na fase inferior

Φ_i^T - Fração volumétrica do componente i na fase superior

Φ_j^B - Fração volumétrica do componente j na fase inferior

Φ_j^T - Fração volumétrica do componente j na fase superior

w_{iP} - Par potencial de energia entre o componente i e o soluto particionado

w_{ij} - Par potencial de energia entre os componentes i e j

% (w/w) ou **% (m/m) –** Porcentagem massa/massa

ρ - Número de sítios reticulares por unidade de volume (densidade numérica total)

$\mathbf{\Delta H_{ap-int}}$ – Variação da entalpia de interação aparente

$\mathbf{C_P^I}$ ou $\mathbf{C_P^B}$ – Composição de polímero na fase inferior

$\mathbf{C_P^S}$ ou $\mathbf{C_P^T}$ – Composição de polímero na fase superior

$\mathbf{C_S^I}$ ou $\mathbf{C_S^B}$ – Composição de sal na fase inferior

$\mathbf{C_S^S}$ ou $\mathbf{C_S^T}$ – Composição de sal na fase superior

$\mathbf{[P]_B}$ – Concentração de proteína na fase inferior

$\mathbf{[P]_T}$ – Concentração de proteína na fase superior

BSA – Albumina do soro bovino

CFI – composição da fase inferior

CFS – composição da fase superior

CGL – Composição global

CLA ou **TLL** – Comprimento da linha de amarração

EO – Óxido de etileno

FI ou **BP** – Fase inferior

FPLC – Cromatografia líquida rápida para proteínas

FS ou **TP** – Fase superior

GMP – Glico-macropeptídeos

HPLC-RP– Cromatografia líquida de alta performance em fase reversa

HPMC – Hidroxipropilmetilcelulose

Ig's – Imunoglobulinas

$\mathbf{K_P}$ – Coeficiente de partição

Kα – Coeficiente de partição da proteína α-lactoalbumina

K_β – Coeficiente de partição da proteína β-lactoglobulina

L64 – Copolímero L64

LA ou **TL** – Linha de amarração

LB – Linha binodal

LLE – Equilíbrio líquido-líquido

MD – Maltodextrina

M_P – Massa molar do soluto particionado

M_w – massa molar média

n^B - Número total de moléculas na fase inferior

n^T – Número total de moléculas na fase superior

Pc – Ponto crítico

PEO – Poli(óxido de etileno)

pI – Ponto isoelétrico

PO – Óxido de propileno

R – Constante universal dos gases

SABs ou **ATPS**– Sistemas aquosos bifásicos

STL – Inclinação da linha de amarração

T – Temperatura absoluta

V^B – Volume da fase inferior

V^T – Volume da fase superior

w_{L64} – Porcentagem massa/massa de polímero L64

WPC – Concentrado de proteína do soro

w_s – Porcentagem massa/massa de sal

w_w – Porcentagem massa/massa de água

Z - Número de vizinhos

α-la – α-lactoalbumina

β-lg – β-lactoglobulina

$\Delta_{prot\text{-}dil} H_{top}$ – Variação da entalpia de diluição da solução de proteína na fase superior

$\Delta_{prot\text{-}dil} H_{bottom}$– Variação da entalpia de diluição da solução de proteína na fase inferior

$\Delta_{PEO\text{-}P}H°$ - Variação da entalpia de interação entre moléculas de polímero e proteína

$\Delta_{PEO\text{-}S}H°$ - Variação da entalpia de interação entre moléculas de polímero e sal

$\Delta_{PEO\text{-}W}H°$ - Variação da entalpia de interação entre moléculas de polímero e água

$\Delta_{P\text{-}W}H°$ - Variação da entalpia de interação entre moléculas de proteína e água

$\Delta_{S\text{-}P}H°$ - Variação da entalpia de interação entre moléculas de sal e proteína

$\Delta_{S\text{-}W}H°$ - Variação da entalpia de interação entre moléculas de sal e água

$\Delta_{tr}G°$ - Variação da energia livre de Gibbs de transferência

$\Delta_{tr}H°$ - Variação da entalpia de transferência

$\Delta_{tr}S°$ - Variação da entropia de transferência

ε_{ii} – Energia necessária para romper as interações entre duas moléculas semelhantes do tipo i

ε_{ij}- Energia envolvida na formação da interação entre as moléculas i e j

ε_{jj} – Energia necessária para romper as interações entre duas moléculas semelhantes do tipo j

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RESUMO

ANDRADE, Vivianne Molica de, M.Sc., Universidade Federal de Viçosa, Fevereiro de 2011. **Sistemas aquosos bifásicos: determinação de equilíbrio de fase e aplicação para partição de alfa-lactoalbumina e beta-lactoglobulina**. Orientadora: Maria do Carmo Hespanhol da Silva. Coorientadores: Luis Henrique Mendes da Silva e Jane Sélia dos Reis Coimbra.

Este trabalho apresenta, no primeiro capítulo, uma breve revisão sobre obtenção do soro do leite e sua composição, destacando as propriedades nutricionais e tecnológicas das proteínas do soro. Posteriormente, aborda os principais métodos de separação das proteínas do soro, apresentando a utilização de sistemas aquosos bifásicos (SABs) como uma técnica eficiente e economicamente viável de extração líquido-líquido para separação das proteínas α-lactoalbumina (α-la) e β-lactoglobulina (β-lg). O segundo capítulo descreve a obtenção e a determinação do equilíbrio de fase de novos SABs constituídos pelo copolímero L64 + sais orgânicos (citrato de amônio, citrato de sódio, succinato de sódio ou tartarato de sódio) + H_2O em diferentes temperaturas (5, 15 e 25 °C). Medidas microcalorimétricas mostraram que o processo de separação de fases nestes sistemas foi exotérmico e o sal citrato de sódio ($Na_3C_6H_5O_7$) foi mais efetivo em promover a separação de fases que o sal citrato de amônio ($(NH_4)_3C_6H_5O_7$). A capacidade dos diferentes ânions em induzir a formação de SABs com L64 obedeceu à seguinte ordem: citrato ($C_6H_5O_7^{3-}$) > tartarato ($C_4H_4O_6^{2-}$) > succinato ($C_4H_4O_4^{2-}$). Por fim, o terceiro capítulo aborda uma aplicação dos SABs constituídos pelo polímero PEO 1500 + sais orgânicos (citrato de sódio, succinato de sódio ou tartarato de sódio) + H_2O para a partição das proteínas α-la e β-lg. Além disso, os parâmetros termodinâmicos ($\Delta_{tr}H°$, $\Delta_{tr}S°$ e $\Delta_{tr}G°$) associados à transferência das proteínas da fase inferior (FI) para a fase superior (FS) dos sistemas foram determinados. Os resultados obtidos mostraram que a partição da FI para FS é termodinamicamente favorável para α-la ($-6{,}61 < \Delta_{tr}G° < -0{,}77$ kJ mol^{-1}), mas é desfavorável para β-lg ($2{,}68 < \Delta_{tr}G° < 6{,}20$ kJ mol^{-1}). Além

disso, este processo ocorre com perda de entropia configuracional (- 9,23 < $T\Delta_{tr}S°$ < - 1,34 kJ mol^{-1}) e as interações intermoleculares entre moléculas de proteína e polietilenoglicol são mais favoráveis que as interações intermoleculares entre moléculas de proteína e sal (- 7,95 < $\Delta_{tr}H°$ < - 1,42 kJ mol^{-1}), para ambas as proteínas em todos os SABs estudados. Para explicar o comportamento de partição das proteínas, o modelo derivado da teoria de Flory-Huggins foi utilizado e observou-se que a partição da α-la para a FS foi entalpicamente dirigida, enquanto a partição da β-lg para a FI foi entropicamente dirigida.

ABSTRACT

ANDRADE, Vivianne Molica de, M.Sc., Universidade Federal de Viçosa, February, 2011. **Aqueous two-phase systems: determination of phase equilibrium and application for partition of alpha-lactoalbumin and beta-lactoglobulin.** Adviser: Maria do Carmo Hespanhol da Silva. Co-Advisers: Luis Henrique Mendes da Silva and Jane Sélia dos Reis Coimbra.

This work presents, in its first chapter, a brief review on production and compositions of cheese whey, emphasizing the nutritional and technological properties of whey proteins. Subsequently, the main methods of separation of whey proteins are addressed, using aqueous two-phase systems (ATPS) as an efficient and economically viable liquid-liquid extraction technique for separation of alpha-lactalbumin (α-la) and beta-lactoglobulin (β-lg) proteins. The second chapter describes the formation and determination of the phase equilibrium of new ATPS formed by copolymer L64 + organics salts (ammonium citrate, sodium citrate, sodium succinate or sodium tartrate) + H_2O at different temperatures (278.15, 288.15 and 298.15 K). Microcalorimetric measurements showed that the phase separation process in these systems was exothermic and the salt sodium citrate ($Na_3C_6H_5O_7$) was more effective in promoting phase separation than ammonium citrate ($(NH_4)_3C_6H_5O_7$). The capacity of the different anions in inducing ATPS formation with L64 followed the order: citrate ($C_6H_5O_7^{3-}$) > tartrate ($C_4H_4O_6^{2-}$) > succinate ($C_4H_4O_4^{2-}$). Finally, the third chapter describes an application of ATPS formed by polymer PEO 1500 + organics salts (sodium citrate, sodium succinate or sodium tartrate) + H_2O for partition of α-la and β-lg proteins. In addition the thermodynamical parameters ($\Delta_{tr}H°$, $\Delta_{tr}S°$ e $\Delta_{tr}G°$) associated with protein transfer from the bottom phase (BP) to the top phase (TP) were determined. The results showed that the partition from BP to TP is thermodynamically favorable to α-la ($-6.61 < \Delta_{tr}G° < -0.77$ kJ mol^{-1}), but is unfavorable for β-lg ($2.68 < \Delta_{tr}G° < 6.20$ kJ mol^{-1}). Additionally, this process occurs with loss of configurational entropy ($-9.23 < T\Delta_{tr}S° < -1.34$ kJ mol^{-1}) and the

intermolecular interactions between proteins and poly(ethylene glycol) molecules are more favorable than intermolecular interactions between proteins and salt molecules ($-7.95 < \Delta_{tr}H° < -1.42$ kJ mol^{-1}) for both proteins in all ATPS studied. To explain the partition behavior of α-la and β-lg the model derived from the Flory-Huggins theory was used and it was observed that α-la partition to the TP was enthalpically driven, while the β-lg partition to the BP was entropically driven.

Capítulo 1

Revisão de Literatura

1. Introdução

1.1. Soro do leite

O soro do leite é um co-produto resultante da fabricação de queijos, de cor amarelo-esverdeada, obtido pela coagulação do leite. O seu sabor, ligeiramente ácido ou doce, e a sua composição dependem do tipo e do processo de fabricação do queijo, respectivamente. Pode ser obtido em laboratório ou na indústria por três processos principais: a) pelo processo de coagulação enzimática (enzima quimosina), resultando no coágulo de caseínas, matéria-prima para a produção de queijos e no soro "doce"; b) por precipitação ácida no pH isoelétrico (pI), resultando na caseína isoelétrica, que é transformada em caseinatos e no soro ácido; c) por separação física das micelas de caseína por microfiltração, obtendo-se um concentrado de micelas e as proteínas do soro, na forma de concentrado ou isolado proteico [1,2].

A composição do soro é de aproximadamente 93 % de água, 5 % de lactose, 0,9 % de proteínas, 0,3 % de gordura, 0,2 % de ácido láctico e pequenas quantidades de vitaminas [3]. A presença de proteínas com elevado teor de aminoácidos essenciais faz com que o soro do leite tenha alto valor nutricional.

Além das propriedades nutricionais, as proteínas do soro do leite são muito conhecidas pela versatilidade de suas propriedades funcionais tecnológicas como ingredientes em produtos alimentícios, principalmente por sua elevada solubilidade e capacidade de geleificação. Recentemente, têm sido atribuídas às proteínas do soro propriedades funcionais

fisiológicas, capazes de produzir um importante controle na modulação do metabolismo e nos mecanismos de defesa dos organismos animal e humano [4-6].

O soro do leite, por ser pouco aproveitado pelas indústrias, ao ser descartado no ambiente (solo e mananciais de rios) sem tratamento prévio, gera um grave problema de poluição ambiental, principalmente devido ao seu alto teor de matéria orgânica.

No Brasil, a produção de bebidas lácteas é uma das principais opções de aproveitamento do soro do leite, e as mais comercializadas são as bebidas fermentadas, com características sensoriais semelhantes ao iogurte, e bebidas lácteas não-fermentadas. Contudo, o aproveitamento desse co-produto atinge apenas 15% do total de soro produzido, com a produção nacional estimada em 470 milhares de toneladas [7,8].

Devido à vasta aplicabilidade das proteínas do soro do leite, torna-se importante o desenvolvimento de processos de separação e purificação, em larga escala, destas proteínas, e que também sejam viáveis economicamente. Logo, a busca por processos eficientes e econômicos para o processamento de biomoléculas é uma necessidade. As técnicas empregadas em biosseparações devem assegurar que a atividade biológica das moléculas não seja afetada, elevada pureza e altos rendimentos [9]. Neste contexto, é importante o desenvolvimento de novos métodos analíticos para extração e separação das proteínas do soro do leite, agregando valor a este resíduo agroindustrial.

Os principais métodos de separação de proteínas do soro empregam a precipitação seletiva (por adição de sais ou solventes orgânicos) ou cromatografia. A adição de sulfato de amônio ou sulfato de magnésio em solução resultava na precipitação da beta-lactoglobulina (β-lg), restando no sobrenadante a alfa-lactoalbumina (α-la) [10]. Posteriormente descobriu-se que a adição de ácido tricloro-acético [11] promovia a precipitação de todas as proteínas exceto a β-lg, que podia, assim, ser extraída juntamente com a fase líquida em equilíbrio com o precipitado. Em 1988 [12] descobriu-se que o mesmo resultado poderia ser obtido pela

adição do ácido tricloro-acético quando se adicionava cloreto de sódio em pH igual a 3. Além destas técnicas precipitantes, métodos cromatográficos são eficientes na obtenção de proteínas relativamente puras. Blanc [13] e Yoshida [14] utilizaram cromatografia por exclusão molecular para obter amostras de α-la e β-lg enriquecidas em mais de 90 %, enquanto que a aplicação de cromatografia de troca iônica possibilitava rendimentos ainda maiores [15]. Ye *et al.* [16] propuseram um método rápido para isolar as proteínas α-la, β-lg A e β-lg B do soro, sem nenhum tratamento prévio, utilizando cromatografia de troca iônica.

Entretanto, estes métodos possuem desvantagens, pois apresentam alto custo dos equipamentos, no caso da cromatografia e/ou não preservam a forma nativa das proteínas o que ocasiona desnaturação e perda de atividade funcional. Uma alternativa interessante para este contexto é a utilização de sistemas aquosos bifásicos (SABs) como técnica líquido-líquido de separação das proteínas do soro.

1.2. Proteínas do soro do leite

As proteínas do soro do leite apresentam estruturas globulares contendo algumas pontes de dissulfeto, que conferem estabilidade estrutural às macromoléculas. As frações, ou peptídeos do soro, são constituídas de: β-lg, α-la, albumina do soro bovino (BSA), imunoglobulinas (Ig's) e glico-macropeptídeos (GMP). Essas frações podem variar em tamanho, peso molecular e função, fornecendo às proteínas do soro características especiais [17-19]. Presente em todos os tipos de leite, a proteína do leite bovino contém cerca de 80 % de caseína e 20 % de proteínas do soro, percentuais que podem variar em função da raça do gado, da ração fornecida e do país de origem [20]. No leite humano, o percentual das proteínas do soro é modificado ao longo da lactação, sendo que no colostro elas representam cerca de 80 % e, na sequência, esse percentual diminui para 50 % [21].

Dentre as proteínas contidas no soro, as que aparecem em maior concentração são a β-lg e a α-la, correspondendo a cerca de 50 % e 20 %, respectivamente, das proteínas do soro. Os 30 % restantes correspondem a outras proteínas como: soroalbumina, lactoferrina, lisozima e imunoglobulinas.

A proteína β-lg possui estrutura tridimensional globular, apresenta em sua estrutura primária 162 resíduos de aminoácidos, massa molecular de aproximadamente 18,3 kDa, ponto isoelétrico igual a 5,2 e é termosensível. A β-lg é a proteína mais abundante no soro de leite bovino e praticamente não ocorre no leite humano, sendo considerada alergênica e antigênica, uma vez que pode causar alergia em segmentos mais sensíveis da população, principalmente crianças [22,23]. A estrutura particular da β-lg, do tipo lipocalina, forma uma espécie de cálice de caráter hidrofóbico que lhe confere propriedades funcionais de grande aplicação na indústria de alimentos, como capacidade de emulsificação, formação de espuma, geleificação e interação com moléculas responsáveis pelo aroma e sabor do produto [24]. Esta estrutura em forma de cálice contribui para que ela seja uma proteína bastante estável em solução em uma ampla faixa de pH [25].

A proteína α-la possui estrutura tridimensional em forma de um elipsóide, cuja estrutura primária apresenta 123 resíduos de aminoácidos, massa molecular de aproximadamente 14,1 kDa e ponto isoelétrico igual a 4,3. Além disso, a α-la possui forte interação com Ca(II) e outros íons metálicos como Zn(II), Mn(II), Cd(II), Cu(II) e Al(III), sendo a ligação com o cálcio responsável pela estabilização da proteína contra a desnaturação térmica. Em condições fisiológicas, a α-la funciona como uma proteína "modificadora" da especificidade da enzima D-glicose 4-β-galactosil transferase (EC 2.4.2.33), que é responsável pela síntese da lactose nas glândulas mamárias. É rica em triptofano (aproximadamente 6 % em massa) sendo utilizada no preparo de alimentos infantis para torná-los mais próximos da composição do leite humano [26].

1.3. Sistemas Aquosos Bifásicos

Os sistemas aquosos bifásicos (SABs) são misturas ternárias compostas majoritariamente por água que, sob certas condições de composição, temperatura e pressão, apresentam duas fases em equilíbrio termodinâmico. Eles podem ser formados pela mistura de soluções de dois polímeros quimicamente distintos [27,28], pela mistura de soluções de um polímero e um eletrólito [29,30] ou ainda pela mistura de soluções de dois eletrólitos [31]. Os SABs são caracterizados por possuírem duas fases distintas: uma rica em polímero (ou sal), denominada fase superior (FS), e outra rica em sal (ou em outro polímero), denominada fase inferior (FI).

Os primeiros relatos de estudos envolvendo esse tipo de sistema datam do ano de 1896, quando Beijerinck [32,33] descobriu que, ao se misturar soluções aquosas de gelatina e ágar (ou gelatina e amido solúvel), sob dada faixa de temperatura e concentração, formavam-se misturas turvas as quais, quando deixadas em repouso, separavam-se em duas fases límpidas, uma delas (a mais densa) enriquecida em ágar (ou amido) e a outra enriquecida em gelatina, sendo a água o componente majoritário em ambas as fases.

Estudos posteriores realizados por Ostwald e Hertel [34,35] verificaram que amidos provenientes de origens diferentes levavam à formação de SABs com características diferentes, tais como as proporções relativas de seus constituintes.

Mas foi a partir de meados da década de 50, com os trabalhos de Per-Åke Albertsson [36], que ficou evidente a grande aplicabilidade destes sistemas na partição e purificação de materiais biológicos. A Tabela 1 relaciona alguns SABs e os principais biomateriais particionados neles.

Tabela 1. SABs nos quais alguns biomateriais já foram particionados.

Biomaterial	**Sistema Aquoso Bifásico**	
	Polímero 1	**Polímero 2 (ou sal)**
Insulina [37]	Polióxido de etileno (PEO)	Dextrana,tampão citrato-fosfato
Albumina bovina [37]	Dextrana	Ficoll, tampão fosfato
Proteínas anticorpos [38]	PEO	Na_2HPO_4/K_2HPO_4
Lisozima [39]	PEO	Na_2SO_4
Rotavírus [40]	PEO	$(NH_4)_2SO_4$

As vantagens oferecidas pelos SABs residem no fato de eles serem considerados economicamente viáveis e ambientalmente seguros, pois são formados por reagentes de baixo custo e não-tóxicos (polímero, sal e água). Por serem constituídos majoritariamente por água, as propriedades termodinâmicas das fases são semelhantes ao meio aquoso dos seres vivos, o que torna tais sistemas estratégicos na extração de biopartículas, uma vez que evitam a desnaturação, sem perda da atividade biológica [41,42]. Esses sistemas também possuem baixa tensão interfacial, o que viabiliza a transferência de solutos pela interface [43], e por fim podem ser aplicados em larga escala [44].

Entretanto, para aplicar os SABs ao estudo de partição de solutos é importante que se conheça previamente seus diagramas de fases. Estes diagramas representam graficamente a composição na qual se formam duas fases líquidas em equilíbrio termodinâmico, e podem ser apresentados de forma triangular ou retangular, conforme mostrado na Figura 1.

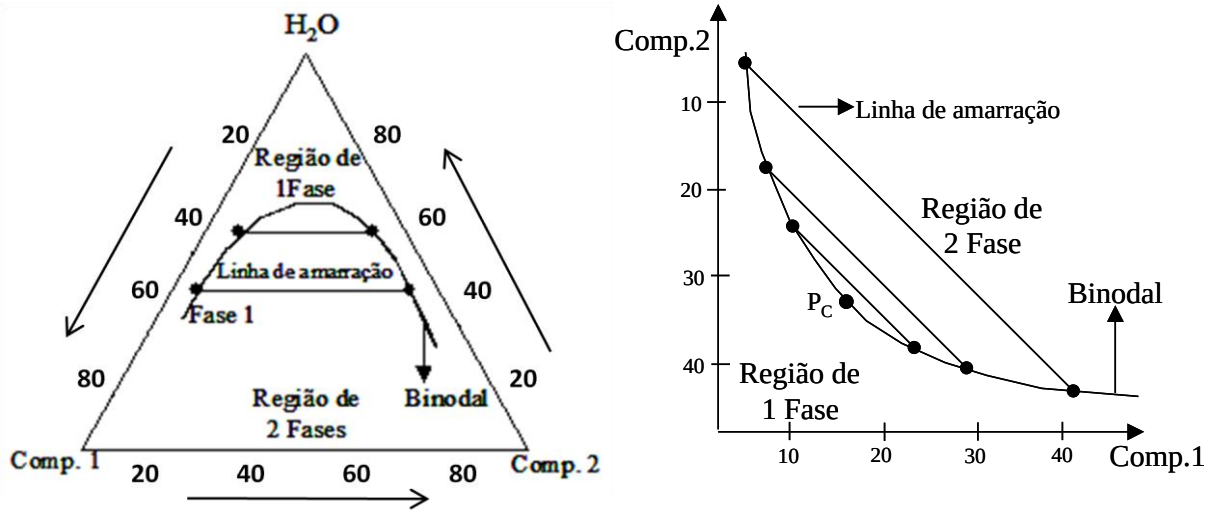

Figura 1. Diagramas de fase expressos em coordenadas triangulares e retangulares.

A Figura 2 representa um diagrama retangular onde o eixo das abscissas representa a concentração de sal do sistema e o eixo das ordenadas corresponde à concentração do polímero. Estas concentrações geralmente são expressas em porcentagem mássica, % (m/m). Neste diagrama encontram-se informações fundamentais para o entendimento e aplicação dos SABs, tais como as composições globais (CGL) nas quais sistemas com uma ou duas fases serão obtidos, a composição da fase superior (CFS) e composição da fase inferior (CFI). A linha binodal (LB) delimita a região homogênea da região bifásica. Esta linha corresponde às composições mínimas dos componentes necessárias para a separação de fases. As composições localizadas acima da LB formam sistemas de duas fases e abaixo desta linha formam sistemas homogêneos. A posição da linha binodal no diagrama pode ser influenciada por fatores como temperatura, natureza do eletrólito, pH do meio, massa molar e hidrofobicidade do polímero. Existem diferentes métodos para a obtenção da LB, entretanto o mais utilizado envolve titulação turbidimétrica e análise das composições das fases [45].

Este diagrama é composto também por linhas de amarração (LA), que são retas que unem as composições de pontos de mistura (ou CGL) com as composições das respectivas fases no equilíbrio termodinâmico. À medida que se caminha para uma LA maior, as

propriedades termodinâmicas intensivas (índice de refração, condutividade, densidade, composição, etc.) das fases (superior e inferior) vão se tornando cada vez mais distintas. Entretanto, sobre uma mesma LA, as composições das fases permanecem inalteradas, mas as propriedades termodinâmicas extensivas (volume, capacidade calorífica, massa, etc.) de cada uma das fases variam. A diminuição sucessiva nos comprimentos das linhas de amarração (CLA) leva de encontro ao ponto crítico (Pc). À medida que as composições das duas fases do sistema se aproximam deste ponto, a diferença entre as propriedades termodinâmicas dessas fases diminui até que, teoricamente, tornam-se iguais.

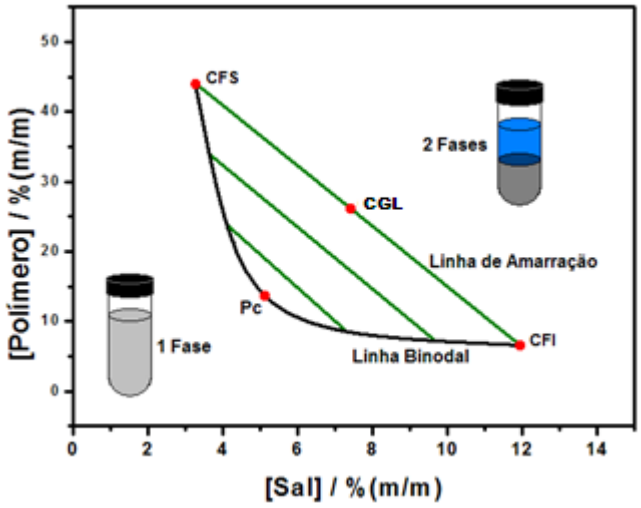

Figura 2. Diagrama de fase expresso em coordenadas retangulares de um SAB constituído por polímero e sal.

Logo, um importante parâmetro a ser analisado em estudos de partição utilizando SABs é o CLA, que é definido matematicamente pela equação 1.

$$\mathrm{CLA} = [(\mathrm{C_P^S} - \mathrm{C_P^I})^2 + (\mathrm{C_S^S} - \mathrm{C_S^I})^2]^{1/2} \quad (1)$$

onde, $\mathrm{C_P^S}$ e $\mathrm{C_P^I}$ são as concentrações de copolímero e $\mathrm{C_S^S}$ e $\mathrm{C_S^I}$ são as concentrações de sal em % (m/m) nas fases superior e inferior, respectivamente. Sendo assim, espera-se que, se um soluto possui maior afinidade por uma das fases do SAB, à medida que se aumenta o CLA,

mais distintas são as propriedades termodinâmicas intensivas entre as duas fases e maior será o coeficiente de partição (K) deste soluto.

A aplicação de SABs para o processamento do soro de queijo teve início em 1989 [46]. Neste trabalho ficou evidente que a partição das proteínas do soro, em geral, era fortemente influenciada pela massa molar do polímero, concentração dos componentes em cada fase, pH, temperatura, propriedades das proteínas (estrutura, hidrofobicidade, massa molar) e a adição de sais que não participavam do processo de separação de fase.

Vários autores estudaram o comportamento de partição das principais proteínas do soro do leite em diferentes SABs. A Tabela 2 sumariza alguns destes trabalhos. Na maioria deles, percebe-se uma tendência da proteína α-la em se concentrar na fase rica em polímero e uma transferência preferencial da proteína β-lg para a fase rica em sal nos sistemas avaliados.

Tabela 2. Partição das proteínas α-la e β-lg em diferentes SABs.

Proteínas	SABs utilizados	Detecção	Ref.
α-la e β-lg	PEO 1500 + fosfato de potássio	FPLC[a]	[47]
α-la e β-lg	PEO (1450, 8000, 10000) + MD[b] (2000, 4000)	Espectrofotometria a 280 nm	[47]
α-la e β-lg	F68 + carbamato de amônio e F38 + carbamato de amônio	Método de Bradford	[48]
α-la e β-lg	PEO 1500 + KH_2PO_4 / K_2HPO_4, pH 7,0	HPLC-RP[c]	[9]
α-la e β-lg	PEO (1000, 1450, 3350) + citrato de sódio, pH (5,2; 6,2 e 8,2)	Espectrofotometria a 280 nm	[49]
α-la e β-lg	PEO (600, 900, 1500) + $(NH_4)_2SO_4$	Método de Bradford	[50]
α-la e β-lg	WPC[d] + HPMC[e], pH 6,5	Método de Kjeldahl	[51]

[a]FPLC: Cromatografia líquida rápida para proteínas; [b]MD: Maltodextrina; [c]HPLC-RP: Cromatografia líquida de alta performance em fase reversa; [d]WPC: Concentrado de proteína do soro; [e]HPMC: Hidroxipropilmetilcelulose

Alves *et al.* [47] obtiveram coeficientes de partição entre 0,29 a 8,0 para α-la e entre 0,005 a 0,056 para a β-lg em sistemas formados por polietilenoglicol (PEO) e fosfato de potássio. O valor de K para a β-lg foi pouco afetado pelo aumento da concentração de polímero, enquanto que para a α-la, o valor de K aumentou com o aumento das concentrações

de polímero e sal no sistema. O comportamento de partição das proteínas α-la e β-lg também foi estudado em SAB PEO / maltodextrina (MD), sendo que os valores de K da β-lg foram menores do que 1 no SAB PEO 8000 / MD 2000, atingindo valores próximos a 1 quando se utilizou o SAB PEO 1450 / MD 4000. O mesmo comportamento foi observado para a α-la, ou seja, o coeficiente de partição da proteína aumentou à medida que a massa molar do PEO diminuiu e/ou a massa molar da MD aumentou.

Oliveira *et al.* [48] encontraram coeficientes de partição variando de 1,0 a 2,5 para a α-la e de 0,1 a 1,0 para proteína β-lg utilizando sistemas formados por carbamato de amônio e os copolímeros tribloco F38 e F68.

Giraldo-Zuñiga *et al.* [9] determinaram o coeficiente de partição das proteínas α-la e β-lg em SAB PEO / fosfato de potássio em pH igual a 7. A partição no SAB PEO 1500 18 % (m/m) + fosfato de potássio 18 % (m/m) forneceu o maior valor de K para α-la (20,55) e o menor para β-lg (0,030).

Boaglio *et al.* [49] avaliaram o comportamento de partição das proteínas α-la, β-lg e soroalbumina bovina (BSA) em SABs formados por PEO / citrato de sódio em diferentes pHs. Observou-se que, com o aumento do CLA, os valores de coeficiente de partição para todas as proteínas decaíram, sendo a partição da α-la mais afetada, pois o valor de K diminuiu cerca de quatro vezes. O aumento da massa molar do polímero também diminuiu a partição das proteínas para a FS do SAB, o que pode ser explicado por uma diminuição do volume livre disponível na fase superior em consequência do aumento na concentração do polímero.

Rodrigues *et al.* [50] estudaram o comportamento de partição das proteínas α-la e β-lg em sistemas formados por PEO / $(NH_4)_2SO_4$. Estes foram considerados uma alternativa econômica, com redução de 50 % dos custos, para a recuperação e separação das proteínas do soro. Além disso, os maiores valores de coeficiente de partição, iguais a 12,8 para α-la e 0,34

para β-lg, foram obtidos quando se utilizou o sistema PEO 900 18 % (m/m) + $(NH_4)_2SO_4$ 14 % (m/m) em pH 7.

Apesar dos inúmeros trabalhos experimentais e teóricos a respeito da partição de proteínas em SABs, ainda não se tem um modelo adequado para a descrição e previsão dos processos de transferência entre as duas fases, sendo necessários estudos para compreender os processos termodinâmicos que governam a partição de solutos em SABs.

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Capítulo 2

Aqueous two–phase systems of copolymer L64 + organic salt + water: Liquid-liquid equilibrium and enthalpic L64–salt interaction

Abstract

Phase diagrams of two–phase systems (ATPS) composed by the triblock copolymer L64 + organic salt (sodium tartrate, sodium succinate, sodium citrate, or ammonium citrate) + water, at different temperatures (278.15, 288.15, and 298.15 K) are presented in this work. Contrary to behavior of ATPS formed by inorganic salts, the study of the temperature influence in the liquid–liquid equilibrium behavior of L64–organic salts ATPS showed an exothermic character for phase separation process. Microcalorimetric measurements showed that this phase separation energy is around – 0.2 kJ mol^{-1} for all organic salts. The slope of tie line (STL) tends to increase with an increase in temperature. The cation nature effect showed that the salt $Na_3C_6H_5O_7$ was more effective in promoting phase separation than $(NH_4)_3C_6H_5O_7$. The capacity of the different anions tested for inducing ATPS formation with L64 followed the order: $C_6H_5O_7^{3-}$ > $C_4H_4O_6^{2-}$ > $C_4H_4O_4^{2-}$. Because the salt–L64 interaction energy to be very similar, the cation and anion effects on the phase separation could be attribute to a process driven by entropy.

Keywords: enthalpic interaction, aqueous two–phase system, triblock copolymer, microcalorimetry

1. Introduction

Separation processes involving macromolecular systems, such as cloud point extraction [1] and polymeric blends [2], are applied frequently in several areas of research and industry. This type of process also occurs through organic solvent/water systems [3–5] and water/water systems, generally called aqueous two–phase systems, or ATPS [6–9]. In the latter case, there are some strategic advantages, including the following: (i) it provides a suitable environment for the preservation of biological activity of some solutes [10]; (ii) low interfacial tensions improve the diffusion process through the interface [11]; and (iii) the possibility of linear scale–up exists [12].

Within this context, ATPS have proven to be an excellent technique in the preconcentration, purification and separation of different solutes, such as nanoparticles [13], proteins [14], cell organelles [15], membranes [16], DNA [17], dye molecules [18], and metallic ions [19]. Under specific thermodynamic conditions [20], ATPS is traditionally formed by the mixture of aqueous solutions of two mutually incompatible polymers [21], by a polymer and a salt [22], or by two salts [23]. This ternary system splits into two water–enriched phases: a polymer–enriched top phase (or enriched with a salt) and a salt–enriched bottom–phase (or enriched with another polymer).

Most ATPS phase diagrams and partitioning studies reported in the literature are relative to systems composed of poly(ethylene oxide) (PEO) [22,24], which are limited for the extraction of water–soluble compounds (hydrophilic solutes). ATPS formed by $(EO)_n$–$(PO)_m$–$(EO)_n$ triblock copolymers are excellent options for PEO-ATPS due to the extent of the aqueous biphasic application for the extraction of hydrophobic solutes into the polymer–enriched phase. Due to their amphiphilic character, triblock copolymer molecules, in aqueous solution and under critical temperature and concentration conditions, go through a self–

assembly process, forming micelles. These aggregates have a hydrophilic crown of ethylene oxide units and a hydrophobic core formed by propylene oxide units [6], which are capable of interacting with hydrophobic compounds. For example, the triblock copolymer L64 ($(EO)_{11}(PO)_{16}(EO)_{11}$, with average molecular weight (M_w) 2900 $g \cdot mol^{-1}$ and 40% ethylene glycol) is known to form aggregates with different morphologies (e.g. vesicles and lamellae) depending on the thermodynamic conditions [25], which allows a wider application of the ATPS.

To the authors' best knowledge, there exists only one work in the literature describing phase diagrams of ATPS composed by the triblock copolymer L64 and inorganic electrolytes [26]. The aim of the present work is to determine the phase diagrams of ATPS composed by L64 + organic salt + water. To investigate the effect of the cations and anions, the organic electrolytes used were sodium tartrate, sodium succinate, sodium citrate, and ammonium citrate. Equilibrium data at 278.15, 288.15, and 298.15 K were determined to study the influence of temperature on the phase composition. Microcalorimetric measurements were used to determine the enthalpy of the interaction between the different salts and the L64 macromolecules.

2. Experimental Section

2.1. Materials. Poly(ethylene glycol)–block–poly(propylene glycol)–block–poly(ethylene glycol) copolymer with the basic backbone $(EO)_n(PO)_m(EO)_n$ was used. The L64, $(EO)_{13}(PO)_{30}(EO)_{13}$, with an average molar mass (M_w) of 2900 $g \cdot mol^{-1}$ and a mass fraction of 40 % EO was purchased from Aldrich. The analytical-grade reagents, $Na_2C_4H_4O_6 \cdot 2H_2O$ (sodium tartrate dihydrate), $Na_2C_4H_4O_4 \cdot 6H_2O$ (sodium succinate hexahydrate), $Na_3C_6H_5O_7 \cdot 2H_2O$ (sodium citrate dihydrate), and $(NH_4)_3C_6H_5O_7$ (ammonium citrate), were

obtained from Vetec (Brazil). Milli–Q II water (Millipore) was used throughout the experiments for the preparation of all aqueous solutions.

2.2. ATPS preparation. As presented elsewhere [26], we have established a general methodology that is used in all equilibrium studies carried out by our group with appropriate adaptations. First, stocks aqueous solutions of L64 and salt were prepared by weighing appropriate amounts of reagents on an analytical balance (Shimadzu, AG 220). We prepared ATPS by mixing appropriate quantities of the aqueous solutions of copolymer and salt in glass vessels according to the global compositions desired. The mixture was manually stirred until the system became turbid. It was allowed to settle for 24 to 72 h at the operational temperature of 278.15, 288.15, or 298.15 K in a temperature–controlled bath (Microquímica, MQBTC 99–20, with an uncertainty of ± 0.1 K). The equilibrium state was characterized by the absence of turbidity in both top and bottom phases. Aliquots of the top and bottom phases were collected with a syringe for analysis.

2.3. Determination of Equilibrium Compositions. The concentrations of salt were determined by conductivity analysis (Schott CG853, Germany) in the mass fraction range of 1.00×10^{-3} to 2.50×10^{-2} %. In this range of dilution of the phases, the salt solutions had the same conductivity in water and diluted polymer solution, showing that the copolymer does not interfere in the conductivity of the samples. The standard deviation of the weight percent of the salt by this method was ± 0.10 %. The copolymer concentration was determined from refractive index measurements performed at 298.15 K using a refractometer (Analytic Jena AG Abbe refractometer, Germany). Due to the copolymer and salt concentration dependence of the refractive index of the phase samples, which is an additive property for the copolymer and salts studied, we obtained the L64 concentration by subtracting the salt concentration

(obtained by conductivity) from the total solution composition (obtained by refractive index) [27]. The standard deviation of the copolymer mass percent was ± 0.006 %. The water content was determined by the difference in the mass (percentage mass) of each component ($w_{H2O} = w_{total} - w_S - w_{L64}$), where w_{total} = 100 % (w/w). All analytical measurements were performed in triplicate.

2.4. Microcalorimetric measurements. The enthalpy changes in the L64 and organic salt interactions were performed in triplicate using a CSC–4200 microcalorimeter (Calorimeter Science Corp.) controlled by ItcRun software. Titrations were carried out at 298.15 K ± 0.002 K by adding aliquots of 1.0 μL of a concentrated solution of one salt (20.0 % (m/m) of sodium succinate, sodium tartrate or sodium citrate and 30.0 % ammonium citrate) into a sample cell containing 1.82 mL of the aqueous mixture formed by L64 and the added salt (19.9 % (m/m) of L64 and 6.70 % (m/m) of sodium succinate; 18.4 % (m/m) of L64 and 7.75 % (m/m) of sodium tartrate; 20.2 % (m/m) of L64 and 6.52 % (m/m) of sodium citrate; or 8.14 % (m/m) of L64 and 29.1 % (m/m) of ammonium citrate). Raw data were analyzed using software supplied by the Calorimetry Sciences Corporation (ITCRun) after subtraction of the blank experiments, which were as follows: i) the addition of 1 μL of salt concentrated solution into a sample cell containing 1.82 mL of water and ii) the addition of 1 μL of water into a sample cell containing 1.82 mL of L64 and the added salt solution. The whole calorimetric procedure was chemically and electrically calibrated by the heat of protonation of (tris(Hydroxymethyl)Aminomethane) and by the joule effect, as recommended [28].

3. Results and Discussion

3.1. Liquid–liquid equilibrium compositions: For macromolecule–salt ATPS, the decrease in the free energy of the system occurs at specific thermodynamic conditions by an exclusion process between the polymer and electrolyte, leading to the formation of two aqueous phases. At lower polymer/salt concentrations, both components of the aqueous solution are miscible forming a single phase. However, above a critical concentration, they will separate into two phases, in which the upper phase is enriched in the polymer and the lower phase is concentrated in the salt component [26]. The composition of each phase depends on the macromolecule and electrolyte nature, opening the possibility to modulate the phase properties by changing the polymer and/or the salt structure.

Tables 1 to 4 present liquid–liquid equilibrium (LLE) data of the upper and lower phases and the tie–line length (TLL) values, expressed in mass percent, for the L64 + sodium succinate + water, L64 + sodium tartrate + water, L64 + sodium citrate + water, and L64 + ammonium citrate + water ATPS at 278.15, 288.15, and 298.15 K. According to the system, four or five tie–lines (TL) were obtained. The tie–line length (TLL) is a thermodynamic parameter that at constant pressure and temperature expresses the difference in intensive thermodynamic functions between the upper and lower phases [7]. TLL is expressed as the difference between polymer and salt concentrations present in the phases and is calculated by the following equation:

$$TLL = [(C_P^T - C_P^B)^2 + (C_S^T - C_S^B)^2]^{1/2} \quad (1)$$

where C_P^T and C_P^B are the polymer concentrations in the top and bottom phases, respectively, while C_S^T and C_S^B are those of the salt. The tie lines were constructed by means of linear regression fitting of the appropriate values of global composition and the compositions of the

upper and lower phases. It was observed that an increase in the overall composition enhanced the segregation between the copolymer and salt, i.e., the concentration of copolymer increased in the upper phase and decreased in the lower phase and that of the salt increased in the lower phase and decreased in the upper phase. This fact was also represented by the increase in TLL. This behavior has been reported previously for other ATPS [20, 26]. Nevertheless, as a consequence of the hydrophobic contribution of the PO segments [29], the top–phase water content is markedly lower than the water concentration observed in the top phase of the PEO–salt ATPS [30–33].

Table 1. Equilibrium Data for L64 (w_{L64}) + Sodium Succinate (w_S) + Water (w_W) System from (278.15 to 298.15) K

system	Overall			top phase			bottom phase			TLL
	100 w_{L64}	100 w_S	100 w_W	100 w_{L64}	100 w_S	100 w_W	100 w_{L64}	100 w_S	100 w_W	
					T = 278.15 K					
1	20.50	9.28	70.22	47.72	3.76	48.52	0.96	13.58	85.46	47.78
2	22.00	9.40	68.60	50.14	3.66	46.20	0.26	14.35	85.39	51.02
3	23.49	9.54	66.97	51.73	3.62	44.65	0.44	14.82	84.75	52.50
4	27.88	9.99	62.13	55.36	3.44	41.20	0.22	18.01	81.77	57.03
					T = 288.15 K					
1	15.93	8.85	75.22	38.52	4.46	57.02	0.23	11.70	88.07	38.97
2	17.92	9.04	73.04	44.76	3.95	51.29	0.27	12.75	86.98	45.34
3	20.48	9.29	70.23	50.19	3.44	46.37	0.22	14.11	85.67	51.10
4	23.49	9.54	66.97	53.98	3.21	42.81	0.33	15.11	84.56	54.95
					T = 298.15 K					
1	12.45	8.50	79.05	16.28	7.68	76.04	0.15	10.96	88.89	16.46
2	14.00	8.65	77.35	27.26	6.67	66.06	0.68	11.45	87.87	27.01
3	15.94	8.85	75.21	34.30	5.73	59.97	0.52	11.90	87.58	34.34
4	17.92	9.05	73.03	36.51	5.44	58.05	0.69	12.21	87.10	36.45

Table 2. Equilibrium Data for L64 (w_{L64}) + Sodium Tartrate (w_S) + Water (w_W) System from (278.15 to 298.15) K

system	Overall			top phase			bottom phase			TLL
	100 w_{L64}	100 w_S	100 w_W	100 w_{L64}	100 w_S	100 w_W	100 w_{L64}	100 w_S	100 w_W	
					T = 278.15 K					
1	17.82	8.23	73.95	27.09	4.71	68.19	0.81	15.95	83.23	28.58
2	20.92	8.26	70.82	32.80	3.58	63.62	0.09	17.53	82.38	35.56
3	22.75	8.47	68.78	35.48	3.42	61.10	0.33	18.30	81.37	38.17
4	26.18	8.26	65.56	39.08	3.01	57.90	0.48	19.29	80.23	41.89
5	28.87	8.71	62.42	42.74	2.63	54.63	0.03	21.14	78.83	46.54
					T = 288.15 K					
1	17.84	8.19	73.97	26.53	6.27	67.20	2.68	12.07	85.25	24.54
2	20.92	8.24	70.84	38.20	4.01	57.79	1.89	13.46	84.65	37.51
3	22.85	8.44	68.71	42.26	3.37	54.37	1.11	14.44	84.45	42.61
4	26.14	8.22	65.64	45.06	2.96	51.97	0.77	15.38	83.85	46.00
5	27.46	8.38	64.16	48.54	2.59	48.87	0.51	16.17	83.32	49.91
					T = 298.15 K					
1	23.2	7.23	69.57	32.28	5.47	62.26	3.51	10.93	85.56	29.28
2	24.83	7.43	67.74	39.27	4.61	56.12	2.65	11.90	85.45	37.34
3	26.46	7.63	65.91	43.03	4.01	52.96	2.70	12.86	84.44	41.28
4	28.09	7.83	64.08	49.26	3.18	47.56	3.17	13.32	83.51	47.20
5	29.72	8.03	62.25	51.61	2.94	45.46	3.42	13.84	82.74	49.41

Table 3. Equilibrium Data for L64 (w_{L64}) + Sodium Citrate (w_S) + Water (w_W) System from (278.15 to 298.15) K

system	Overall			top phase			bottom phase			TLL
	100 w_{L64}	100 w_S	100 w_W	100 w_{L64}	100 w_S	100 w_W	100 w_{L64}	100 w_S	100 w_W	
					T = 278.15 K					
1	19.13	7.17	73.70	27.95	3.83	68.22	0.97	14.32	84.71	28.94
2	22.09	7.25	70.66	33.42	2.86	63.72	1.52	15.45	83.02	34.29
3	22.37	8.55	69.08	37.83	2.35	59.82	0.61	17.68	81.71	40.25
4	25.62	9.1	65.28	44.19	1.87	53.93	0.96	18.83	80.21	46.44
5	28.21	9.29	62.50	47.25	1.62	51.13	1.09	19.87	79.04	49.63
					T = 288.15 K					
1	19.19	7.05	73.76	33.98	3.55	62.47	1.49	11.10	87.40	33.35
2	22.19	7.27	70.54	38.09	3.12	58.79	1.31	12.94	85.75	38.06
3	22.49	8.43	69.08	44.21	2.34	53.46	0.21	14.79	85.00	45.72
4	25.74	8.99	65.27	49.25	1.81	48.95	0.14	16.45	83.41	51.25
5	28.33	9.21	62.46	51.14	1.65	47.21	0.03	17.92	82.06	53.64
					T = 298.15 K					
1	11.53	8.76	79.71	37.15	3.47	59.38	1.28	10.66	88.06	36.58
2	15.06	8.98	75.96	42.99	2.84	54.17	1.37	11.72	86.91	42.56
3	24.16	7.39	68.45	44.76	3.37	51.87	0.33	13.54	86.13	45.58
4	27.56	7.6	64.84	51.43	2.53	46.04	0.08	15.49	84.43	52.96
5	30.89	7.9	61.21	56.31	1.93	41.75	0.35	16.52	83.13	57.83

Table 4. Equilibrium Data for L64 (w_{L64}) + Ammonium Citrate (w_S) + Water (w_W) System from (278.15 to 298.15) K

system	Overall			top phase			bottom phase			TLL
	100 w_{L64}	100 w_S	100 w_W	100 w_{L64}	100 w_S	100 w_W	100 w_{L64}	100 w_S	100 w_W	
					T = 278.15 K					
1	12.73	29.24	58.03	19.76	25.54	54.71	0.28	37.47	62.25	22.84
2	13.91	29.79	56.30	26.83	22.73	50.44	0.65	38.45	60.91	30.54
3	16.2	30.81	52.99	38.79	17.83	43.38	0.50	41.13	58.38	44.83
4	17.45	31.28	51.27	42.10	17.27	40.63	1.08	43.55	55.36	48.71
					T = 288.15 K					
1	12.44	29.56	58.00	27.18	22.21	50.61	0.31	35.73	63.96	30.08
2	13.77	29.75	56.48	31.31	20.92	47.77	0.28	36.88	62.85	34.89
3	14.92	30.25	54.83	35.99	19.47	44.54	0.80	39.11	60.10	40.30
4	15.88	30.74	53.38	41.28	17.29	41.43	0.40	40.38	59.22	46.95
					T = 298.15 K					
1	11.19	28.86	59.95	16.04	27.19	56.76	0.40	35.42	64.17	17.67
2	12.48	29.52	58.00	24.80	23.75	51.45	0.26	36.29	63.45	27.55
3	13.69	29.78	56.53	33.17	20.18	46.65	0.14	37.82	62.03	37.45
4	14.88	30.29	54.83	38.25	18.81	42.94	0.41	38.74	60.85	42.76

The effect of temperature on the binodal position is shown in Figures 1 to 4. For the L64–$(NH_4)_3C_6H_5O_7$ ATPS, the temperature has an insignificant effect on the phase equilibrium compositions (Fig. 4). Nevertheless, a slight increase was observed in the biphasic area with the reduction of the temperature of the systems for L64–$Na_2C_4H_4O_4$, L64–$Na_2C_4H_4O_6$ and L64–$Na_3C_6H_5O_7$ ATPS, indicating that the phase separation process has an exothermic character. Although, most studies found in the literature show that ATPS phase separations are endothermic, Rodrigues et al. [26] have described ATPS composed by the copolymer L64 and sulfate salts as an exothermic phenomenon.

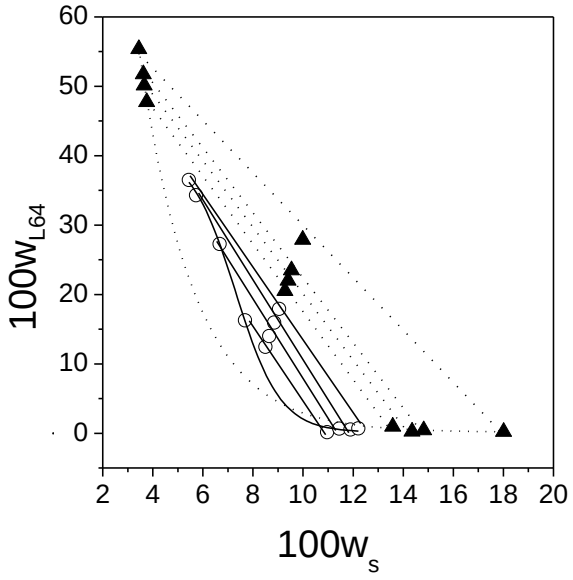

Figure 1. Temperature effect on the phase diagram for the L64 + Sodium Succinate system: ▲, 278.15 K; ○, 298.15 K.

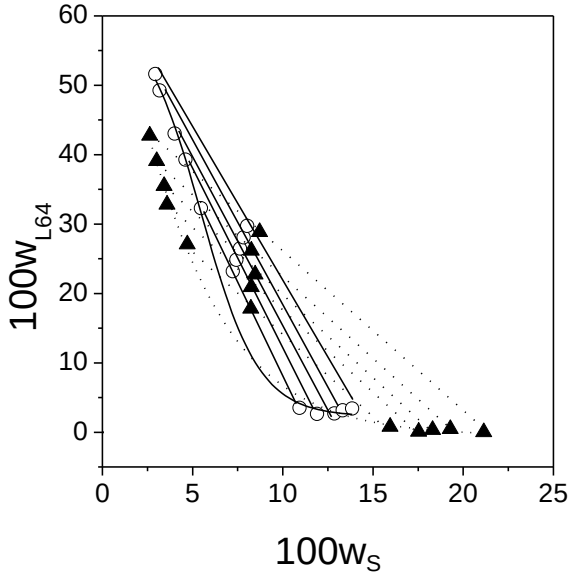

Figure 2. Temperature effect on the phase diagram for the L64 + Sodium Tartrate system: ▲, 278.15 K; ○, 298.15 K.

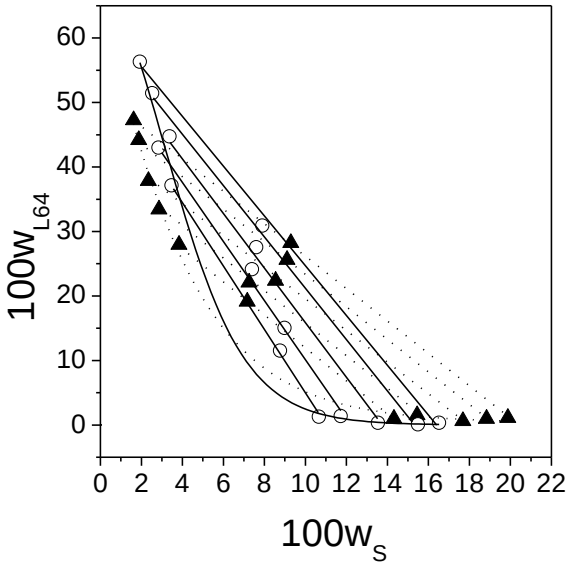

Figure 3. Temperature effect on the phase diagram for the L64 + Sodium Citrate system: ▲, 278.15 K; ○, 298.15 K.

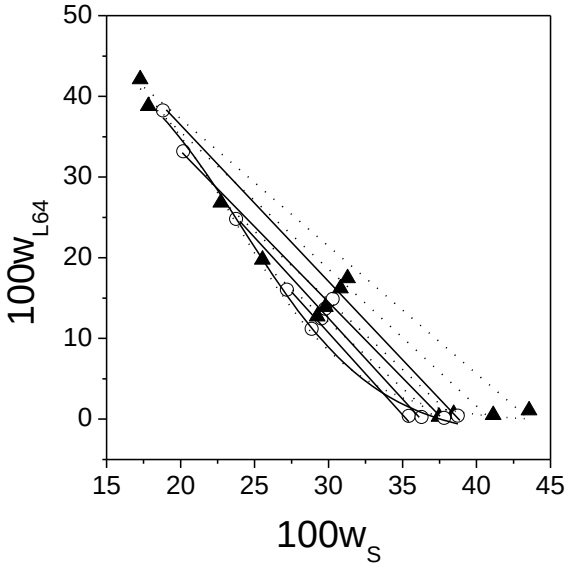

Figure 4. Temperature effect on the phase diagram for the L64 + Ammonium Citrate system: ▲, 278.15 K; ○, 298.15 K.

3.2. Microcalorimetric studies of ATPS formation: Calorimetric measurements were carried out by da Silva and Loh [34] to investigate and explain the ATPS formation process. According to the proposed model, when PEO and salts are mixed, the ions and the polymer interact, releasing solvation water molecules in a process driven by this increase in entropy. The ion binding continues as more electrolytes are added until it reaches a saturation point, after which no more entropy is gained and the phase splitting becomes more favorable. After this saturation point, the addition of more salt would lead to a higher concentration in the bulk than around the polymer. The proposition that phase separation occurs in association with the saturation of the electrolyte–polymer binding may sound contradictory to features of the binodal curves for aqueous two–phase systems, which highlights that at lower concentrations of one component, more of the other component is necessary to induce phase splitting. At this point, it is important to stress that this saturation does not mean physical saturation of the binding sites around the polymer, but that a significant amount of electrolyte is left in solution without interacting with the polymer, destabilizing the system, hence, leading to phase separation.

The microcalorimetric results obtained by da Silva and Loh [34] point out that the interaction between PEO and Na_2SO_4 (or Li_2SO_4) is an endothermic event, and the temperature increment should cause the interaction to be more favorable, leading to a decrease in the amount of salt necessary for phase splitting. To investigate why the phase separation process in L64–organic salts ATPS is exothermic, microcalorimetric measurements were taken to determine the enthalpy change associated with salt–L64 interaction. Figure 5 shows the titration curves obtained in our work, in which the observed apparent molar enthalpy of the interaction, ΔH_{ap-int}, for each injection, is plotted against the injection number. Because the extent of binding (*i.e.*, the number of ions adsorbed on the copolymer chain) is

not known, we cannot calculate the exact molar enthalpy change of the interaction, but instead only the apparent molar enthalpy change, ΔH_{ap-int}.

For the ammonium citrate ATPS salt concentration intervals examined in this work, the ΔH_{ap-int} values are in the range of -2.3 kJ mol^{-1} < ΔH_{ap-int} < -1.8 kJ mol^{-1}, which can explain the minor temperature effect on the phase separation behavior of this ATPS. Different from Li_2SO_4 and Na_2SO_4, the $(NH_4)_3C_6H_5O_7$ has an exothermic interaction with the L64 macromolecules, suggesting that a temperature decrease should make this interaction more favorable, leading to a decrease in the amount of salt necessary for phase splitting. However, this small magnitude of the energy of interaction should explain the insignificant effect of the temperature change on the phase separation process. For the other three salts, sodium citrate, sodium succinate and sodium tartrate, the ΔH_{ap-int} values are endothermic and are in the range of +6.0 kJ mol^{-1} < ΔH_{ap-int} < +21.0 kJ mol^{-1}. Despite these positive values of the interaction energy, the enthalpy change associated with the phase separation process, $\Delta_{p-s}H$, is exothermic for all sodium organic salts, and their values are $Na_2C_4H_4O_4$= – 3.0 kJ mol^{-1}, $Na_2C_4H_4O_6$= – 6.0 kJ mol^{-1} and $Na_3C_6H_5O_7$= – 3.0 kJ mol^{-1}. These negative energies of the phase separation processes are the cause of the decrease in the biphasic area following an increase in temperature.

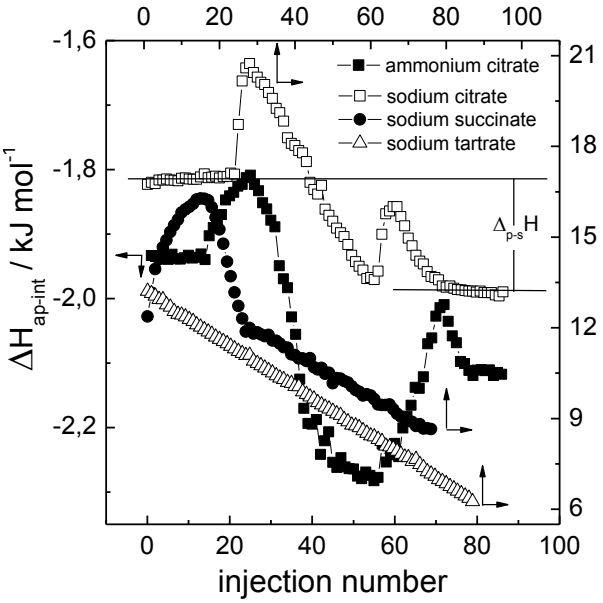

Figure 5. Apparent enthalpic interaction between L64 and different organic salts.

The influence of the temperature on the phase equilibrium also can be analyzed by applying the slope of the tie–line (STL) concept. The STL values, which are reported in Table 5, express the effect of the thermodynamic conditions on system composition. The STL can be calculated as shown by the following equation:

$$STL = \frac{C_P^T - C_P^B}{C_S^T - C_S^B} \quad (2)$$

where C_P^T and C_P^B are the polymer concentrations in the top and bottom phases, respectively, while C_S^T and C_S^B are those of the salt. As shown in Table 5, an increase in the temperature promotes an increase in the STL. Since the binodal position is not influenced significantly by temperature change, a possible explanation for this STL change is the diffusion process of water molecules from the top to the bottom phase, resulting in an increase in the copolymer concentration in the upper phase and a reduction in the salt concentration in the lower phase. This process can be clearly visualized in Table 6, which presents the effect of temperature on the concentration of water in both ATPS phases of some ATPS studied. As shown in Table 6,

the increase in temperature promotes a decrease of the content of water in the top phase and, consequently, an increase of the solvent concentration in the bottom phase.

Table 5. STL Values for L64 + salt + Water Systems

L64+ sodium succinate + water			
system	T/K		
	278.15	288.15	298.15
1	–4.77	–5.27	–4.94
2	–4.67	–5.07	–5.51
3	–4.58	–4.70	–5.47
4	–3.77	–4.51	–5.28
L64 + sodium tartrate + water			
system	T/K		
	278.15	288.15	298.15
1	–2.32	–4.08	–5.27
2	–2.33	–3.84	–5.02
3	–2.35	–3.71	–4.56
4	–2.37	–3.57	–4.54
5	–2.31	–3.53	–4.42
L64 + sodium citrate + water			
system	T/K		
	278.15	288.15	298.15
1	–2.57	–4.30	–4.96
2	–2.53	–3.74	–4.67
3	–2.43	–3.53	–4.33
4	–2.55	–3.35	–3.92
5	–2.53	–3.15	–3.82
L64 + ammonium citrate + water			
system	T/K		
	278.15	288.15	298.15
1	–1.61	–1.99	–1.83
2	–1.66	–1.95	–1.95
3	–1.65	–1.80	–1.88
4	–1.56	–1.78	–1.91

Table 6. Water content in mass percentage in top and bottom phases in function of temperature and tie–line length

T / K; TLL / % (w/w)	top phase water content / % (w/w)	bottom phase water content / % (w/w)
	sodium citrate	
278.15; 38.17	61.10	81.37
288.15; 37.51	57.79	84.65
298.15; 37.34	56.12	85.45
	sodium tartrate	
278.15; 34.29	63.72	83.02
288.15; 33.35	62.47	87.40
298.15; 36.58	59.38	88.06

Figure 6 presents the influence of anions on the phase diagram of the L64 + sodium salt systems at 278.15 K. For salts in which the cation is Na^+, the ability of the three anions to promote the formation of ATPS followed the order: citrate > tartrate > succinate. Because sodium citrate has the higher endothermic energy of interaction with L64 and its phase separation energy is not the most negative, we can conclude that the higher efficiency of sodium citrate in promoting the phase separation can be associated with the entropic aspect of the splitting phase process, i.e., the positive entropic change due to the increase of translational entropy of water molecules that are released during the biphasic systems formation. The cation effect on the binodal position is also shown in Figure 6. As can be seen, lower $Na_3C_6H_5O_7$ concentrations were required to generate an ATPS as compared to $(NH_4)_3C_6H_5O_7$, an indication that Na^+ cations are more capable of inducing ATPS formation than NH_4^+ cations. This observed behavior can be attributed to different interaction energies between cations and EO segments in the copolymer molecule. The L64–$Na_3C_6H_5O_7$ interaction energy is higher than that of the L64–$(NH_4)_3C_6H_5O_7$ apparent enthalpic interaction, which suggests that for the sodium organic salt, there is a large increase in the translational

entropy of water molecules released during the formation of such interactions, similar to the study by da Silva and Loh [34].

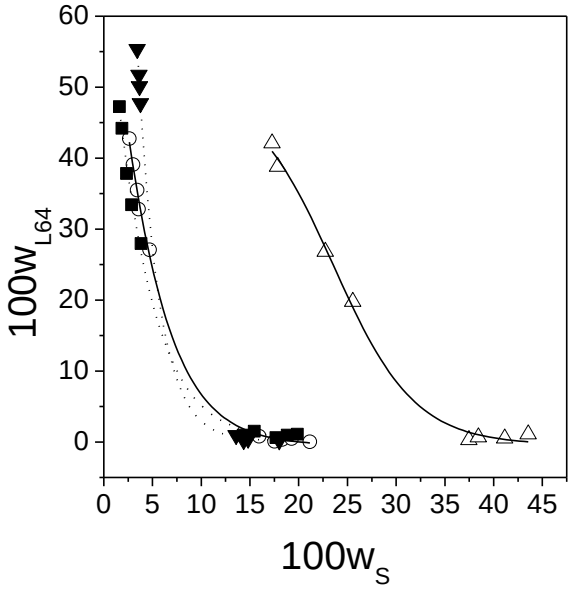

Figure 6. Influence of electrolytes on the phase diagram of the L64 + Salt systems at 278.15 K: Δ, Ammonium Citrate; ■, Sodium Citrate; ▼, Sodium Succinate; ○, Sodium Tartrate.

4. Conclusions

The liquid–liquid equilibrium data for the L64 + sodium succinate + water, L64 + sodium tartrate + water, L64 + sodium citrate + water, and L64 + ammonium citrate + water were obtained at T = 278.15, 288.15 and 298.15 K. The effect of temperature on the phase–forming ability for the system was also studied, and it was observed that the area of the biphasic region decreased slightly with an increase in temperature, except for the L64–$(NH_4)_3C_6H_5O_7$, in which the influence of temperature was insignificant. This temperature effect was attributed to the ion–L64 enthalpic interaction, which produced negative ΔH_{ap-int} values, demonstrating that a decrease in temperature favors this kind of interaction. An increase in the STL with increasing temperature was also observed. The ability of anions to

promote the phase separation of the studied ATPS followed the order: citrate > tartrate > succinate. Moreover, the salt $Na_3C_6H_5O_7$ was more capable of inducing ATPS formation than the salt $(NH_4)_3C_6H_5O_7$.

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Capítulo 3

Partitioning behavior of α-lactoalbumin and β-lactoglobulin in PEO – organics salts – H_2O ATPS: a termodynamic approach

Abstract

Partitioning behavior of α-lactoalbumin and β-lactoglobulin was studied in aqueous two-phase systems (ATPS) formed by poly(ethylene glycol) with an average molecular weight of 1500 g mol^{-1} (PEO 1500) and organics salts (sodium citrate, sodium succinate and sodium tartrate) in pH 4.0, at 298.15 K. α-la was concentrated in the phase rich in poly(ethyleneglycol), while β-lg was concentrated in the salt-rich phase of the systems studied. ATPS formed by PEO 1500 + sodium citrate + H_2O showed the best separation capability between the proteins. To explain the partition behavior of proteins, a simple model derived from the Flory-Huggins theory was used. In addition, the thermodynamical parameters ($\Delta_{tr}H°$, $\Delta_{tr}S°$ and $\Delta_{tr}G°$) associated with protein transfer from the bottom phase to the top phase were determined. The partitioning of α-la protein to the top phase was enthalpically driven mainly due to a specific interaction between PEO molecules and this protein. On the other hand, for the β-lg protein, the entropic contribution predominates as opposed to the enthalpic contribution, resulting in partition of this protein to the bottom phase of systems examined.

Keywords: α-lactoalbumin; β-lactoglobulin; aqueous two-phase systems; partition; purification; thermodynamical parameters

1. Introduction

Cheese whey is a co-product from cheese making which has been largely disposed of by industry. Because of its high organic content, cheese whey disposal constitutes a serious environmental problem mainly for its high chemical oxygen demand. In this respect, in the last years, many alternatives have been studied to develop new strategies that add value to whey [1,2].

The composition of whey is approximately 93 % water, 5 % lactose, 0.9 % protein, 0.3 % fat, 0.2 % lactic acid and small amounts of vitamins [3]. Within this context, whey proteins have received special attention for their excellent nutritional properties, potential biological activity and unique functional properties [3,4]. Among these proteins, α-lactalbumin (α-la) and β-lactoglobulin (β-lg) are the main constituents, and the interest in the development of new techniques for their separation and purification increased in the last years [5].

The common techniques used in purification of proteins are precipitation with salts or organic solvents and chromatographic techniques [6-8], wich are expensive and present enormous difficulties to be used in large-scale applications [9]. As an alternative to this problem, the use of aqueous two-phase systems (ATPS) in extraction and purification of these proteins has proved to be of great interest since the pioneering work of Albertsson in the mid 1960s [10].

The ATPS are systems composed of two immiscible phases, consisting of a mixture of aqueous solutions of two chemically distinct polymers [11,12] or a polymer and a salt [13,14] or two different salts [15] in critical thermodynamic condition. The phase split results in a polymer-enriched top phase and a salt-enriched bottom phase (or one enriched with the other polymer), or the reverse. Additionally, these systems present higher water content in both phases.

The high concentration of water in these systems (between 65 % and 90 % in mass) ensures biomolecules separation under mild conditions and a suitable environment, which allows for preservation of their structures. This fact makes ATPS highly advantageous when compared with traditional liquid extraction systems or any technique that use organic solvents.

In this sense, many works have been developed to study the partition behaviors of α-la and β-lg in ATPS [9,11,16-20], however without determining the thermodynamic driving force of this partition. In addition, to our best knowledge, there is no article reported in the literature that uses organic salt in ATPS to the extraction of α-la and β-lg. Since understanding how Thermodynamics controls this process is very important in the prediction of protein partitioning, the objectives of the proposed work were to evaluate the use of ATPS composed of polyethylene glycol (PEO 1500), salt (sodium citrate, sodium succinate or sodium tartrate) and water, in pH 4.0, as a separation technique for α-la and β-lg; and to obtain, for the first time, the thermodynamical parameters ($\Delta_{tr}H°$, $\Delta_{tr}S°$ and $\Delta_{tr}G°$) associated with protein partition processes in ATPS.

2. Experimental

2.1. Materials

Poly(ethylene glycol) with average molar mass (M_w) of 1500 $g \cdot mol^{-1}$ (PEO 1500) was purchased from Synth (Brazil). The analytical-grade reagents $Na_2C_4H_4O_6.2H_2O$ (sodium tartrate dihydrate) was obtained from Merck (USA), $Na_2C_4H_4O_4.6H_2O$ (sodium succinate hexahydrate) and $Na_3C_6H_5O_7.2H_2O$ (sodium citrate dihydrate) were obtained from Vetec (Brazil). α-lactoalbumin and β-lactoglobulin were purchased from Sigma (USA). All reagents

used were of analytical grade requiring no further purification. Distilled water was used throughout the experiments for the preparation of all aqueous solutions.

2.2. Preparation of the aqueous two-phase system

ATPS were prepared by mixing appropriate quantities of the aqueous solutions of polymer (PEO 1500) and salt (sodium tartrate or sodium succinate or sodium citrate), in centrifuge tubes, according to the global compositions desired. These global compositions data were obtained from literature [21,22]. The mixture was manually stirred, until the system became turbid. It was allowed to settle for a minimum of 24 h at 298.15 K in a temperature-controlled bath (Microquímica, MQBTC 99-20, with an uncertainty of ± 0.1 K) to reach thermodynamic equilibrium. The equilibrium state was characterized by the absence of turbidity in both top and bottom phases. The distilled water used to prepare the solutions was previously adjusted to pH 4.0 by addition of appropriate quantities of HCl solutions.

2.3. Determination of partition coefficient of proteins

After ATPS achieved the thermodynamic equilibrium, the top and bottom phases were collected with syringes. Subsequently, (2.50 ± 0.01) g of each phase were mixed with (0.100 ± 0.001) g of protein solution at 20.0 mg g^{-1} in a glass tube. The systems obtained were slowly mixed and left in the thermostatic water bath at 298.15 K for a minimum of 48 h to reach thermodynamic equilibrium. Then, aliquots of the top and bottom phases were collected. After adequate dilution, the protein concentration was determined in each phase by measuring the absorption at 280 nm, in a Shimadzu digital double beam spectrometer UV-2550. Diluted phases without the protein were used as blanks. Distilled water at pH 4.0 was utilized for all dilutions. All analytical measurements were performed in duplicate.

The partition coefficient (K_P) of the proteins (α-la or β-lg) between the two phases was defined as:

$$K = \frac{[P]_T}{[P]_B} \quad (1)$$

where $[P]_T$ and $[P]_B$ are the equilibrium concentrations of the partitioned protein in the top and bottom phases, respectively. The molar absorptivity of both proteins in the top and bottom phases was the same. The relative standard deviation for the K values was less than 10.0%.

2.4. Determination of the enthalpy of transfer

The enthalpy of transfer ($\Delta_{tr}H°$) was determined directly by isothermal titration microcalorimetry. This technique shows some advantages including the lower amount of reagent necessary for the experimental determinations.

Measurements were performed at a constant temperature of 298.150 ± 0.001 K by using a CSC-4200 microcalorimeter (Calorimeter Science Corp.) controlled by ItcRun software with a 1.82 mL reaction cell (sample and reference). The sample and reference cells were loaded with the upper phase or the lower phase of the systems. The titrations were carried out by consecutive injections (1 µL) of a 120.0 mg g^{-1} protein titrant solution with a gastight Hamilton syringe (25 µL), controlled by the instrument, with intervals of 10 min between each injection. The solution was titrated in the sample cell with stirring at 300 rpm using a helix stirrer. The whole calorimetric procedure was chemically and electrically calibrated by the heat of protonation of (tris(hydroxymethyl)aminomethane) and by the Joule effect as recommended [23].

The enthalpy of transfer ($\Delta_{tr}H°$) of the protein between the bottom and top phases, was defined as:

$$\Delta_{tr}H° = \Delta_{prot\text{-}dil}H_{top} - \Delta_{prot\text{-}dil}H_{bottom} \quad (2)$$

where, $\Delta_{prot\text{-}dil}H_{top}$ and $\Delta_{prot\text{-}dil}H_{bottom}$ are the enthalpy change of dilution of the protein solution in the top and bottom phase respectively.

3. Results and discussion

3.1. Influence of the tie line length (TLL) on partition behavior of α-la and β-lg

The TLL is an important thermodynamic parameter that expresses the difference between the intensive thermodynamic properties of ATPS phases. At constant pressure and temperature, it is dependent on the difference of salt and macromolecules concentration, in % (w/w), present in the bottom and top phases, respectively, and it is commonly used as a variable that determines the processes of solute partition. The difference between the intensive thermodynamic properties is enhanced with the increase in the TLL value, which is calculated by the following equation:

$$TLL = \sqrt{(C_P^T - C_S^B)^2 + (C_P^T - C_S^B)^2} \quad (3)$$

where C_P and C_S are the polymer and salt concentrations in % (w/w), and T and B indicate the top and bottom phases, respectively.

Generally, the unequal solute distribution increases as TLL values increase. This distribution of bioparticles between the ATPS phases is the result of an intricate and delicate balance of interactions between proteins and the ATPS components (polymers, water and electrolyte) present in the two phases that coexist in equilibrium [24]. In this work, the partition coefficient of proteins was determined in five different TLL for all ATPS, except for the PEO 1500 + $Na_2C_4H_4O_4$ + H_2O system, for which only four TLL were acquired and examined.

The partition behavior of the α-la and β-lg proteins in the system formed by PEO 1500 + sodium citrate + H_2O is presented in Fig. 1. As noted, the values of K_P for the α-la protein

were higher than K_P for the β-lg protein. In addition, it was observed that the partition coefficients of both proteins increase as TLL increases. In Fig. 2, similar results were observed for the other systems (PEO 1500 + sodium tartrate + H_2O and PEO 1500 + sodium succinate + H_2O) studied. The large difference between K_α and K_β values in the systems studied indicates the high potential of the PEO + organic salt + H_2O ATPS in the separation of these proteins.

These results are consistent with the literature [16,19], however Alves *et al.* [9] reported that the partition coefficient for β-lg shows a weak dependence on the PEO concentration in the system. Boaglio *et al.* [18] evaluated the influence of molecular mass (1000, 1450 or 3350 g mol^{-1}) and concentration of PEO, and pH (5.2, 6.2 or 8.2) in the partitioning behavior of milk whey protein in PEO + sodium citrate + H_2O ATPS. It was found that, as TLL increases, the values of partition coefficient for all proteins decrease, as opposed to the results obtained in this work and in all other literature data.

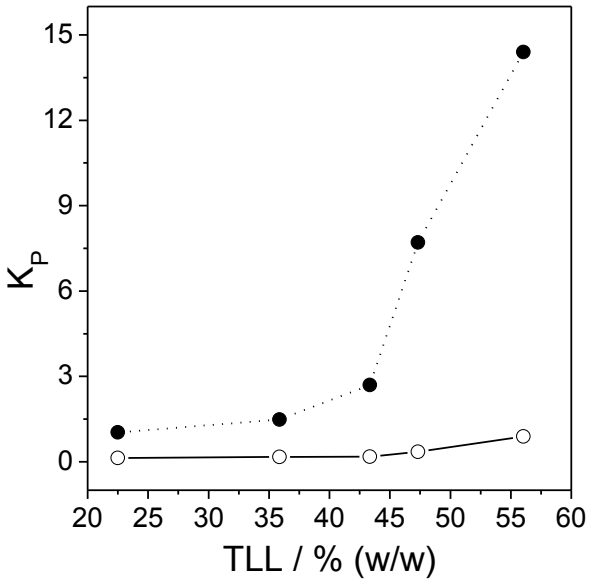

Fig. 1. Partition behavior of the proteins (●) α-la and (○) β-lg in ATPS formed by PEO 1500 + sodium citrate + H_2O, in pH 4.0, at 298.15 K.

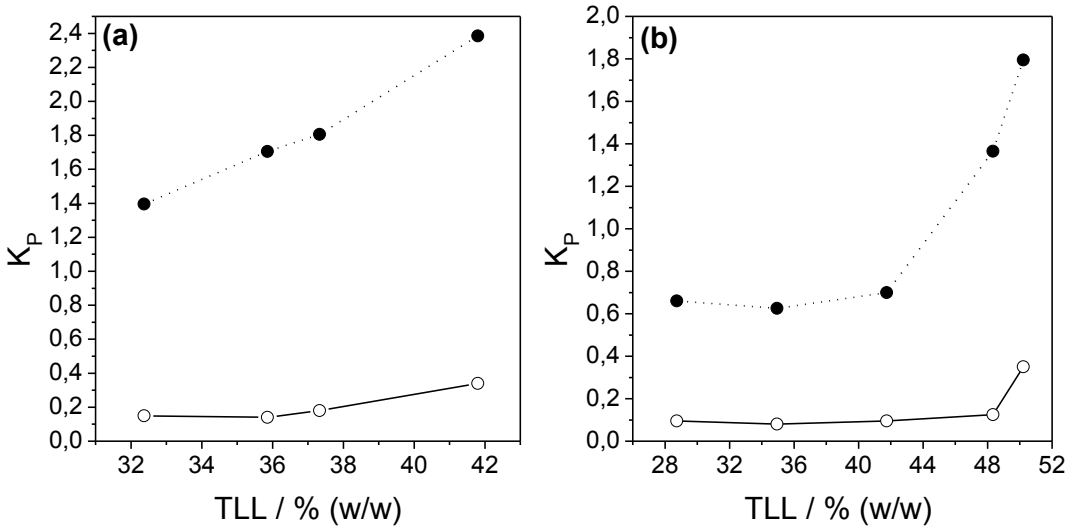

Fig. 2. Partition behavior of the proteins (●) α-la and (○) β-lg in ATPS formed by PEO 1500 + **(a)** sodium succinate and **(b)** sodium tartrate + H_2O, in pH 4.0, at 298.15 K.

To explain the partition behavior of proteins, a simple model derived from the Flory-Huggins theory was used. This model was developed by Haynes *et al.* [25] and consists of a set of analytical equations that express the partition coefficient of solutes, in ATPS, in terms of enthalpic and entropic contributions.

In the Haynes model the entropic contribution to the partition coefficient is predicted by equation (4):

$$\ln K_P = \frac{M_P}{\rho}\left(\frac{n^T}{V^T} - \frac{n^B}{V^B}\right) \tag{4}$$

where M_P is the molar mass of the partitioning solute (α-la or β-lg); ρ is the number of lattice sites per unit volume of the system; and n^T and n^B are the total number of molecules in the top and bottom phases, respectively, which, divided by the phase volume, V^T and V^B, produce the phase numerical density.

This equation shows that, in the absence of enthalpic effects, the solute is transferred to the phase with higher numerical density, where the number of molecules by unit volume is higher. Since, in all ATPS studied in this work, the phase with the higher numerical density is

the salt-rich phase (bottom phase), mainly due to the higher water content in this phase as shown in Table 1, according to equation (4) there must be a strong entropic force driving the solute to move into this phase. Thus, the proteins concentration in the bottom phase leads to an increase in the entropy of the system.

Table 1. Difference between H_2O concentration in the top and bottom phases of ATPS, at 298.15 K.

	$\Delta[H_2O] = [H_2O]_{\text{bottom phase}} - [H_2O]_{\text{top phase}}$ % (w/w)		
TLL	PEO 1500 + $Na_3C_6H_5O_7$	PEO 1500 + $Na_2C_4H_4O_4$	PEO 1500 + $Na_2C_4H_4O_6$
1	10.00	17.99	13.40
2	15.20	20.32	15.19
3	17.70	21.01	16.95
4	19.78	22.13	18.25
5	21.49	-	18.54

This prediction is in agreement with experimental data obtained in the transfer process of β-lg protein, whereby this protein remains in the salt-rich phase, but does not explain the partition of α-la protein to the top phase. Based on the Haynes's model, the α-la transfer to the polymer-rich phase could only be due to an enthalpic contribution, which is expressed by:

$$\ln K_P = -\frac{M_P}{RT}\left[\sum_{i=1(i\neq P)}^{m}\left(\Phi_i^T - \Phi_i^B\right)w_{iP} - \sum_{i=1(i\neq P)}^{m-1}\sum_{j=i+1(j\neq P)}^{m}\left(\Phi_i^T\Phi_j^T - \Phi_i^B\Phi_j^B\right)w_{ij}\right] \quad (5)$$

where Φ_i and Φ_j are the volume fraction of the ATPS-forming compounds (water, salt or polymer). The superscripts T and B refer to the top and bottom phases, respectively, and the subscript P refers to the partitioned solute (α-la or β-lg); w_{ij} is the effective pair-wise interchange energy defined according to equation (6):

$$w_{ij} = z\left[\varepsilon_{ij} - \frac{1}{2}(\varepsilon_{ii} + \varepsilon_{ij})\right] \quad (6)$$

where *z* is the number of nearest neighbors and ε_{ij} is the potential energy of one i-j pair.

In equation (5) there are two terms that reflect the enthalpic contribution to the partition of the solute. The first term $\left(\sum_{i=1(i\neq P)}^{m} \left(\Phi_i^T - \Phi_i^B \right) w_{iP} \right)$ represents an energetic contribution due to the difference in all interactions between the protein with each component present in the top and bottom phases. This term tell us that the proteins transfer is enthalpically more favorable to the phase which is enriched in component "i", with which protein molecules interact more strongly.

The double summation $\sum_{i=1(i\neq P)}^{m-1} \sum_{j=i+1(j\neq P)}^{m} \Phi_i^T \Phi_j^T w_{ij}$, in the second term, is the self-energy of the top phase and provides the total enthalpy in this phase formation (in the absence of partitioned solute) divided by the number of lattice sites in the top phase. Insertion of protein (α-la or β-lg) into a phase requires breaking interactions between the original components of the phase to create a cavity into which the protein fits. Although this process is dependent on the proteins volume, da Silva *et al.* [26] demonstrated that the phase self-energy does not contribute very much to the partitioning behavior of solutes.

So, the increase in K_P values as TLL increases for both proteins indicates that the protein-macromolecules interaction is more favorable than the protein-salt interaction, since the increase in TLL leads to an increase in the amount of the component with which proteins interact more favorably (macromolecules in the top phase). Thus, the transfer of α-la protein to the macromolecular-rich phase is attributed to enthalpic molecular interactions between this protein (α-la) and poly(ethylene glycol). However, the preferential permanence of β-lg in the bottom phase indicates that the entropic factor is more important that the enthalpic factor, and therefore, the partition behavior of β-lg protein is entropically driven.

3.2. Thermodynamical transfer parameters

Table 2 shows the thermodynamical parameters ($\Delta_{tr}H°$, $T\Delta_{tr}S°$ and $\Delta_{tr}G°$) obtained for α-lactalbumin and β-lactoglobulin proteins in ATPS formed by PEO1500 + $Na_3C_6H_5O_7$ + H_2O, PEO1500 + $Na_2C_4H_4O_4$ + H_2O and PEO1500 + $Na_2C_4H_4O_6$ + H_2O, in pH 4.0, at 298.15 K.

Table 2. Thermodynamical parameters obtained for α-lactalbumin and β-lactoglobulin in ATPS formed by PEO1500 + $Na_3C_6H_5O_7$ + H_2O, PEO1500 + $Na_2C_4H_4O_4$ + H_2O and PEO1500 + $Na_2C_4H_4O_6$ + H_2O, in pH 4.0, at 298.15 K.

Tie-line %	$\Delta_{tr}G°$ (kJ mol^{-1})		$\Delta_{tr}H°$ (kJ mol^{-1})		$T\Delta_{tr}S°$ (kJ mol^{-1})	
(w/w)	α-la	β-lg	α-la	β-lg	α-la	β-lg
PEO1500 + $Na_3C_6H_5O_7$ + H_2O						
22.49	-0.06	5.12	-1.42	-2.31	-1.36	-7.43
56.04	-6.61	0.29	-8.35	-8.13	-1.74	-8.42
PEO1500 + $Na_2C_4H_4O_4$ + H_2O						
32.37	-0.83	4.68	-5.22	-4.21	-4.39	-8.89
41.80	-2.16	2.68	-4.63	-6.45	-2.47	-9.13
PEO1500 + $Na_2C_4H_4O_6$ + H_2O						
28.73	1.03	5.85	-1.69	-2.43	-2.72	-8.28
50.21	-1.45	2.59	-5.22	-6.64	-3.77	-9.23

The free energy of transfer ($\Delta_{tr}G°$) is defined as the free molar energy associated with the protein transfer process from the saline phase to the polymeric phase and is calculated by the thermodynamic equation

$$\Delta_{tr}G° = - RTlnK \quad (7)$$

where R is the universal gas constant, T is the absolute temperature and K is the partition coefficient.

The Fig. 3 shows the relationships between variables $\Delta_{tr}G°$ and TLL for the ATPS studied. For both proteins transfer processes, a reduction in the free energy of the system was

observed with an increase in TLL; however for β-lg protein the transfer from the bottom phase to the top phase is an unfavorable thermodynamic process, i.e., $\Delta_{tr}G° > 0$.

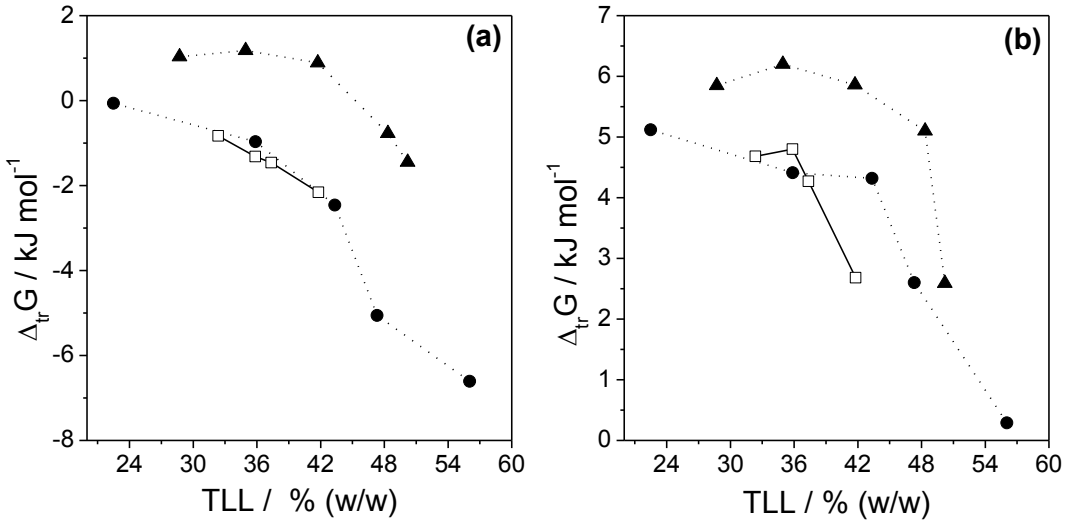

Fig. 3. Influence of the TLL on $\Delta_{tr}G°$ for **(a)** α-la and **(b)** β-lg in ATPS formed by PEO 1500 + (●) sodium citrate, (□) sodium succinate or (▲) sodium tartrate + H_2O, in pH 4.0, at 298.15 K.

Thermodynamically, $\Delta_{tr}G°$ could be divided in two contributions, an enthalpic ($\Delta_{tr}H°$) and another entropic ($\Delta_{tr}S°$). To investigate the contribution of the enthalpic molecular interaction to proteins transfer, $\Delta_{tr}H°$ was measured using the microcalorimetric technique.

The proteins enthalpy of transfer may be rationalized as a sum of six independent processes, according to:

$$\Delta_{tr}H° = \Delta_{S\text{-}W}H° + \Delta_{S\text{-}P}H° + \Delta_{P\text{-}W}H° + \Delta_{PEO\text{-}W}H° + \Delta_{PEO\text{-}P}H° + \Delta_{PEO\text{-}S}H° \quad (8)$$

where, $\Delta_{i\text{-}j}H$ is the enthalpy of interaction between the components i and j. The components are: protein (P), water (W), salt (S) and macromolecule (PEO). In Eq. (8), the term $\Delta_{P\text{-}W}H°$ can be ignored, since the number of protein-water interactions broken in the bottom phase are similar to the number of protein-water interactions formed in top phase. The same can be applied to the term $\Delta_{PEO\text{-}S}H°$, because the main difference in composition between the two phases are salt (enriched in bottom phase) and macromolecules (enriched in top phase), since

the number of macromolecule-salt interactions is negligible in both phases. So, the main contributions to proteins enthalpy of transfer are $\Delta_{S\text{-}P}H°$, $\Delta_{PEO\text{-}W}H°$, $\Delta_{PEO\text{-}P}H°$ and $\Delta_{S\text{-}W}H°$ with the first two terms positive, *i.e.* the system should absorb energy to break the protein-salt and water-macromolecule interactions in the proteins partition process from the bottom phase to the top phase. The last two terms are negative, since the system releases energy in formation of protein-macromolecule and water-salt interactions. Thus, $\Delta_{tr}H° < 0$ in the Table 2 means that $|\Delta_{PEO\text{-}P}H° + \Delta_{S\text{-}W}H°| > \Delta_{S\text{-}P}H° + \Delta_{PEO\text{-}W}H°$.

For most systems studied, as the TLL increases, the enthalpy of transfer for both proteins become more negative, thus rendering the transfer process more favorable. This favorable enthalpy, according to equation (5), has two contributions: one due to the molecular interaction between the protein and the components of each phase and the other due to the self-energy of the phases. Increasing the TLL, the PEO concentration increases in the upper phase that causes the rise in the number of molecular interactions between protein and the polymer, and therefore more energy is released. In addition, with the increase in PEO concentration, the self-energy of the upper phase also increases (less energy absorbed to separate the PEO-water bond than water-salt bond), favoring the partition of the proteins to this phase.

From the values of $\Delta_{tr}G°$ and $\Delta_{tr}H°$, it was possible to obtain the entropy change associated with this transfer process by using the classical thermodynamic relationship, $\Delta_{tr}G° = \Delta_{tr}H° - T\,\Delta_{tr}S°$ (9)

As noted in Table 2 the partitioning of proteins from bottom phase to top phase occurs with a decrease in the system's entropy, which is greater for the β-lg protein than for the α-la protein. Based on equation (4), this occurs because the proteins molecules move from a region with the highest numerical density (bottom phase) to another (top phase) where the numerical density is lower, leading to a decrease in the configurational entropy of the system.

Furthermore, this entropic force is more pronounced for solutes with higher molecular weight. Since the β-lg protein has higher molecular weight (18,300 g mol^{-1}) than the α-la protein (14,100 g mol^{-1}), the partition of the β-lg protein is more influenced by the entropic contribution.

In Table 2, for α-la protein, it is also important to note that, in PEO 1500 + $Na_3C_6H_5O_7$ + H_2O system, the value of the entropic term in equation (9) is almost constant for both TLL. However in PEO 1500 + $Na_2C_4H_4O_4$ + H_2O system, the $T\Delta_{tr}S°$ values decreases with an increase in TLL from 32.37 to 41.80 % (w/w). Besides the lower numerical density of the ATPS top phase, these different behaviors of the entropy reduction of the cited systems can be explained through other processes that can be occurring in the protein partition, such as: conformational change of the protein chain and configurational change of the ions in the protein electric double layer.

The partitioning of α-la protein to the top phase is enthalpically driven mainly due to a specific interaction between PEO molecules and the protein, which agrees with the observed negative values of $\Delta_{tr}H°$, *i.e.*, the enthalpic contribution predominates over the loss of configurational entropy caused by the lower numerical density of the top phase. On the other hand, for the β-lg protein, the entropic contribution predominates with regard to the enthalpic contribution, resulting in partition of this protein to the bottom phase of the systems investigated.

3.3. Effect of the electrolyte nature on the partition of proteins α-la and β-lg

The partition of α-la and β-lg proteins in systems formed by PEO 1500 + sodium citrate + H_2O, PEO 1500 + sodium tartrate + H_2O and PEO 1500 + sodium succinate + H_2O is shown in Fig. 4. As observed, the electrolyte nature strongly influences the partition coefficient values. For lower values of TLL, there is not significant difference between the

partition coefficients; however, for higher values of TLL, the K_P values become more distinct and the increase in the K_P values follows the order: K_P (tartrate) < K_P (succinate) ≈ K_P (citrate).

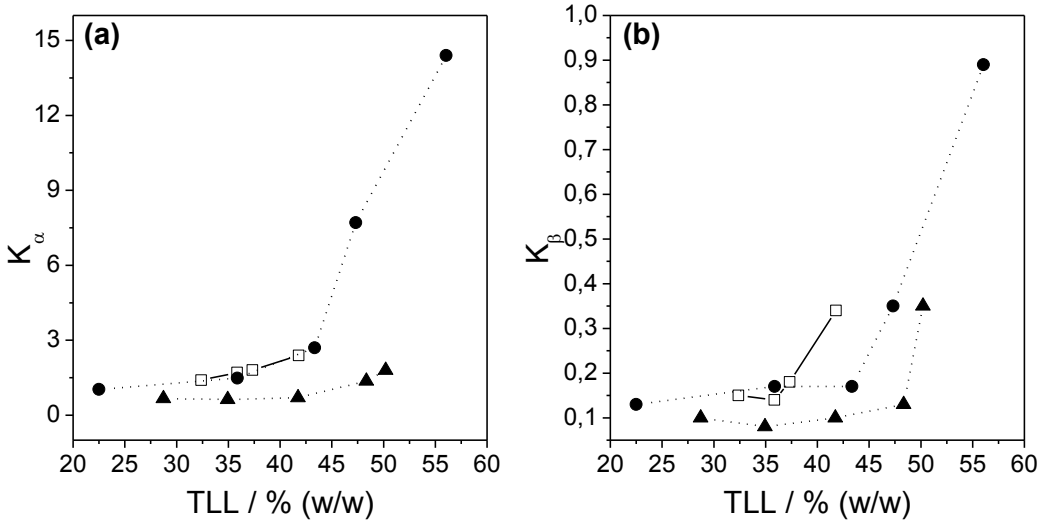

Fig. 4. Effect of the electrolyte nature on the partition of protein **(a)** α-la and **(b)** β-lg in ATPS formed by PEO 1500 + (●) sodium citrate, (□) sodium succinate and (▲) sodium tartrate + H_2O, in pH 4.0, at 298.15 K.

ATPS formed by PEO 1500 + sodium citrate + H_2O was the system that showed more favorable partition into the top phase.

According to Table 2, the α-la protein partition in the PEO 1500 + $Na_3C_6H_5O_7$ + H_2O system causes a smaller change in entropy. This shows that the protein transfer from the bottom phase to top phase is enthalpically driven and occurs with release energy of to the system. This energy is due to different intermolecular interactions of the protein when present in the top or in the bottom phases. So, the citrate-protein interaction is less enthalpically favorable than the succinate-protein interaction, which is less favorable than the tartrate-protein interaction, explaining the different partition behaviors.

4. Conclusion

Thermodynamic studies about the partitioning of two proteins, α-la and β-lg, used as model proteins, were performed in different ATPS, giving insights about the driving force of the proteins transfer process in ATPS. Based on calorimetric measurements, for α-lactalbumin protein, the transfer process was enthalpically driven, while for β-lactoglobulin, the partition was entropically driven.

The partition coefficient of proteins was strongly influenced by the nature of electrolyte and tie-line length. The ATPS tested can be utilized in the separation of α-lactalbumin and β-lactoglobulin since the results obtained in this work showed that α-lactalbumin tends to concentrate in the top phase, and β-lactoglobulin separates into the bottom phase, in all ATPS studied.

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