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EQUILÍBRIO DE FASES EM SISTEMAS AQUOSOS BIFÁSICOS: POLÍMERO/POLÍMERO E POLÍMERO/SAL

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Mestre em Engenharia de Alimentos

PARECER

Este exemplar corresponde à redação final da tese defendida por Luiza Helena Meller da Silva, aprovada pela Comissão Julgadora em 28 de abril de 2000.

Campinas, 28 de abril de 2000.

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ÍNDICE GERAL

Página

ÍNDICE DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

CAPÍTULO 4

CAPÍTULO 5

ÍNDICE DE FIGURAS

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

CAPÍTULO 4

CAPÍTULO 5

RESUMO

Neste trabalho foram determinados 15 diagramas de equilíbrio de fases líquido-líquido a 25 °C divididos em três tipos de sistemas aquosos bifásicos: polietileno glicol (PEG)/ maltodextrina (MD)/água, polipropileno glicol (PPG)/MD/água e PEG/fosfato de potássio/uréia/água . Nos sistemas polímero/polímero estudou-se a partição das proteínas α-Lactoalbumina (α-La), β-Lactoglobulina (β-Lg) e Albumina de Soro Bovino (BSA) e nos sistemas PEG/sal a partição das proteínas Lisozima, Catalase e β-Galactosidase. Os sistemas PEG/MD foram formados pela combinação de PEGs 1450, 8000 e 10000 e MDs 2000 e 4000. Nestes sistemas observou-se que a concentração de PEG na fase rica em MD foi muito baixa (< 1%), enquanto que a concentração de MD na fase rica em PEG foi relativamente alta (≈ 20%) quando comparadas à dextrana ou sal nos sistemas tradicionais PEG/Sal, PEG/Dextrana. Nos sistemas PPG/MD formados pela combinação de PPGs 400 e 3500 com MDs 1000 e 2000 a concentração de PPG na fase rica em PPG foi muito elevada (> 50%). Os sistemas PPG400/MD foram selecionados para estudar a partição das proteínas. Nos sistemas PEG/MD o coeficiente de partição (K) das proteínas β-Lg e BSA aumenta à medida que o comprimento da linha de amarração (CLA) aumenta, já para a-α-La K diminui quando o CLA aumenta. Nos sistemas PPG/MD a partição de BSA, α-La e β-Lg diminui com o aumento do CLA. Na maioria dos casos, nos sistemas PPG/MD, K foi maior que 1, mostrando a preferência das proteínas estudadas pela fase rica em PPG. A partir dos dados experimentais dos sistemas polímero/polímero, ajustou-se o modelo NRTL mantendo os parâmetros binários, comuns aos sistemas, constantes. O modelo, expresso em fração mássica, mostrou representar bem o equilíbrio líquido-líquido com baixos valores de desvio em relação aos dados experimentais. Os sistemas PEG/sal/uréia foram formados por PEG (1450, 3350 e 8000)/fosfato de potássio (pHs 7 e 9)/ uréia (6%). A partição foi estudada nas mesmas condições variando a concentração de uréia (3 e 6%). O efeito da adição de uréia no equilíbrio deslocou a curva binodal para concentrações maiores de PEG/sal. Na maioria dos casos a pH 9, K foi maior que 1 mostrando a preferência das proteínas pela fase rica em PEG. Em geral K diminui com o aumento do peso molecular de PEG e com o aumento da concentração de uréia.

SUMMARY

This is an experimental study conducted at 25 °C on the phase equilibrium behaviour of polyethylene glycol (PEG)/maltodextrin/water (MD), polypropylene glycol (PPG)/MD/water and PEG/potassium phosphate/urea/water systems. The partitioning behaviour of Bovine Serum Albumin (BSA), α-Lactoalbumin (α-La) and β-Lactoglobulin (β-Lg) were studied in the polymer/polymer systems and the partitioning behavior of Lysozyme, Catalase and β-Galactosidase were studied in PEG/salt systems. The PEGs (molecular weights 1450, 8000 and 10000) and MD (molecular weights 2000 and 4000) were combined to form the systems under analysis. In all systems the PEG concentration in MD rich phase is very small and the MD concentration in PEG rich phase is relatively high when compared to dextran or salt in systems formed with PEG/dextran or PEG/salt. PPG/MD systems were combinations of PPGs (molecular weights 400 and 3500) and MD (molecular weights 1000 and 2000). In all systems the PPG concentration in PPG rich phase is relatively high when compared to systems formed with PEG/dextran, PEG/MD or PEG/salt. In PEG/MD systems the partition coefficient for α-La and BSA decreased as the molecular weight of PEG increased. The partition coefficient for α-La were markedly higher than those for β-Lg in the PEG 1450 system. The partition coefficients for BSA were the lowest for all the PEG molecular weights studied in the present work. In the PPG/MD systems most of the cases the proteins partitioned preferentially to the top phase (K_p>1) wich is the PPG-rich phase. In the PPG 400/MD 1000 system the partition coefficients of α-La, β-Lg and BSA decreased as the TLL increased. The NRTL model was adjusted to the experimental data for the polymer/polymer systems, keeping constant the binary parameters that are common to all systems. This model, in mass fraction, described well the liquid-liquid equilibrium showing litlle deviation from experimental data. PEG/salt systems were combinations of PEGs (1450, 3350 and 8000), potassium phosphate (pH 7 and 9) and urea (6%). The lisozyme, β-Galactosidase and catalase partitioning were studied in the same conditions at two urea concentrations (3 and 6%). The addition of urea displaced the binodal curves towards higher concentrations of PEG/salt. In most cases at pH 9, the proteins partitioned preferentially to the top phase (K_p>1) wich is the PEG-rich phase. In general K decreased as PEG molecular weight and urea concentrations increased.

INTRODUÇÃO

A necessidade de pesquisar e desenvolver o campo de biosseparações reside no fato de que 50-90% do custo da produção de biocompostos fica por conta da técnica de purificação utilizada. Existe portanto a necessidade de uma técnica eficiente, efetiva e economicamente viável que apresente alta pureza e alta recuperação, mantendo intacta a atividade da molécula. Uma alternativa promissora de separação é aquela obtida com os Sistemas Aquosos Bifásicos (SAB) (Diamond and Hsu, 1992).

O fenômeno de separação de fases em sistemas aquosos foi observado pela primeira vez em 1896 pelo microbiologista holandês Beijerinck (Diamond e Hsu, 1988). Mas somente anos mais tarde, Albertsson (1960) descreveu sistemas aquosos novamente e investigou suas propriedades.

Quando pares de polímeros solúveis em água ou um polímero solúvel e um soluto de baixa massa molar são misturados e uma certa concentração é excedida, um sistema aquoso bifásico é formado. Devido à alta presença de água, estes sistemas formam um ambiente favorável a células, organelas celulares e proteínas biologicamente ativas (Albertsson, 1986).

Sistemas aquosos bifásicos diferem do tradicional sistema de extração líquido-líquido porque ambas as fases contém uma alta porcentagem de água (70 - 95%) (King *et alii*, 1988). Em concentrações tão altas de água, todos os sais e grupos ionizáveis presentes nas proteínas são dissociados em função do pH, dificultando estudos com relação a modelagem.

A extração nestes sistemas oferece vantagens sem igual para processos em grande escala, como alto rendimento de extração, forte aproximação do equilíbrio, fácil ampliação de escala e emprego em processos contínuos. Além disso, permite o uso de

equipamentos desenvolvidos para a extração líquido-líquido tradicional (Coimbra *et alii*, 1995; Coimbra *et alii*, 1998; Kula, 1990; Porto *et alii*, 1997).

O trabalho apresentado a seguir reúne artigos escritos e submetidos à publicação em revistas durante o desenvolvimento desta pesquisa. Como esta alternativa de redação de tese ainda é pouco utilizada optou-se por fornecer informações adicionais que facilitam o entendimento e esclarecem principalmente detalhes experimentais:

- ao final de cada capítulo estão apresentados, na forma de Anexos, os resultados não explicitados nos artigos, e

- nos Apêndices, ao final do trabalho, estão resultados das análises de umidade e dispersão dos polímeros e o pH das fases dos sistemas Polietileno Glicol (PEG)/Maltodextrina (MD) e PEG/fosfato de potássio.

OBJETIVOS

1. Construir diagramas de equilíbrio para diferentes sistemas aquosos bifásicos, compostos por PEG/MD/água, PPG/MD/água e PEG/fosfato de potássio/uréia/água;

2. Modelar os dados de equilíbrio dos sistemas polímero/polímero utilizando o modelo NRTL;

3. Determinar o coeficiente de partição (K) das proteínas α-Lactoalbumina, β-Lactoglobulina e Albumina de soro bovino nos sistemas polímero/polímero e Lisozima, β-Galactosidase e Catalase nos sistemas PEG/sal.

JUSTIFICATIVAS

A maltodextrina foi escolhida para substituir a dextrana nos sistemas polímero/polímero devido a seu baixo custo. Como ela é um polímero comercial produzido em grande quantidade seu valor comercial é muitas vezes menor que a dextrana (R$ 1,40/Kg MD; R$ 400,00/kg dextrana)

Estas proteínas foram selecionadas porque cobrem uma grande faixa de pesos moleculares (14.000 Da para lisozima a 250.000 Da para catalase) e diferentes propriedades de superfície (lisozima é altamente hidrofílica enquanto BSA é bem conhecida por ser uma proteína hidrofóbica). As proteínas do leite α-La e β-Lg foram selecionadas por já serem utilizadas em outras pesquisas no Laboratório de Separações Físicas (LASEFI).

O modelo NRTL foi adotado por ser muito utilizado em cálculos de engenharia e predizer bem o equilíbrio em sistemas aquosos bifásicos O modelo UNIQUAC não foi considerado devido aos parâmetros r e q necessários para o cálculo da parte combinatorial, já que os sistemas estudados foram compostos por polímeros com alta dispersão molecular. Este modelo é mais adequado para sistemas puros, com baixa dispersão.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1.1. TIPOS DE SISTEMAS AQUOSOS BIFÁSICOS

As Tabelas 1 e 2 apresentam uma lista extensa de sistemas aquosos bifásicos e seus componentes, desenvolvida por Albertsson (1986). Entre a variedade de sistemas aquosos bifásicos, os sistemas PEG/dextrana e PEG/ fosfato de potássio são os mais estudados para a partição de proteínas.

Tabela 1 – Classe dos SABs formados por dois polímeros.

Sistemas	
Polipropileno glicol	Metoxi-propilenoglicol
	Polietileno glicol
	Álcool polivinílico
	Polivinilpirrolidona
	Hidroxipropil dextrana
	Dextrana
	Hidroxipropil amido
	Maltodextrina
Polietileno glicol	Álcool polivinílico
	Polivinilpirrolidona
	Dextrana
	Ficoll
Metilcelulose	Hidroxipropil dextrana
	Dextrana
Etilhidroxietilcelulose	Dextrana
	Hidroxipropil amido
Hidroxipropil dextrana	Dextrana
Ficoll	Dextrana

Fonte: Albertsson, 1986.

Recentemente, devido às suas vantagens em comparação aos sistemas PEG/dextrana, a aplicação de sistemas PEG/sal vem crescendo em especial para processos de separação em grande escala. Além do menor custo e maior seletividade, a separação nestes sistemas ocorre mais rapidamente, devido à grande diferença de densidade e de viscosidade entre as fases, facilitando o emprego de extratores contínuos no processo (Lei *et alii*, 1990; Vernau e Kula, 1990; Greve, 1990; Cheluget *et alii*, 1994).

Tabela 2 – Classe dos SABs formados por um polímero e um soluto de baixa massa molar

Sistemas	
Polipropileno glicol	Fosfato de potássio
	Glicose
	Glicerol
Polietileno glicol	Fosfato de potássio
	Aminoácidos
	Mono/dissacarídeo
	Peptídeo/proteína
	Cloreto de sódio
Metoxi-propilenoglicol	Fosfato de Potássio
Dextrana	Alcool propílico

Fonte: Albertsson, 1986.

1.2. DIAGRAMA DE EQUILÍBRIO

Chamamos de diagrama de equilíbrio a representação utilizada para expressar as concentrações de um sistema de fases. Para os sistemas aquosos bifásicos (SAB), onde a região de interesse se concentra na parte do diagrama com elevado teor de água, a forma de eixos cartesianos que expressa apenas duas das concentrações do sistema é a mais utilizada.

Um diagrama de equilíbrio (Figura 1) é formado pelas linhas de amarração

(LA) e pela curva binodal, ambas determinadas pela análise química das composições do sistema. As linhas de amarração são obtidas através da união dos pontos que representam as concentrações dos componentes nas fases superior e inferior com o ponto da concentração mássica global do sistema (A, B, C). A curva binodal (D) divide o diagrama de equilíbrio em duas regiões, monofásica e bifásica e é traçada pela união dos pontos extremos das linhas de amarração. Chama-se de ponto crítico o ponto que divide a parte da binodal que reúne as fases ricas em polímero àquela parte contendo as fases rica em sal, no caso do sistema abaixo (Silva, 1994).

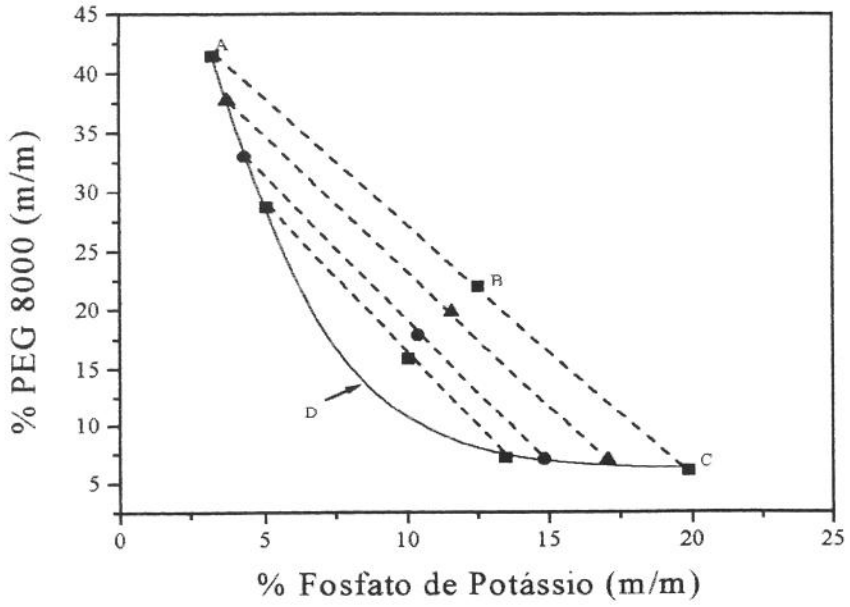

Figura 1 - Esquema representativo de um diagrama de equilíbrio (---) linhas de amarração, (—) binodal (Fonte: Silva *et alii*, 1997).

1.3. COEFICIENTE DE PARTIÇÃO

A base para a separação de substâncias com o emprego de sistemas aquosos bifásicos está na distribuição seletiva dos mesmos entre as fases formadas. O dado relevante neste caso é o coeficiente de partição da substância de interesse, definido na equação 1 pela

relação das concentrações dessa substância em mol/L nas fases superior (C_S) e inferior (C_I) respectivamente (Baskir *et alii*, 1988).

$$K = \frac{C_S}{C_I} \qquad (1)$$

Na separação de proteínas em sistemas líquidos, a pressão osmótica do sistema não precisa ser considerada. Neste caso, os sistemas compostos por um polímero e um sal têm sido mais utilizados, já que apresentam maior seletividade e menor custo em relação aos formados por dois polímeros (Kula, 1990; Chen, 1992; Coimbra *et alii*, 1995; Wang *et alii*, 1992).

O coeficiente de distribuição da substância de interesse depende principalmente, do sistema de fases escolhido (do seu diagrama de equilíbrio), da concentração da substância, da temperatura e do pH, sendo a seleção do sistema de fases o passo chave do trabalho de partição. Outros aspectos que afetam a partição são: tamanho da proteína, massa molar dos polímeros e concentração do sal utilizado etc.

A massa molar do polímero utilizado afeta diretamente a partição do biomaterial, alterando o diagrama de fases e mudando o número de interações polímero-proteína. Em geral, mantida todas as condições constantes, se a massa molar de um dos polímeros for aumentada, a concentração da biomolécula será maior na fase oposta, ou seja, a proteína é fortemente atraída por polímeros menores e repelida por polímeros maiores (Baskir *et alii*, 1988).

É bem conhecido que em sistemas PEG/dextrana o coeficiente de partição aumenta quando o peso molecular de dextrana é aumentado e diminui quando o peso molecular de PEG é aumentado (para proteínas com baixo peso molecular este efeito é

pequeno, não muito significativo) (Forcinit e Hall, 1991; Franco, 1992). A adição de sais nestes sistemas torna este efeito mais pronunciado devido à diferença de potencial eletrostático entre as fases, causada pela adição de sais, que se distribuem preferencialmente na fase rica em dextrana (Bamberger *et alii*, 1984).

Jonhansson *et alii* (1996) estudaram o efeito da adição de íons hidrofóbicos na partição de proteínas em sistemas PEG/copolímeros de PPG. Concluíram que a hidrofobicidade da proteína exerce uma forte influência no comportamento da partição e que a adição de íons hidrofóbicos aos sistemas direciona a partição para a fase de topo, que pode ser facilmente separada da fase pela técnica de indução de temperatura.

Na partição da biomolécula o efeito da temperatura geralmente é indireto e se manifesta através de mudanças na forma da curva binodal (diagrama de fases), de alterações na estrutura da proteína ou através de sua desnaturação, fatores que interferem na partição da mesma (Baskir *et alii*, 1988). Quando o coeficiente de partição da proteína é menor que 1, ele aumenta com o aumento da temperatura. O efeito da temperatura é mais pronunciado em proteínas com pequena massa molar do que em proteínas com alta massa molar (Forciniti *et alii*, 1991)

O estudo do pH é de fundamental importância para o conhecimento do comportamento de partição, pois sua variação acarreta mudanças complexas e ainda pouco conhecidas na estrutura e na carga da proteína. Adicionalmente, existem poucos trabalhos neste aspecto (Forciniti *et alii*, 1992).

Em geral o efeito do pH é maior quanto mais distante ele estiver do ponto isoelétrico (PI) da proteína (carga neutra), pois fora desta região a proteína possui carga (carga + < PI < carga -). O pH afeta a estrutura conformacional da proteína o que pode fazer com que a partição ocorra de diferentes formas ($K>1$ ou $K<1$) no mesmo sistema somente pela mudança de pH.

1.4. MODELOS TERMODINÂMICOS

Em um sistema bifásico em equilíbrio, o potencial químico (μ) de cada componente é igual nas duas fases. O problema de modelagem destes sistemas é justamente relacionar o potencial químico com o conjunto de fatores que interferem no equilíbrio. Apesar do progresso significativo que tem sido feito modelando termodinamicamente a distribuição de proteínas nestes sistemas, ainda faltam estudos fundamentais da partição da biomolécula entre as duas fases. E, em alguns casos, falta uma modelagem mais apropriada mesmo para os dados de equilíbrio do próprio sistema empregado (Baskir *et alii*, 1988).

Duas teorias vem sendo aplicadas com freqüência na modelagem de sistemas aquosos bifásicos: a teoria "Lattice" e a expansão osmótica do virial. A expansão osmótica de virial, derivada a partir do conhecimento da pressão osmótica de um solvente em solução, vem sendo usada em várias versões pelos mesmos autores (Kang and Sandler 1987; King *et alii*, 1988; Haynes *et alii*,1989; Grossmann *et alii*; 1993; Cabezas *et alii*, 1989) com bons resultados na descrição do comportamento de fases de sistemas aquosos bifásicos polímero/polímero e no coeficiente de partição de substâncias bioativas.

Como uma extensão destes modelos, Wu *et alii*, (1998) desenvolveram um modelo modificado de Pitzer, que incorpora o conceito das interações dos coeficientes de virial e pode predizer simultaneamente o equilíbrio líquido-líquido de uma série de sistemas aquosos bifásicos PEG/sal (para diferentes pesos moleculares de PEG) com uma boa precisão, correlacionando os parâmetros do modelo a partir dos dados das misturas binárias e ternárias no equilíbrio líquido-vapor.

Os modelos mais tradicionais usados para correlacionar e/ou predizer o equilíbrio líquido-líquido também vem sendo empregados com relativo sucesso em sistemas aquosos bifásicos. Eles podem ser divididos em: moleculares (NRTL - "Non-Random, Two-Liquid / Renon e Prausnitz, 1968; UNIQUAC - "Universal Quasi-Chemical" / Abrams e

Prausnitz, 1975, etc.) e de contribuição de grupos (ASOG – "Analytical Solution of Groups" / Kojima e Tochigi, 1979; UNIFAC – "UNIQUAC Functional Activity Coefficients" / Fredenslund *et alii*, 1975).

Para representar o equilíbrio de fases de sistemas aquosos polímero/polímero pode-se encontrar trabalhos na literatura empregando os modelos de Flory-Huggins (Walter *et alii*, 1985; Kang e Sandler, 1987), UNIQUAC (Kang e Sandler, 1987) e UNIFAC (Tan *et alii*, 1994). No entanto estes modelos quando utilizados para o cálculo do equilíbrio em sistemas Polímero/sal, não apresentam bons resultados para a predição de diagramas homólogos (variando somente peso molecular do polímero) ou necessitam utilizar parâmetros diferentes para sistemas homólogos de uma mesma série.

Outras alternativas de modelagem, em particular redes neurais, vem sendo propostas na literatura (Kan e Lee, 1996). Tais alternativas observam que para um sistema particular, um conjunto específico de parâmetros satisfaz o modelo termodinâmico, mas que sua utilização para predizer o equilíbrio de outro sistema nem sempre gera bons resultados. Deste modo, os autores concluem que um conjunto de parâmetros específico obtido para um sistema particular é válido somente para aquele sistema particular.

Mas é o modelo NRTL que vem sendo amplamente utilizado (Peng *et alii*, 1994; Peng *et alii*, 1995; Mishima *et alii*, 1995; Zafarani-Moatar and Salabat, 1998; Wu *et alii*, 1998; Wu *et alii* 1999) para a correlação de sistemas aquosos bifásicos com melhores resultados, a razão mais provável para isso talvez seja a utilização de três parâmetros para o cálculo do equilíbrio. As equações do modelo NRTL original, na forma apresentada por Novàck *et alii* (1987), são transcritas abaixo:

$$\frac{g^E}{RT} = \sum_{k=1}^{C} x_k \frac{C_k}{J_k} \qquad (2)$$

Onde o coeficiente de atividade é dado pela equação :

$$\ln \gamma_i = \frac{C_i}{J_i} + \sum_{k=1}^{C} \frac{x_k G_{ik}}{J_k} \left(\tau_{ij} - \frac{C_k}{J_k} \right) \tag{3}$$

Onde

$$C_k = \sum_{j=1}^{C} x_i G_{jk} \tau_{jk} \tag{4}$$

$$J_k = \sum_{j=1}^{C} x_j G_{jk} \tag{5}$$

$$G_{ij} = \exp\left(-\alpha_{ij} \tau_{ij}\right) \tag{6}$$

$$\tau_{ij} = \frac{A_{ij}}{T} \tag{7}$$

1.5. COMPONENTES DOS SISTEMAS

1.5.1. Polietileno Glicol (PEG) (α-Hydro-ω-hydroxypoly(oxy-1,2-etanodil)

Polímeros sólidos ou líquidos denominados polioxietilenos, com fórmula geral $H(OCH_2CH_2)_nOH$, onde n é maior ou igual a 4; são compostos de grande importância comercial e como tais, produzidos mundialmente em grande quantidade e em várias massas

molares. Arbitrariamente, a designação PEG é usada para materiais com baixas massas molares (abaixo de 20000), facilmente determinados pelos grupos hidroxilas, enquanto a designação PEO, óxido de polietileno, é frequentemente restrita para materiais com altas massas moleculares (maior que 100000), que não evidenciam a funcionalidade da hidroxila. Os materiais com alta e baixa massas moleculares se distinguem pelo método de preparo; PEG é feito da polimerização de óxido de etileno com um catalisador ácido ou básico, enquanto PEO emprega catalisadores heterogêneos. É difícil preparar PEG com massa molecular acima de 10000 pela fácil desidratação do terminal hidroxietileno, dando um alqueno na polimerização (Bamberger *et alii*, 1985).

Os polietileno glicóis com massa molecular acima de 10000 são frequentemente obtidos pela ligação de cadeias de baixa massa molar. Este processo de ligação pode introduzir uma ramificação ou um sítio hidrofóbico numa molécula, que certamente pode afetar suas propriedades moleculares. Polietileno glicóis são altamente higroscópicos e absorvem uma quantidade significativa de água se expostos a altas umidades.

Os PEGs são líquidos claros e viscosos ou sólidos brancos que se dissolvem em água formando soluções transparentes. Também são solúveis em benzeno, diclorometano e tetrafurano; com um pequeno aquecimento são solúveis em metanol, acetona, etanol e tolueno; são insolúveis em hexano e éter etílico (Bamberger *et alii*, 1985).

Os polietileno glicóis são moléculas relativamente estáveis e atóxicas. São muito utilizados na indústria alimentícia, cosmética e farmacêutica. São bastante estáveis na forma de solução, pó seco ou flocos, como geralmente são vendidos (em baixas massas moleculares têm a forma líquida ou de pasta) (Walter *et alii*, 1985).

As formas de análise de PEG vão desde a simples determinação do ponto de fusão à complexa espectroscopia de ressonância magnética nuclear (NRM). Mais recentemente vem crescendo o uso de técnicas como índice de refração e cromatografia

líquida (HPLC). Dependendo do fabricante, o polietileno glicol pode ser conhecido como: Poliglicol E, Carbowax, Pluracol E, Macrogol, PEG, Jeffox, Nycoline, Poly G ou Solbase.

1.5.2. Polipropileno Glicol (PPG)

Polímeros líquidos não voláteis e não corrosivos, com fórmula geral $CH_3CHOH(CH_2OCHCH_3)_nCH_2OH$. Comparáveis aos polietilenos glicóis, porém mais solúveis em óleo e substancialmente menos solúveis em água. Comercialmente são encontrados nas massas moleculares de 425, 1025, 2025 e 3500 mas somente os de baixa massa molecular são solúveis em água. São solventes para óleos vegetais, ceras e resinas. Usado em: fluídos hidráulicos, lubrificantes de borracha, agentes antiespumantes, adesivos, formulações de tintas (The Condensed Chemical Dictionary, 1956).

1.5.3. Maltodextrina

A maltodextrina é um produto obtido da fécula de mandioca, por hidrólise parcial enzimática e beneficiada através de processo tecnológico adequado. É classificada de acordo com a dextrose equivalente (DE), que é uma medida dos açúcares redutores presentes expressos como porcentagem da matéria seca. Quando a DE diminui, a solubilidade da maltodextrina também diminui. A Figura 2 apresenta sua estrutura química (Ninni, 1999):

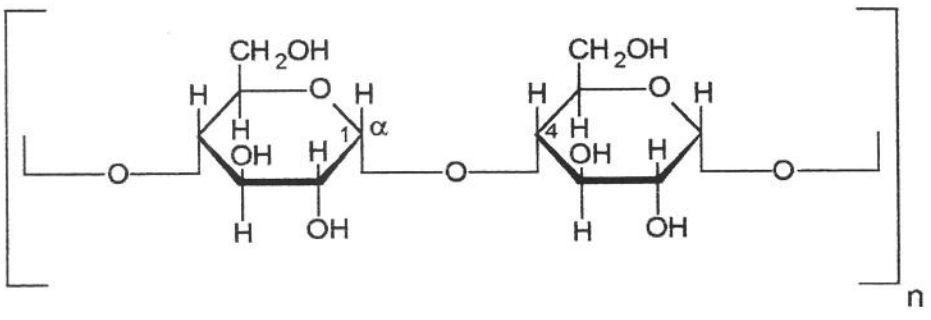

Figura 2 – Estrutura química da molécula de maltodextrina.

1.5.3.1. Maltodextrina LOREMALT 2030

É recomendada para aplicações alimentícias onde se deseja o aumento da concentração de sólidos solúveis sem ter um aumento da viscosidade da solução.

Na fabricação de embutidos encontra uma aplicação especial no processo de cura pelo aumento da concentração dos sólidos solúveis, diminuindo o tempo de cura, conferindo brilho e realçando o sabor. Aplicada no preparo de recheios de biscoitos e bolachas evita o desenvolvimento de arenosidade do açúcar. É recomendada no preparo de misturas de pós como veículo de vitaminas, aminoácidos e minerais, quando se deseja uma maior concentração de açúcares livres (Cia Lorenz - Fabricante).

Propriedades Físico-Químicas:	
pH (solução 10% à 25 °C)	4,7-5,2
Umidade máxima	5,0%
Dextrose equivalente	29,0 a 32,0

1.5.3.2. Maltodextrina LOREMALT 2001

Foi especialmente desenvolvida para aplicações alimentícias como formulações infantis, pós para preparo de bebidas, produtos dietéticos e energéticos, formulados sólidos, encapsulante de aromas, chocolate e derivados, panificação e produtos de confeitaria, biscoitos, sobremesas congeladas e produtos embutidos.

É usada como veículo de vitaminas, minerais e aminoácidos no preparo de formulações em pó, para regular o grau de doçura. Devido à sua característica de pó inerte, não interfere no sabor original, e em alguns casos pode até melhorar o tempo de prateleira do produto formulado, e, por suas características de solubilização e dispersão, fornece ao produto pronto para uso, uma alta concentração de sólidos dissolvidos. Na produção de aromas na

forma de pó, atua como agente encapsulante além de veículo estável. É aplicada no preparo de cremes para recheio e cobertura de tortas e recheio de biscoitos. Nas sobremesas e produtos congelados inibe a formação de grandes cristais de gelo. Em produtos como doce de leite e leite condensado inibe a cristalização da lactose (Cia Lorenz - Fabricante).

Propriedades fisico-químicas:	
pH (solução 10% à 25 °C)	4,7-5,2
Umidade máxima	5,0%
Dextrose equivalente	19,0 a 23,0

1.5.4. Fosfato de Potássio (Monobásico e Dibásico)

Fosfatos são sais inorgânicos, amplamente conhecidos. Em sistemas PEG/sal, são utilizados com o intuito de fornecer uma grande diferença de potencial eletrostático entre as fases, aumentando o coeficiente de partição de proteínas quando adicionadas ao sistema.

O fosfato de potássio monobásico, também chamado de fosfato diácido de potássio, tem massa molecular 136,09 e sua fórmula molecular é KH_2PO_4. Em solução a 5% e temperatura de 25°C seu pH fica entre 4,1-4,5. O fosfato de potássio dibásico anidro, tem massa molecular 174,18 e sua fórmula molecular é K_2HPO_4. Em solução a 5% e temperatura de 25°C seu pH fica entre 8,5-9,6 (MERCK INDEX, 1983)

1.5.5. Uréia

Possui fórmula química CH_4N_2O, massa molar 60,06 g/mol (C 20,0%, H 6,71%, N 46,65%, O 26,64%. É produto do metabolismo de proteínas. Solúvel em água (50g/100ml) e etanol (16g/100ml), praticamente insolúvel em clorofórmio. Usada em fertilizantes e rações animais como fonte de nitrogênio e na indústria de papel para amaciar a

celulose. Nomes comerciais: Carbamida, Aquacare, Aquadrate, Basodexan, Pastaron, Ureaphil, Nutraplus (MERCK INDEX, 1983).

1.5.6. Proteínas

Proteínas são compostos heteropoliméricos constituídos geralmente por combinações de 18 aminoácidos, podendo este número, contudo, ser superior a 20. Para que seja considerado proteína, um polímero de aminoácidos deve atingir um peso molecular mínimo arbitrário de 6.000 daltons (Da) e ter um limite superior de 1.000.000 de Da. Todas as enzimas até agora conhecidas, possuem massas molares que vão de 12.000 até 1.000.000 Da.

1.5.6.1. Albumina de Soro Bovino (BSA)

De acordo com sua estrutura e composição química, albuminas são proteínas solúveis em água e soluções salinas diluídas, com massa molar 67.000 Da. Desnaturam e precipitam facilmente com o calor.

O fenômeno de solubilidade de uma proteína deve ser visualizado como a capacidade de um número substancial de grupos polares localizados na superfície da mesma, se solvatar na água através de pontes de hidrogênio.

Mudanças de pH no meio, desde que não sejam extremas (± 1 ou 1,5 unidades) em torno do pH natural, causam desnaturação reversível nas estruturas terciárias da maioria das proteínas (Farfan, 1990).

A albumina, quando em pH entre 5 e 8, tem sua forma nativa (N); em pH 8 há uma transição da forma N para a forma B, que corresponde a um leve decréscimo em sua hélice. Acima de pH 9 e a baixas temperaturas, possui forma A (proteína desnaturada). Em

pHs ácidos ela agrega, forma E; 4 a 4,5 (abre parcialmente), abaixo de 4 (forma colóide). O coeficiente de partição da albumina tem um valor mínimo a pH 5,6 e seu ponto isoelétrico é igual a 4,6 (Forciniti *et alii*, 1992).

1.5.6.2. Lisozima

É uma proteína pequena, massa molar 14.300 Da, altamente hidrofílica e com ponto isoelétrico igual a 10,5. Tende a desnaturar em pH alcalino e altas temperaturas e a polimerizar a pH alcalino e baixas temperaturas. É muito estável - não muda sua conformação interna- em pHs entre 1,2-11,3 em soluções diluídas de sal a temperatura ambiente (Forciniti *et alii*, 1992).

1.5.6.3. Catalase

É uma proteína grande, massa molar 250.000 Da, hidrofóbica e com ponto isoelétrico igual a 5,6. Dissocia-se em subunidades em pH alcalino e temperatura ambiente. Perde sua atividade em pH alcalino devido à dissociação parcialmente reversível em moléculas de menor peso molecular; uma molécula de catalase se quebra em 3 ou 4 subunidades em pH acima de 10 (Forciniti *et alii*, 1992).

1.5.6.4. β-Galactosidase (Lactase)

Catalisa a hidrólise da lactose, açúcar do leite, em glicose e galactose e na indústria de alimentos é empregada para evitar a cristalização da lactose nos tratamentos de leites e derivados. Pode ser utilizada para transformar o soro de queijo em xarope de lactose, que pode servir como substituto de xaropes de sacarose, glicose e milho para uso na panificação, refrigerantes, laticínios e sobremesas. De origem microbiana é formada

intracelularmente em leveduras e bactérias e excretada por fungos, por isso sua massa molecular varia de acordo com a fonte (90.000 a 270.000 Da) (Silva, 1999).

1.5.6.5. α-Lactoalbumina (α-la)

A α-la é susceptível ao tratamento térmico, desnatura-se em pH 6,7 a 65°C, com reversibilidade de 80 a 90% sob resfriamento. Estruturalmente assemelha-se à lisozima do ovo (Morr & Ha, 1993).

Tabela 3 - Propriedades físico-químicas da α-la (Eigel *et alii,* 1984)

Massa molar (daltons)	14.000
Ponto isoelétrico (PI)	4,2-4,5
Hidrofobicidade média (kcal/resíduo)	1020
Total de aminoácidos (mol)	123
Resíduos apolares (mol)	44
Resíduos de cisteína (mol)	8
Resíduos de disulfito (mol)	4
Resíduos de lisina (mol)	12
Resíduos de ácido glutâmico (mol)	8
Resíduos de ácido aspárgico (mol)	9

1.5.6.6. β-Lactoglobulina (β-lg)

A β-lg é sensível à temperatura e ao pH, desnaturando reversivelmente em temperaturas abaixo de 65°C, com extensa mudança conformacional. Entre pH 5,2-7,5 existe como dímero, entre 3,5-5,2, polimeriza-se como um octômero e abaixo de 3,5 ou acima de 7,5 dissocia-se nos monômeros correspondentes (Morr & Ha, 1993).

Tabela 4 - Propriedades físico-químicas da β-lg (Eigel *et alii*, 1984)

Massa molar (daltons)	18.000 monômero, 36.000 dímero
Ponto isoelétrico (PI)	5,2
Hidrofobicidade média (kcal/resíduo)	1075
Total de aminoácidos (mol)	162
Resíduos apolares (mol)	54
Resíduos de cisteína (mol)	5
Resíduos de disulfito (mol)	2
Resíduos de sufidrila (mol)	1
Resíduos de lisina (mol)	15
Resíduos de ácido glutâmico (mol)	16
Resíduos de ácido aspárgico (mol)	10

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CAPÍTULO 2

PHASE EQUILIBRIUM IN POLYETHYLENE GLYCOL/MALTODEXTRIN AQUEOUS TWO-PHASE SYSTEMS

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ABSTRACT

The polysaccharide maltodextrin (MD) can provide a low cost alternative to the fractionated dextran for the use with polyethylene glycol (PEG) in aqueous two-phase, two-polymer systems. The physical characteristics of these MD/PEG systems are similar in many respects to dextran/PEG systems. These systems' behaviour was studied at 25 °C and local atmospheric pressure (727 mm Hg) under several concentration ratios and molecular weights of MD/PEG. Molecular weight of PEG was 1450, 8000 and 10000 and MD molecular weight was 2000 and 4000. This paper establishes phase diagram data, subsequently adjusting the NRTL model for the calculation of activity coefficients. The results are satisfactory and show the good descriptive quality of the model.

Keywords :
Liquid-liquid equilibrium, aqueous two-phase systems, maltodextrin, PEG, NRTL

2.1. INTRODUCTION

Aqueous two-phase systems can be formed by combining either two "incompatible" polymers or a polymer and a salt in water, above a certain critical concentration (Szlag *et alii*, 1990). Many authors have tested systems like that and determined their phase diagrams (Kula, 1979; Walter, 1985, Snyder *et alii*, 1992; Cheluget *et alii*, 1994; Silva *et alii*, 1997). Comprehensive reviews have been compiled by Albertsson (1986) and Zaslavsky (1995). PEG/salt systems have been used in large scale for protein separation. These systems are attractive because of low-cost, rapid phase disengagement and the availability of commercial separators, which allow faster continuous protein separation (Coimbra *et alii* 1994, 1995, 1998). Polymer/salt systems, on the other hand, are not very selective, can damage fragile proteins and in some cases, when high salt concentration is used, became a waste disposal problem. In contrast polymer/polymer systems can be more selective

by incorporating appropriate ions or ligands in the system. The most common polymer/polymer system is composed by dextran and PEG, but this system is very expensive for scale up. This problem can be solved by the use of alternative polymers (Szlag *et alii*, 1990, Atkinson and Johns, 1994; Christian *et alii*, 1998).
Here the possibility of using low-cost starch derivatives (maltodextrin) as replacements for dextran was investigated.

Several attempts have been made in the literature to describe the liquid-liquid equilibrium in aqueous two-phase systems (Kang and Sandler, 1987; Wu *et alii*, 1996; Wu *et alii*, 1998; Wu *et alii*, 1999). The thermodynamic description of this phase equilibrium is important as it could provide the basis for extrapolating experimental data and predicting phase compositions when such data are not available. The thermodynamic representation of phase equilibrium could also aid the design and process optimisation of the aqueous two-phase extraction technique. In this paper the NRTL model for the activity coefficients was used to correlate the equilibrium data for PEG/MD systems.

2.2. MATERIALS AND METHODS

MD 2000 and 4000 (commercial name Loremalt 2030 and Loremalt 2001 respectively) were kindly supplied by Companhia Lorenz (Blumenau, SC, Brazil). Polyethylene glycols 1450, 8000 and 10000 were purchased from SIGMA.

The polymers were analysed by Gel Permeation Chromatography (GPC) in a Waters chromatograph using the following experimental conditions: water as the movel phase at a rate of 0.8 µl/min, injection temperature of 40 °C, refraction index detector, sample injection 100 µl. This methodology was used in order to obtain the polymers' molecular distribution and polydispersity index (Mw / Mn). The results are shown in Table 1.

The water content of each polymer was determined through Karl Fisher titration using a Metrohm equipment (Swiss). The water content was taken into account for preparing the stock solutions.

Table.1 – Polymer characterisation by GPC.

Product*	Average Molecular Weight	Polydispersity Index (Mn/Mw)
Loremalt 2030	2017	1.31
Loremalt 2001	4004	1.98
PEG 1450	1468	1.03
PEG 8000	8768	1.09
PEG 10000	11589	1.10

*Commercial Name

2.2.1. Phase diagrams

Stock solutions were prepared by the addition of Milli Q water to a known quantity of polymer containing (55g MD / 100g total and 50g PEG / 100g total). The diagrams for PEG (1450, 8000 and 10000) and MD (2000 and 4000) were determined in centrifuge tubes. Mixtures consisting of known weights of polymer stock solutions were prepared on an analytic balance (A200 S Sartorius, Germany), accurate to 0.0001g. Typically 15g of a system were prepared. Systems were mixed for 10 minutes and then centrifuged (BR4i model, Jouan, France) at 2900 g for 40 minutes at 25 °C. The tubes were brought to equilibrium in a thermostatic bath (Viscotherm VT2, Physica, Germany) at 25 °C ± 0.1 and local atmospheric pressure (727 mm Hg) for 5 hours. After this treatment, the two phases became clear and transparent, and the interface was well defined. Samples of the two phases were analysed.

2.2.2. Analysis of phase compositions

The phase compositions were determined using a combination of polarimetry and freeze drying, according to the methodology suggested by Christian *et alii* (1998) for a polysaccharide/PEG system. The MD concentration was determined by polarimetry (Carl Zeiss Jena, POLAMAT A model, equipped with a mercury lamp at 546 nm, Germany), since PEG is optically inactive. The water concentration was determined by freeze drying (EZ DRY model, FTS Systems, New York, USA) at -54 °C and 100 m Torr for 48 h. The PEG concentration was determined by difference. The PEG concentration was further checked by refratometry (Polskie Zaklady Optyczne (PZO), Minska 25, 03-808 Warszawa-RL3, Germany). The average standard deviations of the phase concentrations (analysed in triplicate) were ± 0.004% for water, ± 0.006% for MD and ± 0.007% for PEG.

2.3. RESULTS

Several equilibrium diagrams were built for the system PEG/MD containing different polymer's molecular weight. The PEG 1450/MD 2000 mixture does not form aqueous two-phase system at the concentrations selected here. All the results are expressed as weight percentage. Four tie lines were determined for each polymer combination, except for the system PEG 1450/MD 4000, for which only three tie lines were determined. The experimental compositions for all systems are given in Tables 2-3.

The complete diagram for the PEG 1450/MD 4000 system at 25 °C is shown in Figure 1. It can be seen that good linear fittings were obtained for the experimental data. The tie lines were determined by linear regression of each corresponding set of total, bottom phase, and top phase concentrations. The tie lines compositions were confirmed, within an experimental error of 3%, by performing mass balances on the top and bottom equilibrium compositions to determine the amounts of PEG and MD used to generate the total mixture.

These mass balance deviations are in good agreement with results reported in the literature for other systems (Silva *et alii*, 1997; Albertsson, 1986; Cheluget *et alii* , 1994; Snyder *et alii*, 1992).

Table 2 – Phase compositions for PEG/MD 2000 systems

System	Total system (% w/w)			Top phase(%w/w)			Bottom phase (%w/w)		
	MD	PEG	Water	MD	PEG	Water	MD	PEG	Water
PEG 8000/MD 2000	34.80	11.22	53.97	28.65	15.59	55.75	50.42	0.81	48.75
	36.81	12.97	50.21	27.19	18.57	54.23	54.99	0.60	44.40
	38.78	15.05	46.15	24.36	25.42	50.20	59.21	0.53	40.25
	40.50	16.69	42.80	23.27	29.08	47.64	63.87	0.89	35.23
PEG 10000/MD 2000	34.63	10.72	54.64	27.50	14.64	57.84	50.59	0.45	48.94
	35.77	12.38	51.84	25.61	18.04	56.34	54.99	0.73	44.27
	38.79	15.15	46.05	23.47	26.09	50.42	59.23	0.91	39.84
	40.26	16.81	42.91	22.47	29.60	47.92	60.91	0.90	38.17

Table 3 – Phase compositions for PEG/MD 4000 systems

System	Total system (%w/w)			Top phase (%w/w)			Bottom phase (%w/w)		
	MD	PEG	Water	MD	PEG	Water	MD	PEG	Water
PEG 1450/MD 4000	33.82	10.96	55.21	30.86	12.68	56.44	43.48	5.92	50.59
	36.18	12.54	51.26	27.18	17.76	55.04	50.46	4.31	45.21
	43.18	13.75	43.05	25.91	24.59	49.49	61.42	2.29	36.28
PEG 8000/MD 4000	34.98	11.02	53.99	18.55	18.84	62.60	53.54	0.00	46.45
	36.71	13.23	50.04	17.70	23.15	59.13	58.12	0.00	41.87
	38.93	15.05	46.00	15.43	30.19	54.37	60.15	0.00	39.84
	40.11	16.81	43.06	14.97	36.53	48.49	63.91	0.12	35.96
PEG 10000/MD 4000	34.02	10.61	55.36	18.15	18.75	63.08	53.13	0.00	46.86
	35.77	12.52	51.69	17.31	23.02	59.66	57.60	0.00	42.39
	37.84	14.55	47.60	15.17	30.14	54.68	58.11	0.04	41.84
	39.86	16.43	43.70	14.56	32.70	52.72	63.40	0.50	36.08

For most systems the PEG concentration in bottom phase is very small, and in some cases PEG is almost excluded from this phase. Similar results were reported by Szlag *et alii* (1990). The only exception in this behaviour was observed for the PEG 1450/MD 4000 system. All PEG/MD systems were characterised by the presence of considerable quantities of MD in the top phase. This quantity increased markedly as the molecular weight of the polymers decreased. The same behaviour was observed by Atkinson and Johns (1994). In all systems studied in the present work the MD concentration in top phase is relatively high when compared to dextran or salt in systems formed with PEG/dextran or PEG/salt (Albertsson, 1986; King *et alii*, 1988; Silva *et alii*, 1997; Zaslavsky, 1995).

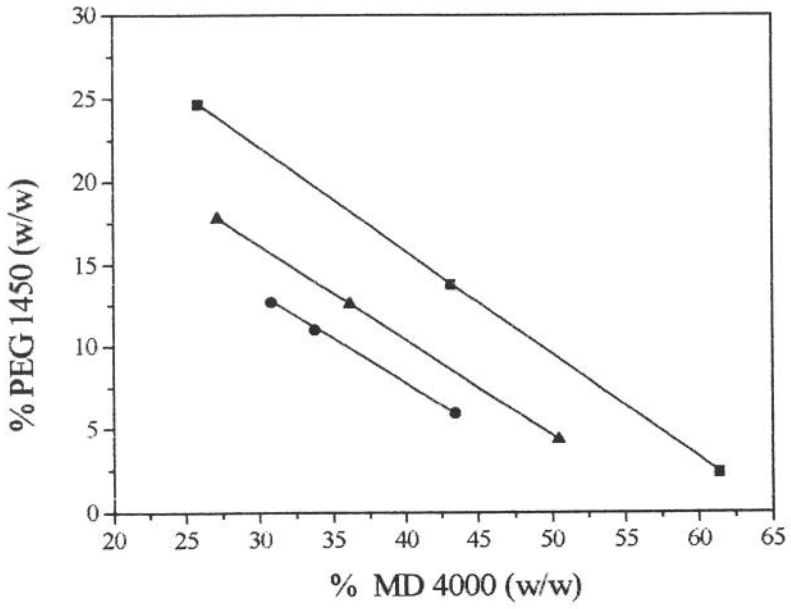

Figure 1 - Experimental tie lines for the system PEG 1450/MD 4000 at 25 °C

The concentration of MD required to form two phases with PEG is much greater than that of dextran or salt. The systems with the lowest PEG molecular weight required particularly large concentrations of both polymers to exhibit phase splitting. For this reason more concentrated stock solutions (PEG 55 %, MD 60%) were used in this case.

Retrogradation of MD has been reported to be a further disadvantage of its use as a phase-forming aqueous two-phase system polymer (Szlag *et alii*, 1990). In this work, no retrogradation was observed due to the relatively low MD molecular weight. MD is produced by Companhia Lorenz using a combination of both acid and enzymic hydrolysis. Such processing strategy reduces the tendency to retrogradation (Atkinson and Johns, 1994). This gives to the low molecular weight MD a distinct cost advantage over other specifically modified starch derivatives, used to retard retrogradation.

Figure 2 shows the binodal curves for the PEG/MD 4000 systems. These curves were determined by fitting a sigmoidal (Boltzmann) equation to the experimental data. The decrease in PEG molecular weight caused a decrease on the two-phase region, a behaviour already reported in the literature for similar systems (Szlag *et alii*, 1990, Albertsson, 1986). The advantage of using less PEG of higher molecular weight is, however, offset by the higher viscosity of such solutions.

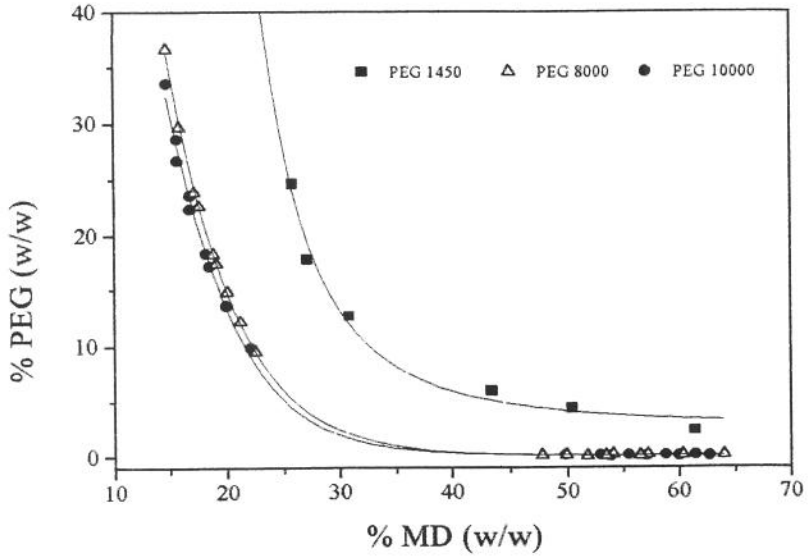

Figure 2 - Binodal curves for the systems PEG/ MD 4000 at 25 °C

2.4. MODELING

The experimental tie line data were utilised in adjusting the parameters of the NRTL model for the activity coefficients. The weight fraction was used as the unit of concentration due the large difference in molecular weights between the components in the systems. The same procedure was suggested by Oishi and Prausnitz (1978) for modelling solvent activities using the UNIQUAC and UNIFAC models.

The equation 1 shows the NRTL model expressed in weight fraction:

$$\ln\gamma_i = \frac{\sum_j^C \frac{\tau_{ji} G_{ji} w_j}{M_j}}{\sum_j^C \frac{G_{ji} w_j}{M_j}} + \sum_{j=1}^C \left[\frac{w_j G_{ji}}{M_j \sum_k^n \frac{G_{kj} w_k}{M_k}} \left(\tau_{ij} - \frac{\sum_k^C \frac{\tau_{kj} G_{kj} w_k}{M_k}}{\sum_k^C \frac{G_{kj} w_k}{M_k}} \right) \right] \tag{1}$$

where

$$G_{ij} = \exp\left(-\alpha_{ij}\tau_{ij}\right) \tag{2}$$

$$\tau_{ij} = A_{ij}/T \tag{3}$$

$$\alpha_{ij} = \alpha_{ji} \tag{4}$$

Following the procedure developed by Stragevitch and d'Avila (1997), adjustments of the parameters were made by minimisation of the maximum likelihood objective function. Table 4 shows the parameters estimated from the experimental data.

Table 4 – Adjusted parameters of the NRTL model.

Parameters	A_{ij} (K)	A_{ji}(K)	$\alpha_{ij} = \alpha_{ji}$
12[a]	-1415.9	2876.7	0.27937
13	-4800.2	-548.03	0.42858
14	-4419.3	-188.24	0.46957
23	-1588.0	-3997.9	0.20001
24	695.67	-3095.5	0.29902
52	-1449.4	3059.30	0.27937
53	-4441.1	-148.26	0.42858
54	-2129.7	-656.49	0.47000
56	-3228.3	-150.42	0.47000
62	-5000.1	-2295.5	0.20125

[a)]MD 2000 (1), Water (2), PEG 8000 (3), PEG 10000 (4), MD 4000 (5), PEG 1450 (6)

Figures 3 and 4 show the experimental and calculated tie lines and the calculated binodal curves for the systems PEG 8000/MD 4000 and PEG 8000/MD 2000. For these aqueous two-phase systems the correlation was successful. The experimental data were compared to the calculated values by liquid-liquid flash using the adjusted parameters. The deviation between the experimental and calculated weight fractions for each system is given in Table 5, calculated according to Equation 5:

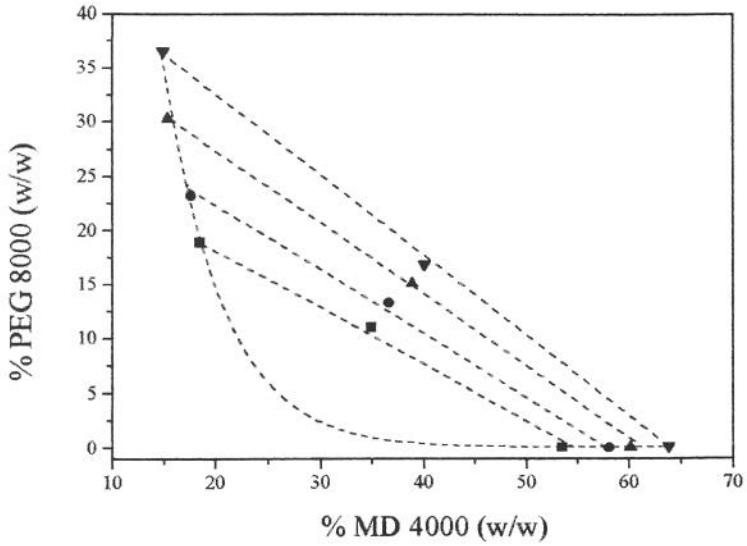

Figure 3 - Experimental and calculated tie-lines and the calculated binodal curve for the PEG 8000/MD 4000 System

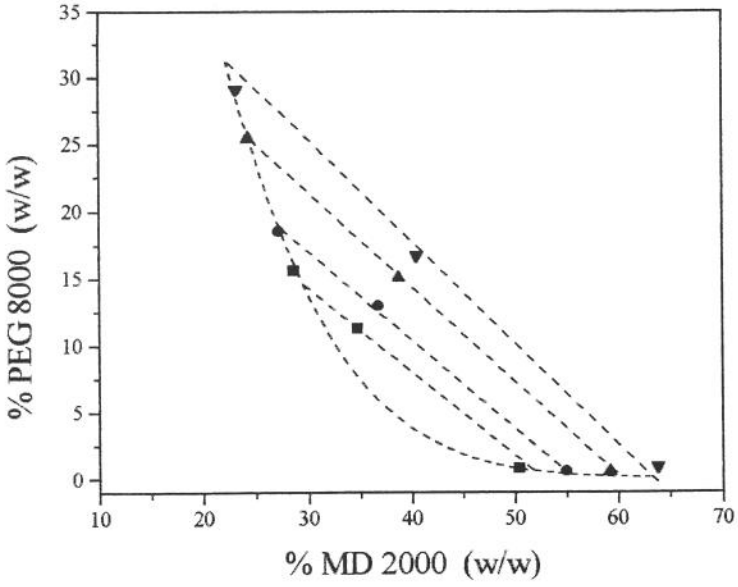

Figure 4 - Experimental and calculated tie lines and the calculated binodal curve for the PEG 8000/MD 2000 System

$$\Delta w = 100\sqrt{\frac{\sum_{n=1}^{N}\sum_{i=1}^{C}\left[\left(w_{n,i}^{I,ex} - w_{n,i}^{I,calc}\right)^2 + \left(w_{n,i}^{II,ex} - w_{n,i}^{II,calc}\right)^2\right]}{2NC}} \quad (5)$$

The best results were obtained for the system PEG 8000/ MD 4000. For the system PEG 1450/ MD 4000 the results shown are not satisfactory if compared with other systems. The low average deviation of the NRTL model for the MD/PEG/Water systems shows that it is possible to obtain a significative set of parameters, which describes well such systems.

Table 5 – Percent deviation of experimental to calculated weights fractions

MD/PEG System	Δw %
2000/8000	0.7777
2000/10000	0.6992
4000/1450	2.6272
4000/8000	0.3430
4000/10000	0.6271
Average deviation	1.0148

2.5. CONCLUSIONS

The present work analysed the equilibrium phase behaviour of MD and PEG systems at 25°C and atmospheric pressure, under several conditions of concentrations and molecular weights of the polymers. The PEGs with molecular weights 1450, 8000 and 10000 and MD with molecular weights 2000 and 4000 were combined to form the systems.

The PEG 1450/MD 2000 mixture does not form an aqueous two-phase system at the concentrations selected in this work. For the other systems, the PEG concentration in bottom phase is very small, and in some cases PEG is almost excluded from this phase. The only exception in this behaviour was observed for the PEG 1450/MD 4000 system, where the molecular weight of MD is higher than PEG.

In all systems the MD concentration in top phase is relatively high when compared to systems formed with PEG/dextran or PEG/salt. The concentration of MD required to form two phases with PEG is much greater than that of dextran or salt. The decrease in PEG molecular weight caused a decrease in the two-phase region.

The NRTL model for the MD/PEG/Water systems showed that it was possible to obtain a significative set of parameters that well describes such systems.

2.6. ACKNOWLEDGMENT

This work was supported financially by research grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - Proc. 1997/10630-7), CNPq (Proc. 521011/95-7) and CAPES/PICDT.

2.7. NOMENCLATURE

A - Parameter of NRTL model

C - Number of components

G - Parameter of NRTL model

M - Molecular weight

N - Total number of tie-lines in a given group of data

T – Absolute Temperature (K)

w - Weight fraction

2.7.1. Superscripts/subscripts

calc - Calculated data

ex - Experimental data

i, j, k - Components

I, II - Phases

2.7.2. Greek letters

α - NRTL parameter

γ - Activity coefficient

τ - NRTL parameter

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ANEXO 2.1: DIAGRAMAS DE EQUILÍBRIO DOS SISTEMAS PEG/MD

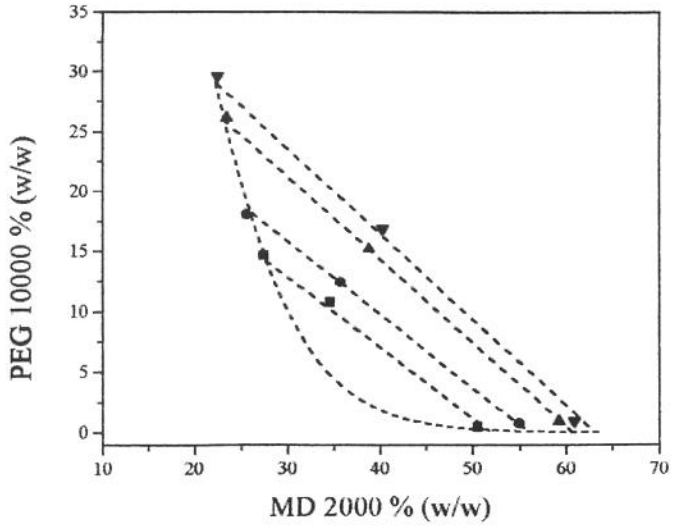

Figure 5 - Experimental and calculated tie lines and the calculated binodal curve for the PEG 10000/MD 2000 System

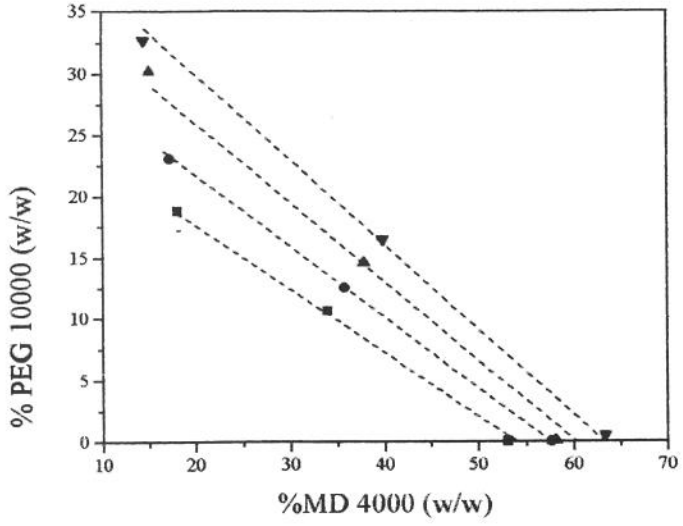

Figure 6 - Experimental and calculated tie lines and the calculated binodal curve for the PEG 10000/MD 4000 System

CAPÍTULO 3

BOVINE SERUM ALBUMIN, α-LACTOALBUMIN AND β-LACTOGLOBULIN PARTITIONING IN POLYETHYLENE GLYCOL/MALTODEXTRIN AQUEOUS TWO-PHASE SYSTEMS

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ABSTRACT

The polysaccharide maltodextrin (MD) can provide a low cost alternative to substitute the fractioned dextran for the use with polyethylene glycol (PEG) in aqueous two-phase, two-polymers systems. In this work we have studied the partitioning of Bovine Serum Albumin (BSA), α-Lactoalbumin (α-La) and β-Lactoglobulin (β-Lg) in PEG/MD aqueous two-phase systems. The experiments were performed at local atmospheric pressure (727 mm Hg) and 25 °C. The influence of the polymers molecular weights and tie line length were investigated.

Keywords : Partitioning, Aqueous two-phase systems, BSA, α-Lactoalbumin, β-Lactoglobulin

3.1. INTRODUCTION

The industrial production of biological compounds is strongly dependent upon the separation technique applied. Liquid-liquid extraction is increasingly used for the isolation and purification of proteins and enzymes (Coimbra *et alii*, 1995; Peng *et alii*, 1995; Coimbra *et alii*, 1998; Johansson *et alii*, 1998; Rito-Palomares and Hernandez, 1998). Over the last three decades many research groups have been using aqueous two-phase system technique for the separation of proteins, cells and other biological materials in laboratory scale (Johansson, 1985; Fisher, 1981; Atkinson and Johns 1994; Christian *et alii*, 1998; Zaslavsky , 1995).

The most common aqueous two-phase systems used for biomolecule separation are polyethylene glycol (PEG)/dextran or PEG/salt systems. A major set back to an ample adoption of PEG/salt systems is that they may damage fragile proteins and also that they present waste disposal problems (Vernau and Kula, 1990). In the case of the PEG/dextran system the high cost of dextran prevents its use in large scale.

Maltodextrin (MD) can provide a low cost alternative to the fractionated dextran. MD is a polysaccharide obtained by the hydrolysis of starch, it is water-soluble and commercially available with polydispersity near that of dextran.

This paper presents the behaviour of the partition coefficients of BSA, α-La and β-Lg in PEG/MD systems at 25°C, with several PEG/MD polymer concentration and different polymer molecular weights.

3.2. MATERIALS AND METHODS

Maltodextrins 2000 and 4000 were kindly supplied by Companhia Lorenz (Blumenau/SC, Brazil). Polyethylene glycols 1450, 8000 and 10000 were purchased from Sigma. The average molecular weight and the polydispersity index of the various polymers used in the present work can be found in Silva and Meirelles (2000). The proteins used in this work were Bovine Serum Albumin (BSA) 96-99%, α-Lactoalbumin and β-Lactoglobulin from bovine milk, electrophoresis grade, all purchased from Sigma.

3.2.1. Proteins Partitioning and Analysis

The tie lines were prepared from polymers stock solutions (55% MD and 50% PEG). Proteins were dissolved in the PEG stock solution. Mixtures of known weights of the stock solutions were made up to a final mass of 12g. All systems contained nearly 50 mg (accurately weighed) of the selected protein. This mixture was gently stirred for 10 minutes at ambient temperature. Complete phase separation was achieved by centrifugation at 2900 g for 40 minutes at 25 °C . After centrifugation the tubes were placed into a thermostatic bath at 25 °C ± 0.1 °C for 5 hours to equilibrate. Visual estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes. The volumes of phases were then used to

estimate the volume ratio (Vr = volume of top (PEG-rich phase)/volume of bottom (MD-rich phase). Aliquots of 5 ml were withdrawn using syringes. The top phase was sampled first, with care being taken to leave a layer of material at least 0,5 cm thick above the interface. The lower phase was withdrawn using a syringe with a long needle. A tiny bubble of air was retained in the needle tip and expelled once in the bottom phase to prevent contamination from top phase material. Protein concentration in the samples was estimated by the Method of Bradford (1976) with BSA as standard. In general, four different tie-line compositions were utilised to determinate the protein partition. The effects of polymers molecular weight and tie line length were studied.

3.3. RESULTS AND DISCUSSION

Protein partitioning in the PEG/MD systems were studied at 25 °C using BSA, α-La and β-Lg. The partition coefficient (K) was calculated as $K = C_T / C_B$ where C_T and C_B are the protein concentrations in mg/g of the top and bottom phases, respectively. The quoted K value is the average of triplicate determinations. The mean standard deviation for the protein concentration was ± 0.0005 mg/g. For the partition coefficients the standard deviation was ± 0.001. The obtained results are given in Table 1. Johansson (1985) reported that the K values for proteins partitioned in systems containing PEG 3350/dextran 500 or PEG 8000/dextran 500 are usually in the range 0.01 – 1.0, similar to most of the results obtained in the present work. But for some of the systems containing MD 4000, α-La and β-Lg concentrated in the top PEG-rich phase (K>1).

3.3.1. Influence of PEG molecular weight

The partition coefficients of α-La, β-Lg and BSA as function of PEG molecular weight are shown in Figure 1. For α-La and BSA the partition coefficient decreases as the

molecular weight of PEG increases. The trend observed here for partitioning in PEG/MD systems agreed well with literature data for PEG/dextran systems (Albertsson, 1986; Johansson *et alii*, 1985). Partition coefficient for α-La was markedly higher than that for β-Lg in the PEG 1450 system, but they show similar values for the systems containing PEG 8000 or 10000. A dramatic decrease in α-La partition coefficient was observed in the PEG molecular weight range of 1450 to 8000. A similar behaviour was reported by Chen (1992) for the partitioning of α-La in PEG/ Potassium Phosphate systems. The partition coefficients for BSA were the lowest for all the PEG molecular weights studied in the present work.

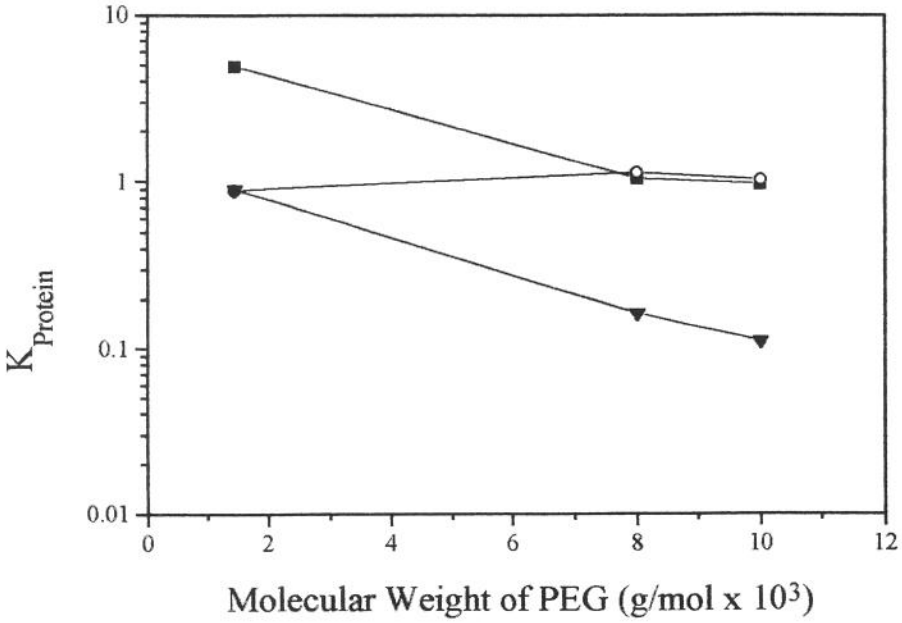

Figure 1 - Effect of PEG molecular weight on the partition coefficients of α-La (■), β-Lg (O), BSA (▼). The system composition was 11% (w/w) PEG / 35% (w/w) MD 4000

For PEG/dextran systems Johansson (1985) observed that in general the protein partition coefficient can be increased by reducing the molecular weight of the polymer in the top phase or by increasing the molecular weight of the polymer in the bottom phase. Our results concerning PEG molecular weight confirm such behaviour, except for β-Lg. In this last

case only small changes are observed and no clear trend is evident (Figure 1). Christian *et alii* (1998) also reported that in the PEG/Arabinogalactan system the PEG molecular has no significant effect upon the partition coefficient of BSA. In contrast, Forciniti *et alii* (1991) observed that in PEG/dextran systems the BSA partition coefficient decreases as PEG molecular weight increases. With regard to the MD molecular weight, only for β-Lg we have observed the same behaviour as reported by Johansson *et alii* (1985) for PEG/dextran.

The PEG 1450/MD 4000 system shows higher partition coefficient values for α-La and β-Lg than those reported for other aqueous two-phase systems: 4.92 for α-La and 0.89 for β-Lg. Chen (1992) quoted partition coefficients of 3.0 for α-La and 0.05 for β-Lg in 14% PEG 1500 / 14% potassium phosphate. Coimbra *et alli* (1995) quoted a partition coefficient of 3.4 for α-La and 0.04 for β-Lg in 14% PEG 1550 / 18% potassium phosphate. Despite the higher values obtained for α-La in PEG/MD systems, the selectivity is better in PEG/salt systems because the partition coefficients for β-Lg are expressively lower for the PEG/salt system.

3.3.2. Effect of tie-line length on partitioning

Phase composition may be usefully characterised by tie-line length (equation 1), since systems sharing a common tie line have the same composition of upper and lower phases and proteins exhibit similar partition coefficients in such systems. The effect of tie-line length (TLL) on partition coefficient was evaluated in all PEG/MD systems (Table 1). Figure 2 illustrates the impact of TLL on the protein partition coefficient in PEG 8000/MD 2000 system. Results show that partition coefficients for BSA and β-Lg increase as TLL increases.

In contrast, the partition coefficient of α-La decreases as TLL increases. The same behaviour was obtained for all proteins in the PEG 10000/MD 2000 system. Albertsson (1986) observed the same trends for serum albumin and phycoerythrin partitioned in

PEG/dextran systems: the partition coefficient of the first protein (serum albumin) increases as TLL increases, while for the last (phycoerythrin) it decreases.

$$\%TLL = \sqrt{\left(PEG_T - PEG_B\right)^2 + \left(MD_T - MD_B\right)^2} \times 100 \qquad (1)$$

Where T and B are the top and bottom concentrations.

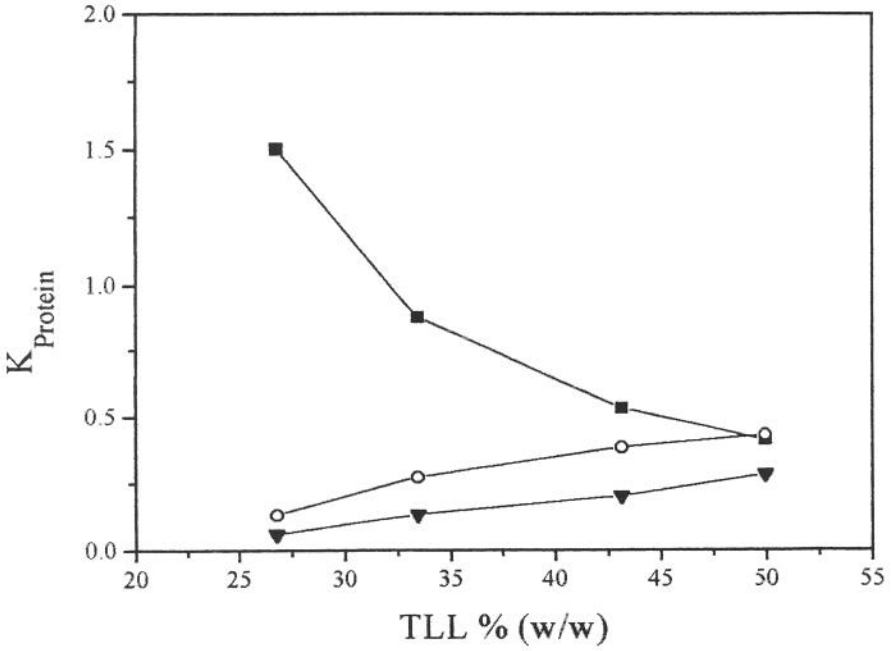

Figure 2 - Effect of tie-line length on partition coefficients in PEG 8000/MD 2000 system. α-La (■),β-Lg (O), BSA (▼).

Table 1 – Partition Coefficients of BSA, α-La and β-Lg in PEG/ MD Systems

System PEG/MD	Total Composition % (PEG/MD)	TLL	Vr	K_{BSA}	$K_{\alpha\text{-La}}$	$K_{\beta\text{-Lg}}$
8000/2000	11.23/34.80	26.78	3.64	0.06	1.50	0.13
	12.97/36.81	33.43	2.71	0.13	0.87	0.27
	15.06/38.78	43.14	2.20	0.20	0.53	0.39
	16.69/40.50	49.94	1.71	0.28	0.41	0.43
10000/2000	10.72/34.63	27.34	3.64	0.13	1.09	0.19
	12.38/35.77	34.47	2.42	0.19	0.62	0.30
	15.15/38.79	44.26	1.67	0.32	0.42	0.37
	16.82/40.26	48.52	1.67	-	0.38	0.46
1450/4000	10.96/33.82	17.82	1.50	0.90	4.75	0.95
	12.55/36.18	29.28	1.68	0.89	4.93	0.89
	13.75/43.18	43.19	1.77	0.89	4.87	0.90
8000/4000	11.02/34.98	39.75	1.45	0.09	1.05	1.14
	13.24/36.71	46.58	1.36	0.16	0.58	1.11
	15.06/38.93	53.96	1.41	0.16	0.68	1.22
	16.81/40.12	61.07	1.33	0.20	-	1.17
10000/4000	10.61/34.03	39.69	1.36	0.08	0.97	1.04
	12.52/35.78	46.41	1.33	0.11	0.65	1.07
	14.55/37.84	52.47	1.27	0.16	0.61	1.28
	16.43/39.86	58.78	1.34	0.13	-	1.15

TLL has no significant effect upon the protein partition coefficients in the PEG 1450/MD 4000 system (Figure 3). For the others PEG/MD 4000 systems the partition coefficients of BSA and α-La exhibit a behaviour similar to that obtained for PEG/MD 2000,

but in this case the effect of the tie-line length is less significant. In such systems β-Lg partitions preferentially to the top phase (K_p>1) and its partition coefficient remains nearly constant as TLL increases (Figure 4).

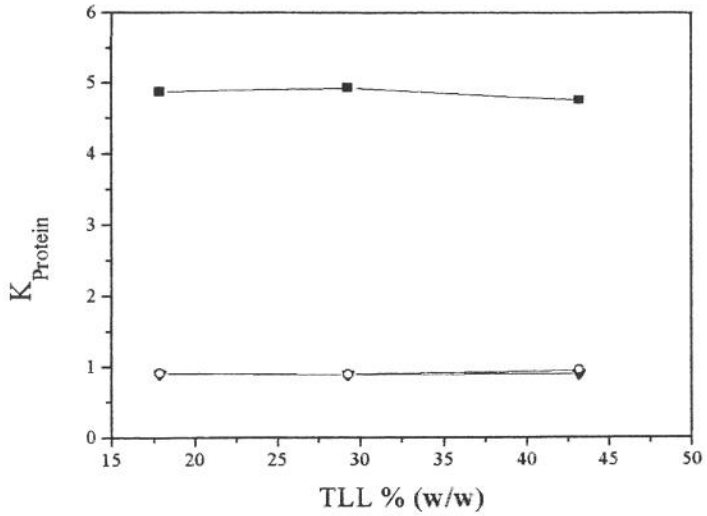

Figure 3 - Effect of tie-line length on partition coefficients in PEG 1450/MD 4000 system. α-La (■), (O) β-Lg, BSA (▼).

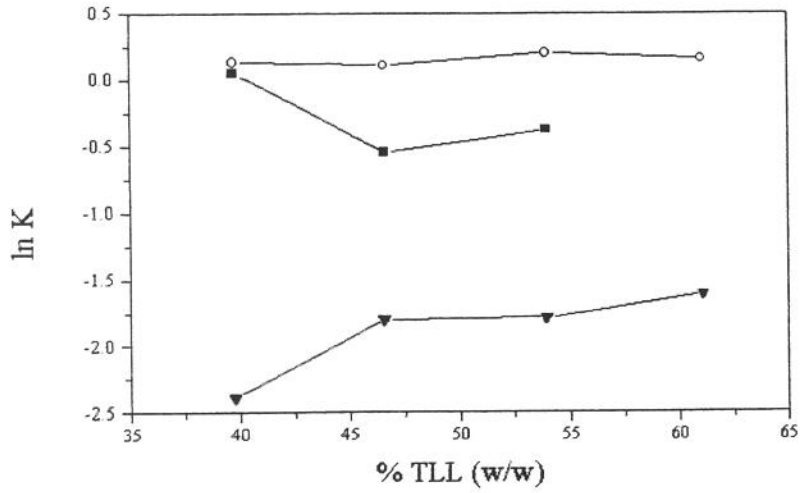

Figure 4 - Effect of tie-line length on partition coefficients in PEG 8000/MD 4000 system. α-La (■), (O) β-Lg, BSA (▼).

In all systems studied in the present work, an increase in the partition coefficients of BSA and β-Lg was accompanied by a decrease of the volume ratio, Vr (see Table 1). A similar trend was observed by Johansson *et alii* (1998) for partitioning of amino acids and proteins in several aqueous two-phase systems. For α-La we have observed an opposite behaviour, which is similar to that reported by Marcos *et alii* (1998) for *penicillin acylase* in PEG/sodium citrate systems.

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ANEXO 3.1 DADOS DE PARTIÇÃO NOS SISTEMAS PEG/MD

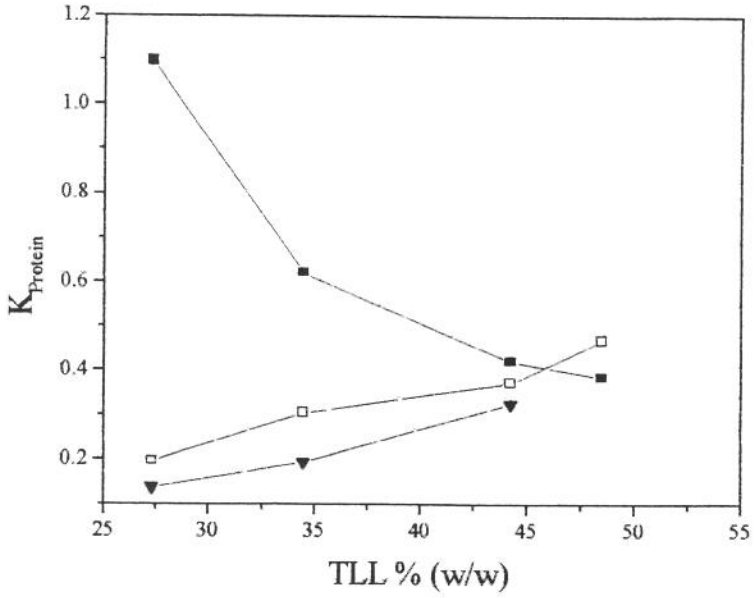

Figure 5 - Effect of tie-line length on partition coefficients in PEG 10000/MD 2000 system. α-La (■), (O) β-Lg, BSA (▼).

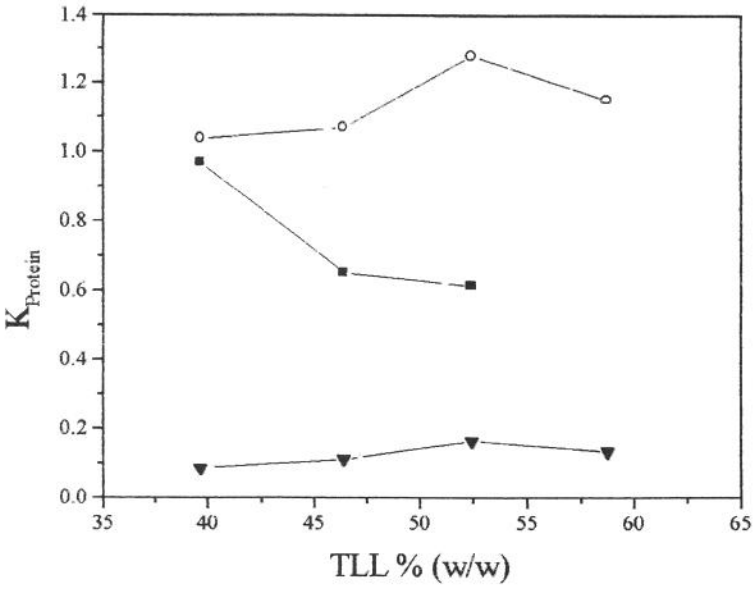

Figure 6 - Effect of tie-line length on partition coefficients in PEG 10000/MD 4000 system. α-La (■), (O) β-Lg, BSA (▼).

CAPÍTULO 4

PHASE EQUILIBRIUM AND PROTEIN PARTITIONING IN AQUEOUS MIXTURES OF MALTODEXTRIN WITH POLYPROPYLENE GLYCOL

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ABSTRACT

Equilibrium data were obtained for polypropylene glycol PPG (400 and 3500)/maltodextrin MD (1000 and 2000) aqueous two-phase systems at 25 °C. Four tie lines were measured for each system. The NRTL model was used to correlate the equilibrium data. The partitioning behaviour of Bovine Serum Albumin (BSA), α-Lactoalbumin (α-La) and β-Lactoglobulin (β-Lg) were studied in PPG 400/MD systems at 25 °C.

Keywords:
aqueous two-phase systems, maltodextrin, polypropylene glycol, NRTL, partitioning, protein

4.1. INTRODUCTION

Aqueous two-phase systems formed by mixtures of two polymers, or one polymer and inorganic salts, are important for separation and purification of enzymes, proteins, nucleic acids, and other substances in biological processes (Albertsson, 1986; Vernau and Kula, 1990). This technology offers the advantages of high capacity, high activity yields and is easy to scale up. For large-scale processes, methods for recycling chemicals have been developed (Hustedt 1986; Greve and Kula, 1991). Many phase diagrams for PEG/salt and PEG/dextran two-phase systems have been reported (Snyder *et alii*, 1992; Zaslavsky, 1995), while liquid-liquid equilibrium data on two-phase systems containing PPG are scarce, thus limiting the potential application of these specific system to biotechnology.

The most common polymer/polymer system is composed by dextran and PEG, but this system is very expensive for scaling up. This problem can be solved by the use of alternative polymers (Szlag *et alii*, 1990, Atkinson and Johns, 1994; Christian *et alii*, 1998). Maltodextrin (MD) is a low-cost starch derivative that can be used as replacement for dextran in aqueous two-phase systems (Silva and Meirelles, 2000a).

Polypropylene glycol is a polymer that is structurally closely related to polyethylene glycol (PEG). Low molecular weights of PPG are completely soluble in water, while high molecular weights are only partially soluble (Molyneux, 1983).

Many authors describe the liquid-liquid equilibrium in aqueous two-phase systems (Kang and Sandler, 1987; Wu *et alii*, 1996; Wu *et alii*, 1998; Wu *et alii*, 1999) using a thermodynamic model. When equilibrium data are not available these models are utilised to provide the basis for extrapolating experimental data and predicting phase compositions. Furthermore, phase diagram data are necessary for the development of models that can predict phase separation

In the present work, we report liquid-liquid equilibrium data for aqueous mixtures of MD (1000 and 2000) and PPG (400 and 3500) and the partition coefficients of the BSA, α-La and β-Lg in PPG 400/MD at 25 °C. The NRTL model for the activity coefficients was used to correlate the equilibrium data for PPG/MD systems.

4.2. EXPERIMENTAL

4.2.1. Materials

The polypropylene glycol, molecular weight 400 and 3500, were purchased from Aldrich. MD 1000 and 2000 were supplied as courtesy by Companhia Lorenz (Blumenau, SC, Brazil). MD is a commercial polymer and therefore can be highly polidisperse. For this reason, this polymer was analysed by Gel Permeation Chromatography (GPC) in a Waters chromatograph (USA). The polydispersity indexes (Mw / Mn) of MDs 1000 and 2000 were found to be 1.22 and 1.74 respectively. The water content of each polymer was determined through Karl Fisher titration using a Metrohm equipment (Swiss). The water content was taken into account for preparing the MD stock solutions. The proteins Bovine Serum Albumin (BSA) 96-99%, α-Lactoalbumin and β-Lactoglobulin from bovine milk, electrophoresis grade, were purchased from Sigma.

4.2.2. Aqueous two-phase equilibrium experiments

Centrifuge tubes, volume 15 cm^3, were used to carry out the phase equilibrium determinations. A stock solution containing 55% of MD was prepared by the addition of Milli Q water. PPG is liquid at ambient temperature and thus was utilised neat. For the determination of the tie lines, mixtures of appropriate amounts of MD stock solution, PPG and water were prepared on an analytic balance (A200 S Sartorius, Germany), accurate to 0.0001g. The systems were mixed for 10 minutes and then centrifuged (BR4i model, Jouan, France) at 2900 g for 40 minutes at 25 °C. The tubes were brought to equilibrium in a thermostatic bath (Viscotherm VT2, Physica, Germany) at 25 °C ± 0.1 for 5 hours. After equilibrium was achieved, phases were sampled with syringes. The top phase was sampled first, with care being taken to leave a layer of material at least 0.5 cm thick above the interface. The bottom phase was withdrawn using a syringe with a long needle.

4.2.3. Protein Partitioning

Proteins were dissolved in the MD stock solution. Mixtures of known weights of MD stock solution, PPG and water were made up to a final mass of 12g. The same procedure utilised to determinate the equilibrium data was adopted to determinate the partition coefficient. All systems contained nearly 50 mg (accurately weighed) of the selected protein. Visual estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes. The volume of phases were then used to find the volume ratio (Vr = volume of top phase (rich PPG)/volume of bottom phase (rich MD)).

4.2.4. Analytical Methods

The concentration of MD was determined by polarimetry (Carl Zeiss Jena, POLAMAT A model, equipped with a mercury lamp at 546 nm, Germany). The standard deviation of the weight percent of MD by this method was ± 0.009%.

The water concentration was determined by freeze drying (EZ DRY model, FTS Systems, New York, USA) at -54 °C and 100 m Torr for 48 h. The standard deviation of the weight percent of water by this method was ± 0.004%.

The PPG concentration was determined by difference. The standard deviation of the weight percent of PPG was ± 0.006%.

The concentrations of proteins (top and bottom) were determined by spectrophotometry. A sample of each phase was mixed with distilled water and its absorbance read at 280 nm using a spectrophotometer (Hach DR-4000U). In all cases the concentrations were analysed in triplicate.

4.3. RESULTS

4.3.1. Liquid-liquid Equilibrium

The experimental liquid-liquid equilibrium results for the aqueous two-phase systems PPG 400/MD and PPG 3500/MD are given in Tables 1-2. All the results are expressed as weight percentage. For each polymer combination four tie lines were determined. The complete phase diagram for PPG 3500/MD 2000 is shows in Figure 1.

For most systems the MD concentration in top phase is very small, and in some cases MD is almost excluded from this phase. For the system PPG 3500/MD this trend is evident. Similar results were reported by Zafarani-Moattar and Salabat (1998) for PPG/Salt systems. All PPG/MD systems were characterised by the presence of considerable quantities of PPG in the top phase. This quantity increased markedly as the molecular weight of PPG increased. The same behaviour is observed by Cheluget *et alii* (1994) in PEG/salt systems. In all systems studied in the present work the PPG concentration in top phase is relatively high when compared to systems formed with PEG/dextran, PEG/salt or PEG/MD (Albertsson, 1986; Silva *et alii*, 1997; Zaslavsky, 1995, Silva and Meirelles, 2000a). The systems PPG/MD

required particularly large concentrations of both polymers to exhibit phase splitting. The very high total polymer concentration necessary for inducing phase separation is most likely due to the very low molecular weight of the polymers.

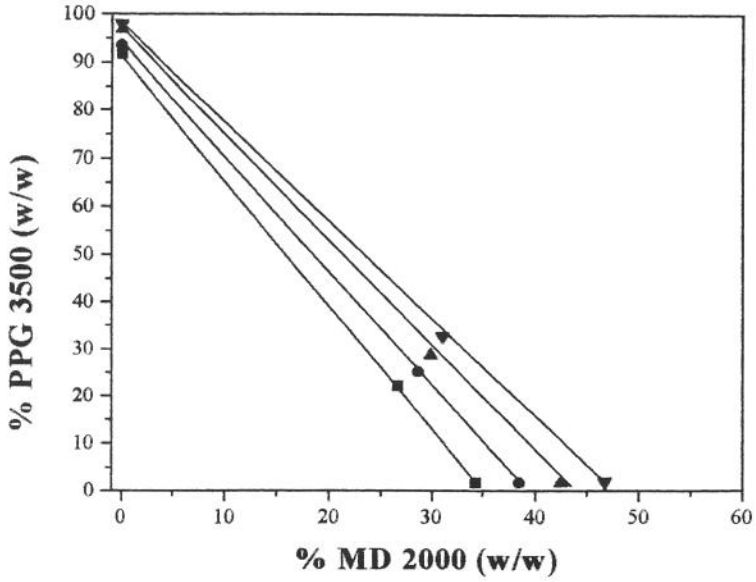

Figure 1 - Experimental tie lines for the system PPG 3500/MD 2000 at 25 °C

The MD produced by Companhia Lorenz is obtained using a combination of both acid and enzymic hydrolysis. Such processing strategy reduces the tendency to retrogradation (Atkinson and Johns, 1994). This process produces low molecular weight MD which exhibits a distinct cost advantage over other specifically modified starch derivatives, used to retard retrogradation.

The effect of increasing the molecular weight of the PPG is to increase the area of two-phase coexistence, a behaviour already reported in the literature for similar systems (Szlag *et alii*, 1990, Albertsson, 1986, Cheluget *et alii*, 1994).

Table 1 – Phase compositions for PPG 400/MD system

System	Total Composition (%w/w)			Top Phase (% w/w)			Bottom Phase (% w/w)		
	MD	PPG	Water	MD	PPG	Water	MD	PPG	Water
PPG 400/MD 1000	16.39	39.59	44.02	1.72	62.82	35.46	32.65	16.40	50.95
	22.50	30.70	46.80	1.88	62.54	35.58	33.73	15.54	50.73
	30.11	26.39	43.50	1.20	67.11	31.69	42.78	11.83	45.36
	30.59	33.61	35.80	0.34	75.94	23.72	52.70	7.20	40.10
PPG 400/MD 2000	26.69	22.10	51.21	11.30	37.32	51.38	33.43	13.88	52.69
	28.45	24.62	46.93	4.75	52.04	43.21	42.41	11.07	46.52
	29.94	28.45	41.61	1.86	64.06	34.08	47.17	9.13	43.70
	31.15	32.45	36.40	0.95	71.93	27.12	51.93	7.11	40.96

Table 2 – Phase compositions for PPG 3500/MD system

System	Total composition (%w/w)			Top Phase (% w/w)			Bottom Phase (% w/w)		
	MD	PPG	Water	MD	PPG	Water	MD	PPG	Water
PPG 3500/MD 1000	16.43	40.07	43.50	0.00	96.59	3.41	27.69	1.74	70.57
	22.51	30.68	46.81	0.00	97.12	2.88	32.60	2.05	65.35
	29.96	26.66	43.38	0.00	96.27	3.73	40.98	2.47	56.55
	32.91	28.57	38.52	0.00	96.56	3.44	46.76	2.80	50.86
PPG 3500/MD 2000	26.72	22.03	51.25	0.06	91.59	8.35	34.34	1.62	64.04
	28.71	24.97	46.32	0.02	93.51	6.47	38.48	1.64	59.88
	29.90	28.56	41.54	0.01	96.76	3.23	42.52	1.66	55.82
	31.07	32.57	36.36	0.01	97.84	2.15	46.76	1.82	51.42

4.3.2. Partition Protein

BSA, α-La and β-Lg were partitioned at 25 °C in the PPG 400/MD systems. The partition coefficient (K) was calculated as $K_{Protein} = C_T / C_B$, where C_T and C_B are the protein concentrations in mg/g of the top and bottom phases, respectively. The partition coefficients are given in Table 3 and the mean deviation for the protein concentration was ± 0.0007 mg/g. For the partition coefficients the standard deviation was ± 0.001. The results given in Table 3 show that for most cases the proteins partition preferentially to the top phase ($K_{protein}>1$), the PPG-rich phase. The partition coefficients obtained for the same proteins in PEG/MD systems by Silva and Meirelles (2000b) are in most cases lower than values reported in the present work.

Table 3 – Partition Coefficients of BSA, α-La and β-Lg in PPG/ MD Systems

System PPG/MD	Total Composition PPG/MD (%w/w)	TLL	Vr	K_{BSA}	$K_{\alpha\text{-La}}$	$K_{\beta\text{-Lg}}$
400/1000	25.78/33.16	69.41	1.52	3.22	1.73	1.56
	27.71/32.78	70.02	1.56	1.99	1.33	1.25
	30.60/33.50	78.57	1.73	1.50	1.09	0.86
	32.69/33.63	92.15	1.80	1.45	1.17	0.90
400/2000	22.14/28.82	43.38	2.34	1.88	1.70	1.12
	24.92/30.83	64.24	1.86	2.66	2.15	1.89
	27.94/31.82	78.46	1.80	2.06	3.28	3.01
	30.48/34.75	88.16	1.78	2.17	1.54	0.75

In partitioning studies it is conventional to express the difference in the composition of the two-phases by the tie line length which is determined by the difference in concentration of the system forming components (PPG and MD) between the phases (Forciniti *et alii* 1991; Belval *et alii*, 1998). Partitioning in PEG/Dextran system is usually carried out at tie line lengths between 10-30 (wt % polymer) while in PEG/MD system is carried out at tie line lengths between 20-60 (wt % polymer) (Silva and Meirelles 2000 b). In the present work we have obtained tie line lengths between 40-90 (wt % polymer) for the PPG/MD system.

The partition coefficients of α-La, β-Lg and BSA as function of tie line length (TLL) for the PPG 400/MD systems are shown in Figures 2-3. In the system PPG 400/MD 1000 (Figure 2) for all proteins the partition coefficient decreases as the TLL increases. The same behaviour was obtained by Albertsson (1986) for serum albumin partitioned in PEG/dextran systems, Peng *et alii* (1995) for lyzozyme in PEG/Phosphate potassium systems and Silva and Meirelles (2000b) for α-La in PEG/MD systems.

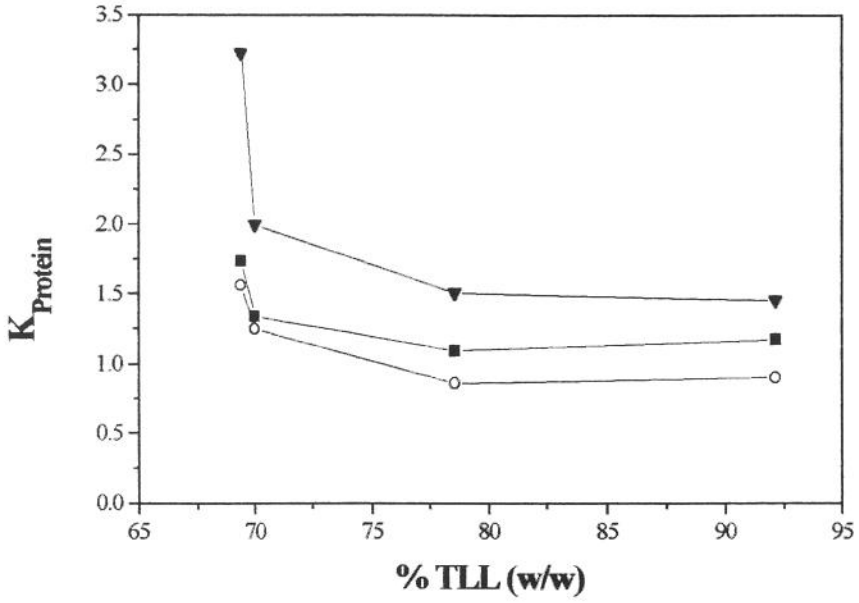

Figure 2 - Effect of tie-line length on partition coefficients in PPG 400/MD 1000 system. BSA (▼), α-La (■),β-Lg (O).

In PPG 400/MD 2000 the partition coefficients of α-La and β-Lg increase as TLL increases until a length of 78.5% (Figure 3). This TLL corresponds to a water concentration of approximately 34% and a PPG concentration of 64% in the top phase. As shown in Table 1, for the system PPG 400/MD 1000 the PPG concentration in top phase is always higher than 62%, and its TLL are generally larger than the corresponding ones for the PPG 400/MD 2000 system. It seems that for very high PPG concentration in the top phase the proteins present there tend to move to the bottom phase, decreasing the partition coefficients. Silva and Meirelles (2000b) observed that the partition coefficients for BSA and β-Lg increase as TLL increase in PEG 8000/MD 2000 system. But for the PEG/MD systems the highest TLL was 61% and the PEG concentration in the top phase was always lower than 37%.

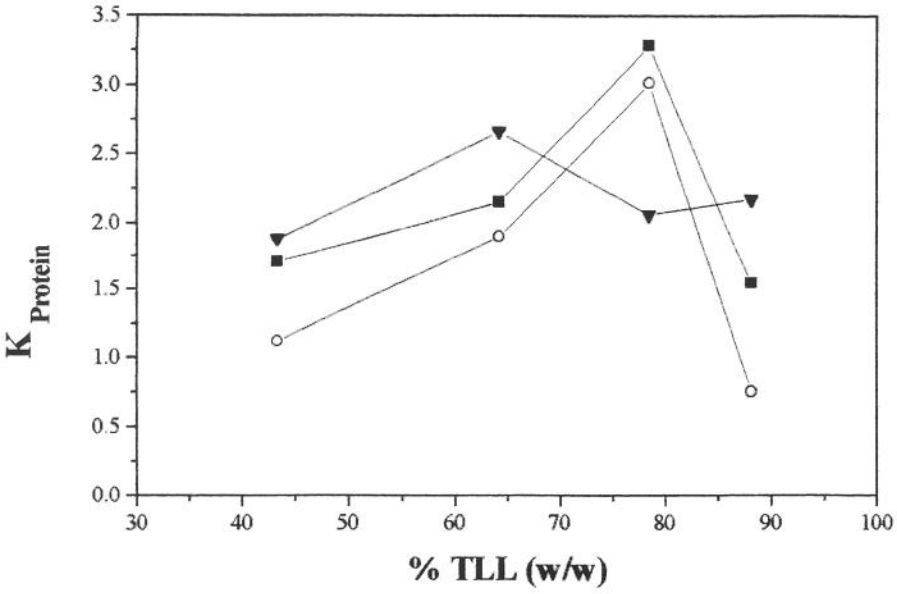

Figure 3 - Effect of tie-line length on partition coefficients in PPG 400/MD 2000 system. BSA (▼), α-La (■), (O) β - Lg.

In PPG 400/MD 1000 system, an increase in the partition coefficients of proteins was accompanied by a decrease of the volume ratio, Vr (see Table 3). These results agree with those obtained by Huddlestone *et alii* (1994) in PEG/Potassium Phosphate systems

and by Johansson *et alii* (1996) for several aqueous two-phase systems. For PPG 400/MD 2000 system we have observed an opposite behaviour, which is similar to that reported by Marcos *et alii* (1998) for *penicillin acylase* in PEG/sodium citrate systems.

4.4. MODELLING

The NRTL equation was utilised for modelling the PPG/MD aqueous two-phase systems. Due to the large difference in molecular weights between the components in the systems the weight fraction was used as the unit of concentration. This procedure was suggested by Oishi and Prausnitz (1978) for the UNIQUAC and UNIFAC models.

Equation 1 shows the NRTL model expressed in weight fraction:

$$\ln\gamma_i = \frac{\sum_j^C \frac{\tau_{ji} G_{ji} w_j}{M_j}}{\sum_j^C \frac{G_{ji} w_j}{M_j}} + \sum_{j=1}^C \left[\frac{w_j G_{ji}}{M_j \sum_k^n \frac{G_{kj} w_k}{M_k}} \left(\tau_{ij} - \frac{\sum_k^C \frac{\tau_{kj} G_{kj} w_k}{M_k}}{\sum_k^C \frac{G_{kj} w_k}{M_k}} \right) \right] \tag{1}$$

where

$$G_{ij} = \exp\left(-\alpha_{ij}\tau_{ij}\right) \tag{2}$$

$$\tau_{ij} = A_{ij}/T \tag{3}$$

$$\alpha_{ij} = \alpha_{ji} \tag{4}$$

Following the procedure developed by Stragevitch and d'Avila (1997), adjustments of the parameters were made by minimisation of the maximum likelihood objective function. Table 4 shows the parameters estimated from the experimental data. Figures 4 and 5 show the experimental and calculated tie lines and the calculated binodal curves for the systems PPG 400/MD 1000 and PPG 400/MD 2000.

Table 4 – Adjusted parameters of the NRTL model.

Parameters	A_{ij} (K)	A_{ji}(K)	$\alpha_{ij} = \alpha_{ji}$
12[a]	-375.35	937.93	0.3801
13	2986.50	544.31	0.2000
23	918.78	-1873.50	0.2884
15	2274.6	-25.01	0.0912
25	2681.30	-1240.30	0.4700
42	-349.20	2292.80	0.4699
43	30.85	-5000.00	0.4185
45	-38.58	1820.80	0.1999

[a)]PPG 400 (1), Water (2), MD 1000 (3), PPG 3500 (4), MD 2000 (5)

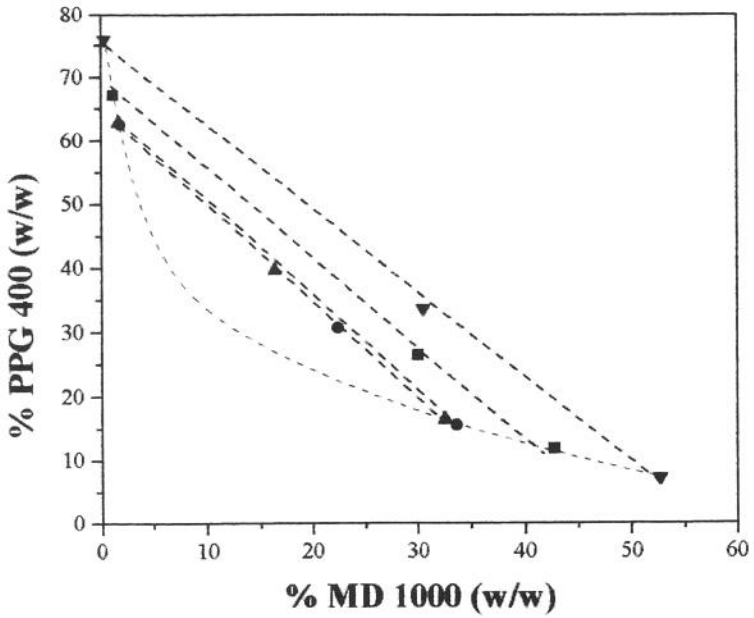

Figure 4 - Experimental and calculated tie lines and the calculated binodal curve for the PPG 400/MD 1000 System.

For these aqueous two-phase systems the correlation was successful. The experimental data were compared to the calculated values by liquid-liquid flash using the adjusted parameters.

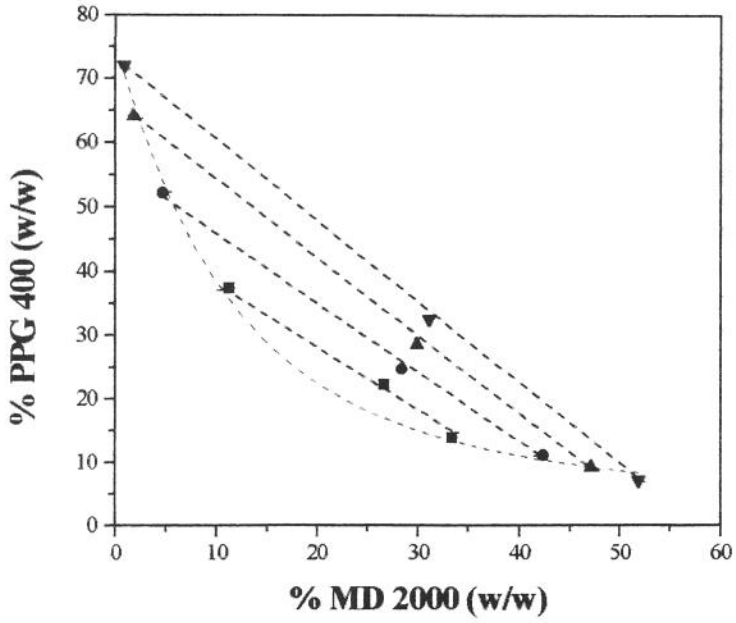

Figure 5 - Experimental and calculated tie lines and the calculated binodal curve for the PPG 400/MD 2000 System.

The deviation between the experimental and calculated weight fractions for each system is given in Table 5, calculated according to Equation 5:

$$\Delta w = 100\sqrt{\frac{\sum_{n=1}^{N}\sum_{i=1}^{C}\left[\left(w_{n,i}^{I,ex} - w_{n,i}^{I,calc}\right)^2 + \left(w_{n,i}^{II,ex} - w_{n,i}^{II,calc}\right)^2\right]}{2NC}} \qquad (5)$$

The best result was obtained for the system PPG 400/ MD 2000. For the system PPG 3500/ MD 1000 the obtained result was not satisfactory if compared with the results for

other systems. The low average deviation of the NRTL model for most PPG/MD/Water systems shows that it is possible to obtain a significative set of parameters, which describes well such systems.

Table 5 – Percent deviation of experimental to calculated weights fractions

PPG/MD System	Δw %
400/1000	0,3840
400/2000	0,3123
3500/1000	3,9563
3500/2000	1,2592
Average deviation	1.3039

4.5. CONCLUSIONS

Equilibrium analysis of phase behaviour of MD and PPG systems at 25°C for different concentrations and molecular weights of the polymers were conducted. These systems were obtained by a combination of PPGs with molecular weights 400 and 3500 and MD with molecular weights 1000 and 2000.

For these systems the MD concentration in top phase is very small, and in some cases MD is almost excluded from this phase. This effect is more sensitive in PPG 3500/MD systems.

In all systems the PPG concentration in top phase is relatively high when compared to systems formed with PEG/dextran, PEG/MD or PEG/salt. A much higher

concentration of MD is also required to form aqueous two-phase systems, as compared to dextran or salt.

BSA, α-La and β-Lg were partioned at 25 °C in the PPG 400/MD systems; for most cases the proteins partition preferentially to the top phase (K_p>1), the PPG-rich phase

In PPG 400/MD 1000 system the partition coefficients of α-La, β-Lg and BSA decreases as the TLL increases. In this system, an increase in the partition coefficients of proteins was accompanied by a decrease of the volume ratio, Vr.

The partition coefficients of α-La and β-Lg in PPG 400/MD 2000 increase as TLL increases until a length of 78.5%; for TLL higher than 78.5% this trend is inverted and the partition coefficients tend to decrease.

The NRTL model for the MD/PPG/Water systems showed that it was possible to obtain a significative set of parameters describing well such systems.

4.6. ACKNOWLEDGMENT

This work was supported financially by research grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - Proc. 1997/10630-7), CNPq (Proc. 521011/95-7) and CAPES/PICDT.

4.7. NOMENCLATURE

A - Parameter of NRTL model

C - Number of components

G - Parameter of NRTL model

M - Molecular weight

N - Total number of tie-lines in a given group of data

T – Absolute Temperature (K)

w - Weight fraction

4.7.1. Superscripts/subscripts

calc - Calculated data

ex - Experimental data

i, j, k - Components

I, II - Phases

n - Tie lines

4.7.2. Greek letters

α - NRTL parameter

γ - Activity coefficient

τ - NRTL parameter

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ANEXO 4.1 – DIAGRAMA DE EQUILÍBRIO DO SISTEMA PPG3500 / MD 1000

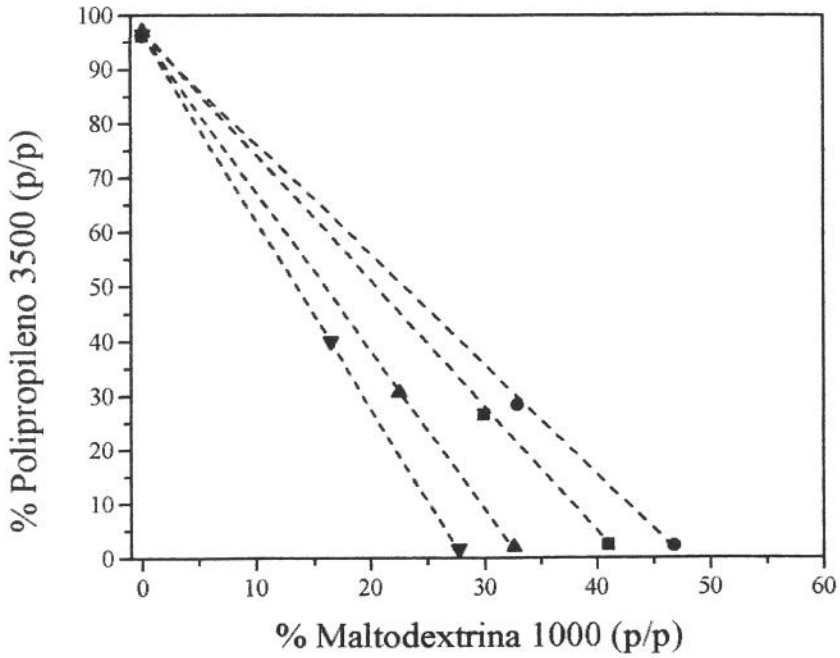

Figure 6 - Experimental tie lines for the system PPG 3500/MD 1000 at 25 °C

CAPÍTULO 5

PEG + POTASSIUM PHOSPHATE + UREA AQUEOUS TWO-PHASE SYSTEMS: PHASE EQUILIBRIUM AND PROTEIN PARTITIONING

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Trabalho submetido à Revista **Journal Chemical Engineering Data**

ABSTRACT

Liquid-liquid equilibria of polyethylene glycol (PEG) + potassium phosphate + urea+water aqueous two-phase systems were studied for different PEG molecular weights and pHs. Experimental techniques and analytical methods are described. Equilibrium data were obtained for PEG (1450, 3350, 10000) + potassium phosphate (pH 7 and 9) + urea (6 wt%) + water aqueous two-phase systems at 25 °C. Four tie lines were measured for each system. The partitioning behaviour of Lysozyme, Catalase and β-Galactosidase were studied in these systems at 25 °C, pH 7 and 9 and at urea concentrations 3 and 6 wt%.

Keywords:
PEG, salt, urea, ATPS, protein, protein partitioning.

5.1. INTRODUCTION

A biocompatible liquid-liquid system can arise when two polymers or a polymer and a salt are dissolved in water, with one of the components predominating in each phase (Walter *et alii*, 1985). An aqueous two-phase system is expected to be a promising mean of separating biological materials because of the mild conditions provided by the phases and their different physical and chemical properties (Mishima *et alii*, 1995).

Polymer/polymer aqueous two-phase systems were widely studied in earlier years. However, electrolyte/polymer aqueous two-phase systems have some advantages such as low price, low viscosity and a short time for phase splitting (Peng *et alii*, 1995). Other advantages of such systems is that important properties, such as ionic strength and pH, can be adjusted conveniently (Grossmann *et alii*, 1998).

Experimental liquid-liquid equilibria (LLE) of systems containing two kinds of polymers have been frequently reported in the literature (Albertsson, 1986; Cabezas *et alii*, 1989; Silva and Meirelles, 2000a). However, LLE of polymer/electrolyte/water systems are relative scarce,

at least for systems containing chaotropic compounds like urea (Voros *et alii*, 1993; Rämisch *et alii*, 1999).

As aqueous two-phase systems become increasingly popular in a number of large-scale and analytical applications, information on biomolecule partitioning in such systems becomes more important. The major factors that influence biomolecule partitioning include the concentration, molecular weight and type of polymers used, pH, ionic strength, temperature, interfacial tension and relative hydrophobicity (Kula , 1979; Zaslavsky *et alii*, 1982; Brooks *et alii*, 1984, Silva and Meirelles, 2000b; Alves *et alii*, 2000).

Aqueous two-phase systems containing urea have been used for the initial recovery steps of biomolecules from cell broth produced by recombinant *Escherichia coli*. For the solubilization of the inclusion bodies formed by the *Escherichia coli* host urea has to be added to the cell broth. High urea concentrations (30-35 wt%, ≅ 5-6 M) are recommended. The further addition of PEG and salts like Na_2SO_4 or Potassium Phosphate to the original system produces an aqueous two-phase system containing a high urea content (Rämsch *et alii*, 1999).

Despite the projected importance of aqueous two-phase extractive techniques for future separation technology, no fully comprehensive theoretical framework exists for predicting protein partition coefficients. One obstacle to these investigations is the lack of an extensive database against which theories can be tested (Forciniti *et alii*, 1991).

Much empirical research has been devoted to the effects of selected independent factors and practical applications. Previous works have studied individual effects, which are important to protein partitioning (Brooks *et alii*, 1984; Diamond and Hsu, 1989; Schluck *et alii*, 1995; Peng *et alii*, 1995; Carlsson, *et alii*, 1995; Huddlestone *et alii*, 1996; Johansson *et alii*, 1996). Further progress in understanding protein partitioning in aqueous two-phase systems will require fundamental thermodynamic data.

In this paper, we present phase diagrams at 25 °C, pH 7 and 9 and urea concentration 6 wt% for the PEG + potassium phosphate + urea + water system, using PEG of molecular weights

1450, 3350 and 8000. The partition coefficients of lysozyme, catalase and β-galactosidase were determined in these systems using two urea concentrations, 3 and 6 wt%.

5.2. EXPERIMENTAL

5.2.1. Materials

Systems - The polyethylene glycol (PEG), molecular weights 1450, 3350 and 8000, were purchased from Sigma. The polymers were analysed by Gel Permeation Chromatography (GPC) in a Waters chromatograph (USA). The polydispersity indexes (Mw/Mn) of PEGs 1450, 3350 and 8000 were found to be 1.03, 1.04 and 1.10 respectively. The potassium phosphate mono and dibasic (minimum purity of 99.8 wt%) and urea (minimum purity of 99.5 wt%) were purchased from MERCK, analytical reagent grade. The water content of each polymer was determined by Karl Fisher titration using a Metrohm equipment (Swiss). This amount of water ranges from 0.36 to 0.75 wt% and it was considered for calculating the water concentrations in the solutions. All the reagents were used without further purification. Distilled water was used in all experiments.

Proteins – Lysozyme (L7773, lot 16H6830) from chicken egg white, Catalase (C9322, lot 88H72501) from bovine liver and β-Galactosidade (G1875, lot 54H7025) from bovine liver grade III crude were purchased from Sigma.

5.2.2. Phase Diagrams

Aqueous two-phase systems were prepared by weighting appropriate quantities of PEG, potassium phosphate, urea and water on an analytic balance (A200 S Sartorius, Germany), accurate to ± 0.0001 g. Liquid-liquid equilibrium cells (Figure 1) were used to carry out the phase equilibrium determinations (Silva *et alii*, 1997). Typically 50 g of a system were prepared. The mixture was magnetically stirred for 50 min, after which the cell was tightly capped and then allowed to attain equilibrium for 5 h at 25 °C and at the desired pH-value. The temperature in the

cell was controlled within ± 0.1 °C in a thermostatic bath (Viscotherm VT2, Physica, Germany). The Henderson-Hasselbach equation was used to determinate the ratio of mono and dibasic salts necessary to bring the pH to 7 and 9. After equilibrium, the two-phases became clear and transparent, and the interface well defined. Samples from top and bottom phases were collected, with the aid of syringes.

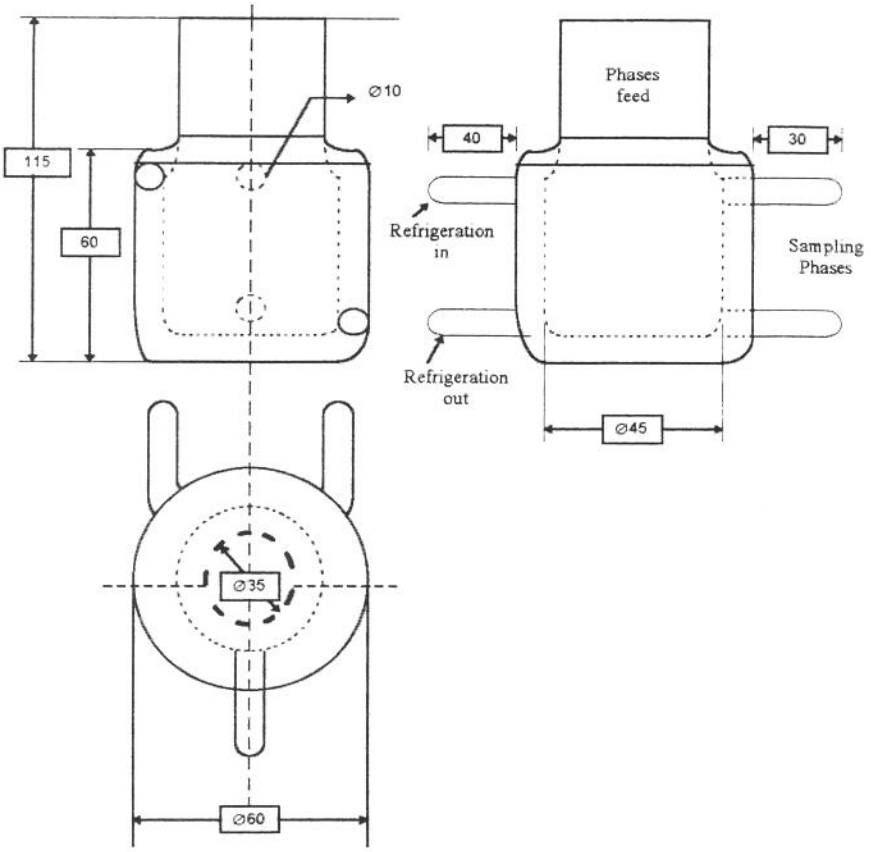

Figure 1 – Scheme of the liquid-liquid equilibrium cell.

5.2.3. Protein Partitioning

Centrifuge tubes, volume 15 cm^3, were used to carry out the protein partitioning experiments. The tie lines were prepared from PEG, salt and urea stock solutions. Proteins were dissolved in the PEG stock solution. Mixtures of known weights of the stock solutions were made up to a final mass of 12 g. All systems contained nearly 50 mg (accurately weighed) of the selected protein. This mixture was gently stirred for 10 minutes at ambient temperature. Complete phase separation was achieved by centrifugation at 2900 g for 40 minutes at 25 °C. After centrifugation the

tubes were placed into a thermostatic bath at 25 °C ± 0.1 °C for 5 hours to equilibrate. Aliquots of 5 ml were withdrawn using syringes. The top phase was sampled first, with care being taken to leave a layer of material at least 0.5 cm above the interface. The lower phase was sampled using a syringe with a long needle (Silva and Meirelles, 2000b).

5.2.4. Analysis of phase compositions

The concentration of salt was determined using simultaneously two methods: phosphate was determined by automated titration, as described in detail by Greve (1989) and the potassium (K) was determined by means of ion-selective electrodes as described by Hustedt *et alii*, (1985). The standard deviation of the salt weight percent by these methods was ± 0.009 %. The concentration of urea was determined following the procedure outlined by AOAC (1984), method 991.20, total nitrogen. The standard deviation of the urea weight percent by this method was ± 0.013 %. The concentration of water was determined by freeze drying (EZ DRY model, FTS Systems, New York, USA) at – 54 °C and 100 mTorr for 48 h. The standard deviation of the water weight percent by this method was ± 0.005%. The PEG concentration was obtained by difference. The standard deviation of the PEG weight percent by this method was ± 0.001 %. All concentrations were analysed in triplicate.

The concentrations of proteins (top and bottom) were determined by spectrophotometry. A sample of each phase was mixed with distilled water and its absorbance read at 280 nm using a spectrophotometer (Hach DR-4000U). The standard deviation for the protein concentration was ± 0.0009 mg/g. The protein concentrations were also analysed in triplicate.

5.3. RESULTS AND DISCUSSION

5.3.1. Liquid-liquid Equilibrium

Phase diagrams for aqueous two-phase systems containing PEG (1450, 3350 and 8000) + potassium phosphate + urea at 25 °C and pHs 7 and 9 were experimentally determined. A typical phase diagram is shown in Figure 2. The black dots represent the total concentrations of each

system, the dashed lines connecting the black circles are the tie lines, and the black circles represent the concentrations in the top and bottom phases in equilibrium. The curved line connecting the circles is the binodal, which separates the homogeneous region from the two-phase region.

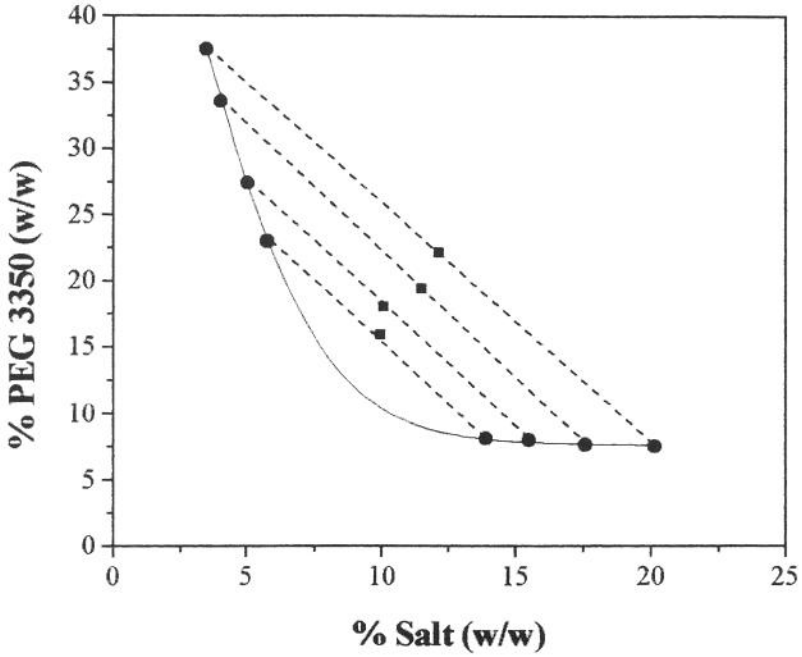

Figure 2 – Phase diagram for the PEG 3350 + potassium phosphate + water at 25 °C, 6 wt% urea and pH=7.

The tie line compositions were confirmed, with an average experimental error of 4%, by performing mass balance based on the equilibrium compositions. These values are in good agreement with results from the literature (Albertsson, 1986; Cheluget *et alii*, 1994; Snyder *et alii*, 1992; Silva *et alii*, 1997).

The experimental liquid-liquid equilibrium results for these aqueous two-phase systems are given in Tables 1-2. All the results are expressed as weight percentage. For each polymer/salt combination four tie lines were determined.

Table 1 - Phase compositions for the PEG + potassium phosphate + water system at 25°C, pH = 7 and urea 6 wt%.

System	Total composition (%w/w)			Top phase (% w/w)			Bottom phase (% w/w)		
	Salt	PEG	Urea	Salt	PEG	Urea	Salt	PEG	Urea
PEG	11.47	20.09	5.93	5.79	31.85	4.65	16.60	10.60	6.07
1450	12.43	22.26	5.99	3.54	39.11	4.39	18.45	10.48	7.43
	13.61	23.39	6.02	2.70	42.98	3.82	20.75	10.12	7.76
	14.61	24.59	6.02	2.47	45.78	3.78	23.71	9.95	9.15
PEG	9.97	15.87	6.04	5.77	22.95	8.19	13.94	8.09	5.33
3350	10.08	17.98	6.04	5.07	27.41	8.40	15.52	7.98	5.87
	11.52	19.36	6.01	4.04	33.56	4.82	17.58	7.64	6.18
	12.16	22.09	6.07	3.49	37.47	4.58	20.16	7.53	9.57
PEG	10.01	15.88	6.07	5.07	28.73	8.38	13.48	7.20	5.54
8000	10.36	17.91	6.03	4.32	33.04	8.57	14.83	7.08	5.81
	11.56	19.80	5.93	3.73	37.79	4.85	17.03	6.95	6.36
	12.53	21.99	6.14	3.25	41.47	3.28	19.84	6.07	12.01

In all systems studied here (PEG/salt/urea) the concentration of PEG and salt required to exhibit phase splitting is higher than in PEG/salt systems without urea, as compared to data reported by Silva *et alii* (1997).

Analysis of the binodal curves from the systems studied with urea, revealed the effect of PEG molecular weight on phase separation. Figure 3 shows the binodal curves for PEG (1450, 3350 and 8000) + potassium phosphate + urea + water. The decrease in PEG molecular weight caused a decrease on the two-phase region, a behaviour already reported in the literature for similar

systems (Szlag *et alii*, 1990; Lei *et alii*, 1990; Vernau and Kula, 1990). The difference in position between the binodal curves begins to decrease at higher molecular weight, with the binodals curves for 3350 and 8000 being very close. In fact, the addition of urea displaced the binodal curve towards higher concentrations (Figure 4), so that these curves for different PEG molecular weights became closer.

Table 2- Phase compositions for the PEG + potassium phosphate + water system at 25°C, pH = 9 and urea 6%.

System	Total composition (%w/w)			Top phase (% w/w)			Bottom phase (% w/w)		
	Salt	PEG	Urea	Salt	PEG	Urea	Salt	PEG	Urea
PEG	10.62	19.95	5.96	4.94	31.71	4.68	15.75	10.46	6.16
1450	12.49	21.99	6.02	3.60	38.84	4.39	18.51	10.21	7.46
	13.67	23.46	6.05	2.76	43.05	3.85	20.81	10.19	7.79
	14.59	24.59	6.00	2.45	45.72	3.76	23.27	9.99	9.13
PEG	9.87	15.63	6.00	4.61	26.70	7.97	12.63	10.39	4.92
3350	10.33	17.88	6.15	3.59	32.26	8.04	15.11	8.33	5.74
	11.31	19.58	6.08	3.31	36.04	4.97	18.06	6.91	6.22
	12.33	21.87	6.12	2.67	43.50	3.39	19.99	6.25	11.06
PEG	10.01	15.95	6.24	3.63	30.69	7.99	14.14	8.63	4.76
8000	10.26	17.80	5.98	3.24	34.44	8.23	15.54	7.29	5.77
	11.39	19.75	5.94	2.74	38.73	4.99	17.64	7.16	6.15
	12.18	21.69	5.99	2.33	42.30	5.42	19.78	7.05	9.51

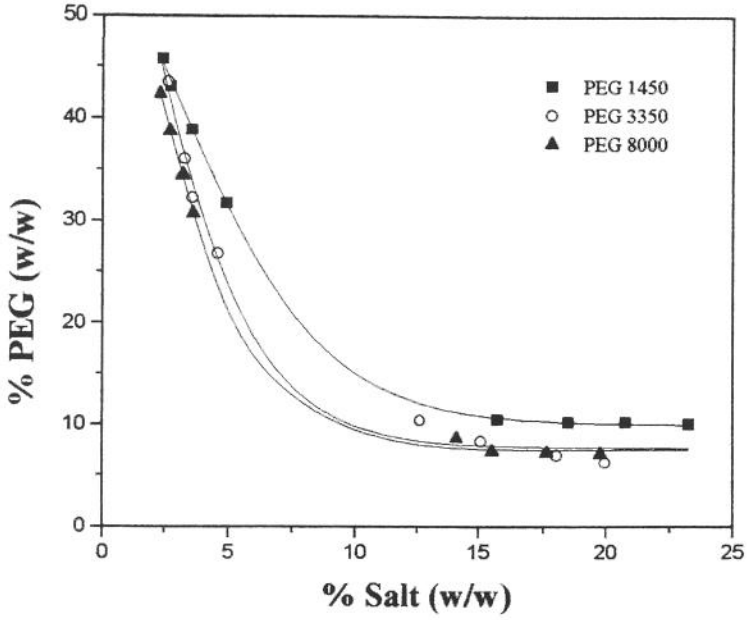

Figure 3 – Binodal curves at 25°C and pH 9 for systems PEG + potassium phosphate + urea. (■) PEG 1450, (O) PEG 3350, (▲) PEG 8000.

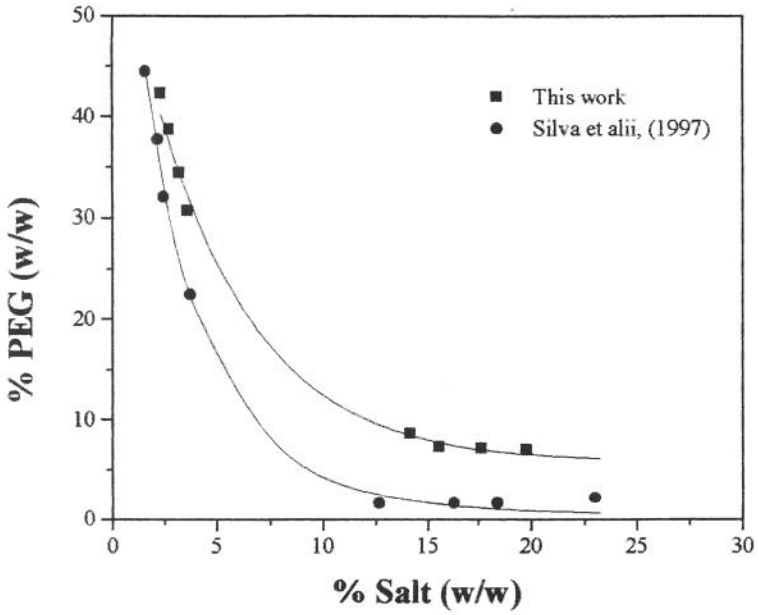

Figure 4 – Binodal curves for systems PEG 8000 + potassium phosphate + urea (■) and PEG 8000 + potassium phosphate (●) at 25 °C and pH 9.

5.3.2. Protein Partitioning

Lysozyme, catalase and β-galactosidase were partitioned at 25 °C in the PEG + potassium phosphate + urea + water systems at different pHs. Two urea concentrations, 3 and 6 wt%, were tested to show the effect of urea concentration on the protein partition coefficients. Partitioning at three different tie lines was determined for each system. The partition coefficient (K) was calculated as $K_{Protein} = C_T/C_B$, where C_T and C_B are the protein concentrations in mg/g of the top and bottom phases, respectively. For the partition coefficients the mean deviation was ± 0.001. Tables 3, 4 and 5 show the partition coefficients of lysozyme, β-galactosidase and catalase at two different pHs in the PEG + potassium phosphate + urea + water systems.

Table 3 - Partition coefficients of lysozyme at 25 °C in PEG + potassium phosphate + urea + water systems

System	%PEG/Salt (w/w)	% urea (w/w)	pH = 7	pH = 9
PEG 1450	24.69/15.28	3.11	1.20	4.15
	25.28/15.16	3.05	1.36	3.14
	26.47/14.92	2.99	1.54	2.92
PEG 3350	24.48/15.33	3.09	1.42	1.81
	25.13/15.14	3.16	1.30	1.77
	26.51/14.73	2.99	1.43	2.14
PEG 8000	24.58/15.29	3.07	0.38	1.04
	25.21/15.24	3.04	0.46	0.91
	26.38/14.90	2.99	0.52	1.67
PEG 1450	21.80/13.62	6.06	1.05	2.05
	22.70/13.64	6.12	1.26	2.21
	24.04/13.59	6.03	1.41	2.48
PEG 3350	21.75/13.57	6.04	0.95	1.10
	22.58/13.58	6.14	1.29	1.13
	23.86/13.65	6.05	1.31	1.21
PEG 8000	21.89/13.63	6.17	0.26	2.37
	22.48/13.67	6.09	0.28	1.35
	23.95/13.59	5.97	0.46	0.66

The highest values for the lisozyme partition coefficients were obtained in PEG 1450/salt/urea 3 % system at pH 9 and the lowest ones were obtained in PEG 8000 + salt + urea 6 % system at pH 7. Table 3 shows that the partition coefficient decreases with increasing PEG molecular weight. In general, the partition coefficient increases as PEG concentration increases, except in the systems PEG 1450+urea 3 wt % and PEG 8000+urea 6 wt%, both at pH 9.

Table 4 - Partition coefficients of β-galactosidase at 25 °C in PEG + potassium phosphate + urea + water systems

System	%PEG/Salt (w/w)	% urea (w/w)	pH = 7	pH = 9
PEG 1450	24.61/15.28	3.15	0.26	3.93
	25.22/15.25	3.02	0.39	1.52
	26.44/14.88	2.97	0.55	1.42
PEG 3350	24.74/15.24	3.06	2.76	3.25
	25.19/15.13	3.08	2.42	2.47
	26.34/14.90	2.97	2.24	3.20
PEG 8000	24.10/15.20	3.12	0.48	1.43
	25.21/15.14	3.01	0.52	1.22
	26.38/14.87	2.92	0.56	2.47
PEG 1450	21.92/13.62	5.98	0.54	1.17
	22.91/13.49	6.06	0.64	1.53
	24.13/13.48	6.10	0.70	1.85
PEG 3350	21.85/13.55	6.12	1.47	2.41
	22.50/13.59	6.04	1.90	2.41
	23.95/13.51	5.95	1.98	2.22
PEG 8000	21.86/13.63	6.09	0.37	1.78
	22.58/13.62	6.08	0.48	0.80
	24.02/13.40	5.99	1.22	0.58

The highest values for β-galactosidase partition coefficients were obtained in PEG 3350 + salt + urea 3 wt% and PEG 1450 + salt + urea 3 wt%systems at pH 9 and the lowest ones were obtained in PEG 1450 + salt + urea 3 wt% system at pH 7. Table 4 shows that the partition

coefficient increases as PEG molecular weight changes from 1450 to 3350 and decreases as the PEG molecular weight changes from 3350 to 8000. In general, the partition coefficient increases as PEG concentration increases, except in PEG 3350 + urea 3 wt % for both pHs and in PEG 1450 + urea 3 wt%, PEG 3350 + urea 6 wt% and PEG 8000 + urea 6 wt% at pH 9.

Table 5 - Partition coefficients of catalase at 25 °C in PEG + potassium phosphate + urea + water systems

System	%PEG/Salt (w/w)	% urea (w/w)	pH = 7	pH = 9
PEG 1450	24.65/15.30	3.12	0.36	4.79
	25.30/15.22	3.09	0.53	3.53
	26.48/14.88	2.95	0.64	3.26
PEG 3350	24.52/15.25	3.04	1.61	2.52
	24.92/15.06	3.11	1.45	2.02
	26.39/14.83	2.94	1.55	2.57
PEG 8000	24.74/14.36	3.07	0.94	3.31
	25.05/15.19	3.04	0.99	3.08
	26.39/14.87	2.92	0.96	3.96
PEG 1450	21.95/13.57	6.11	0.67	3.02
	22.59/13.71	6.13	0.85	3.68
	24.15/13.45	5.94	0.93	3.57
PEG 3350	21.56/13.80	6.08	1.75	1.74
	22.50/13.63	6.05	2.05	1.80
	23.98/13.57	5.92	2.14	1.71
PEG 8000	21.87/13.59	6.02	0.80	5.38
	22.55/13.72	6.02	0.96	1.69
	24.07/13.48	5.93	2.25	1.49

The highest values for catalase partition coefficients were obtained in PEG 1450 + salt + urea 3 wt% and PEG 8000 + salt + urea 6 wt% systems, both at pH 9, and the lowest ones were obtained in PEG 1450 + salt + urea 3 wt% system at pH 7. Concerning the influence of PEG molecular weight the partition coefficients of catalase at pH 7 exhibit a behaviour similar to that

observed for β-galactosidase: the partition coefficients increases as the molecular weight changes from 1450 to 3350 e decreases as the molecular weight changes to 8000. At pH 9 the opposite behaviour was observed in most cases, since the lowest values for the partition coefficient were obtained for the molecular weight 3350. In general, at pH 7 the partition coefficient increases as the PEG concentration increases, except in PEG 3350 + urea 3 wt% and PEG 8000 + urea 3 wt%. At pH 9 this trend was not observed and for the PEG 1450 + urea 3 wt% and PEG 8000 + urea 6 wt% systems the obtained behaviour was exactly the opposite one.

In PEG/salt/urea systems at pH 9, the proteins partition preferentially to the top phase ($K_p>1$) and at pH 7, they partition in most cases preferentially to the bottom phase ($K_p<1$). In general the partition coefficients at pH 9 were higher than at pH 7 (Figure 5).

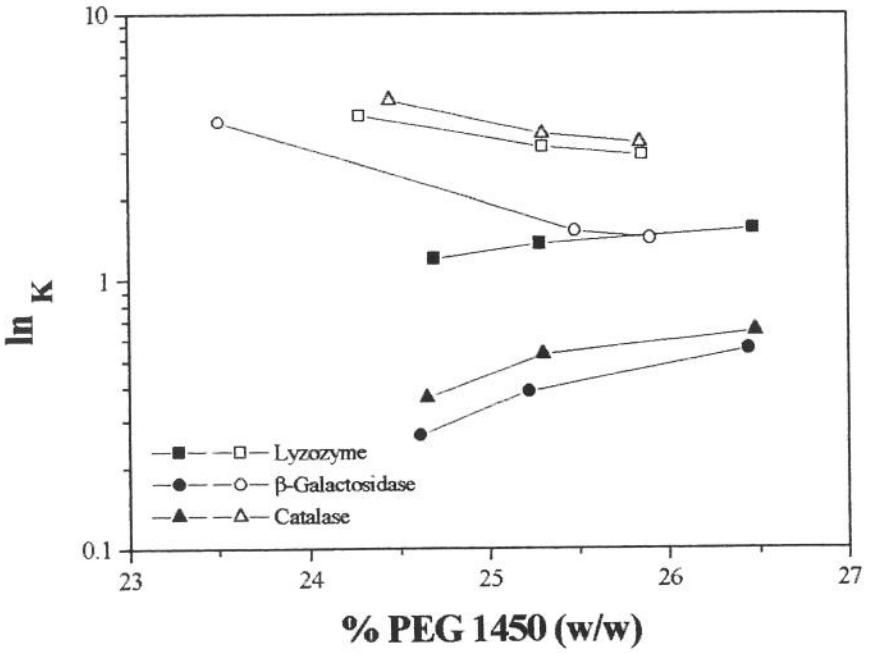

Figure 5 – Proteins partition coefficient in PEG 1450 + potassium phosphate + urea 3%.
(pH = 7 – solid symbols; pH = 9 – open symbols)

In most cases the partition coefficients decrease as the urea concentrattion changes from 3 to 6 wt%, as can be seen in Figure 6.

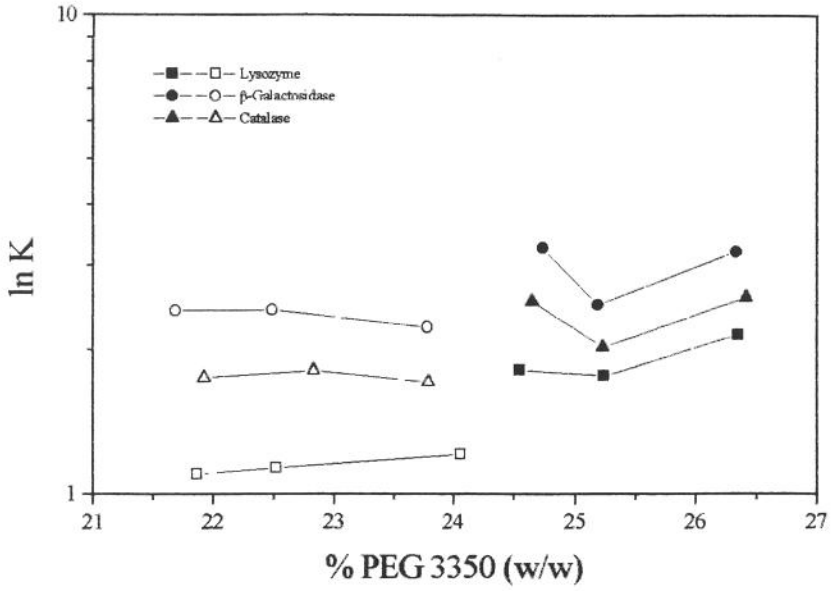

Figure 6 – Partition coefficient of proteins in PEG 3350 / potassium phosphate / urea at pH 9 (urea 3% - solid symbols; urea 6% - open symbols)

5.4. ACKNOWLEDGEMENT

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ANEXO 5.1 – DIAGRAMAS DE EQUILÍBRIO PARA OS SISTEMAS PEG/FOSFATO/URÉIA

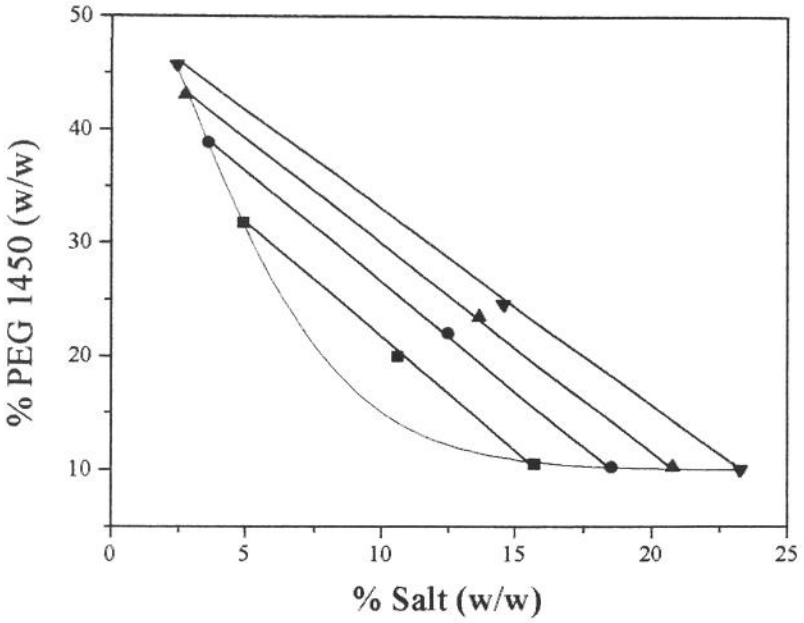

Figure 7 – Phase diagram for the PEG 1450 + potassium phosphate + water + urea 6 %wt at 25 °C and pH 9

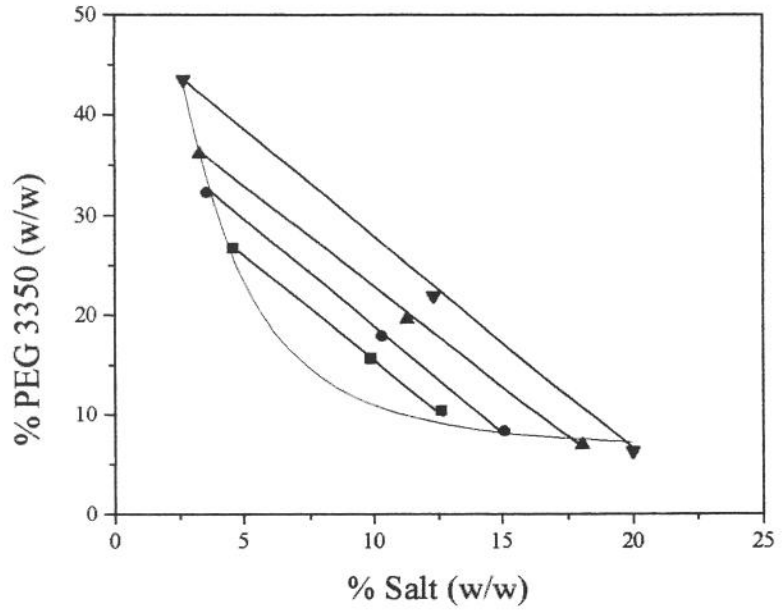

Figure 8 – Phase diagram for the PEG 3350 + potassium phosphate + water + urea 6 %wt at 25 °C and pH 9

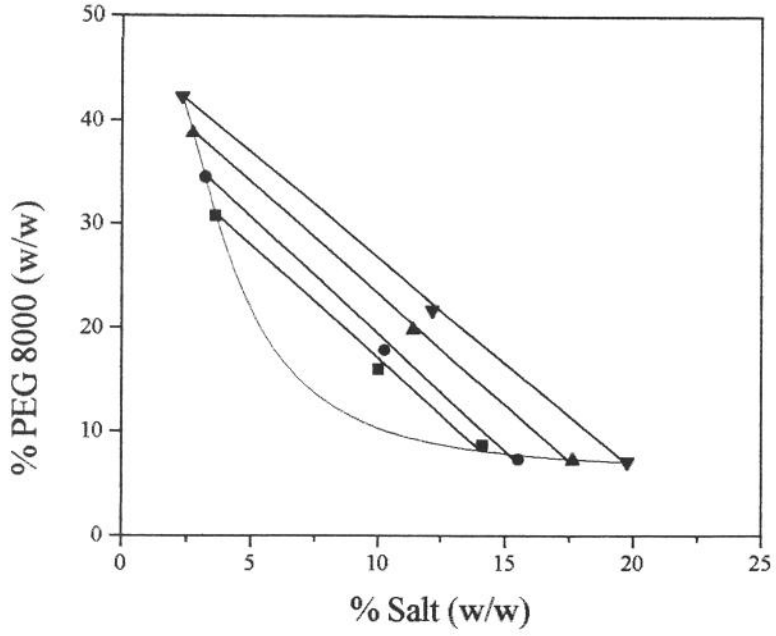

Figure 9 – Phase diagram for the PEG 8000 + potassium phosphate + water + urea 6 %wt at 25 °C and pH 9

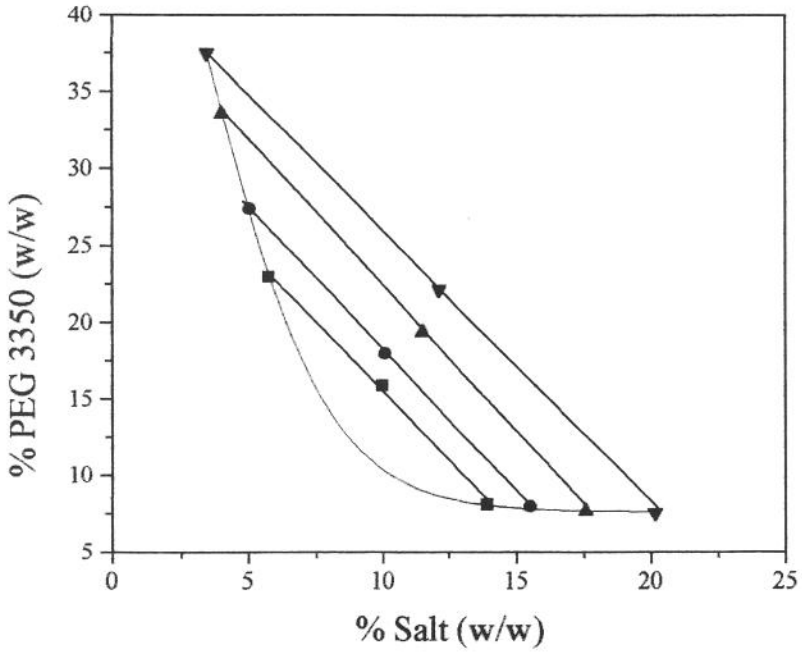

Figure 10 – Phase diagram for the PEG 3350 + potassium phosphate + water + urea 6 %wt at 25 °C and pH 7.

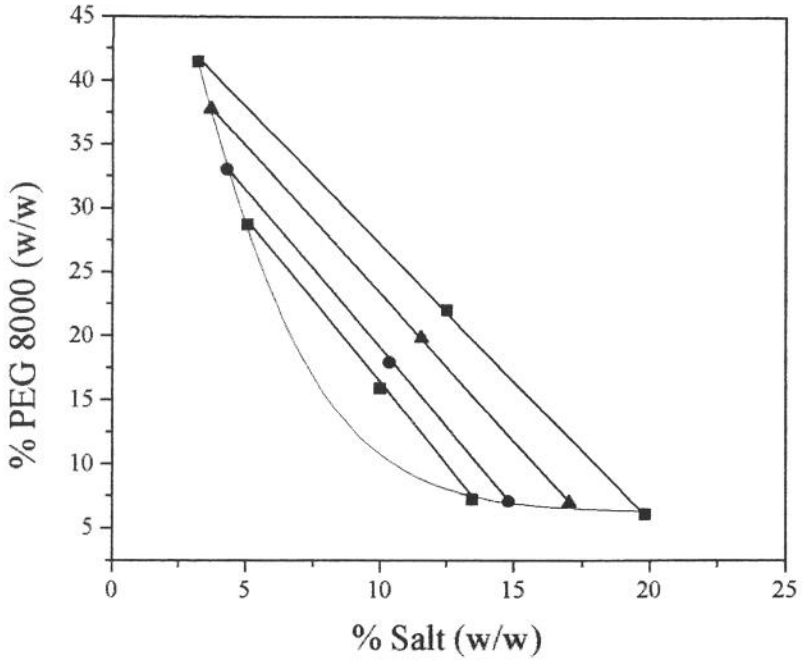

Figure 11 – Phase diagram for the PEG 8000 + potassium phosphate + water + urea 6 %wt at 25 °C and pH 7.

ANEXO 5.2 – PARTIÇÃO DE PROTEÍNAS: EFEITO DA CONCENTRAÇÃO DE URÉIA

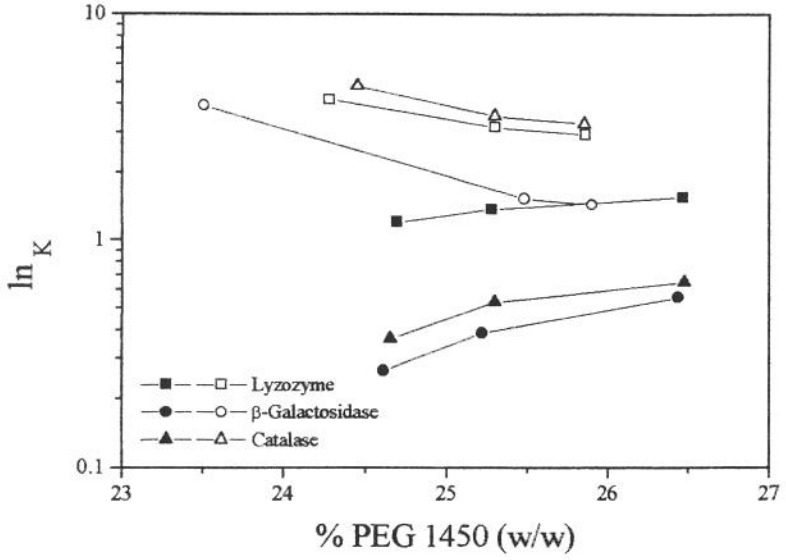

Figure 12 – Effect of urea on partition coefficient of proteins in PEG 1450 + potassium phosphate + urea at pH 7 (urea 3 wt% – solid symbols; urea 6 %wt – open symbols)

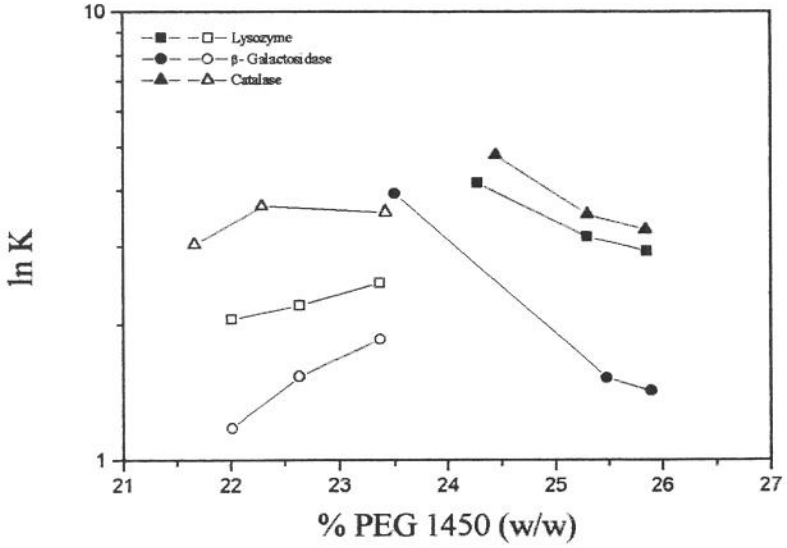

Figure 13 – Effect of urea on partition coefficient of proteins in PEG 1450 + potassium phosphate + urea at pH 9 (urea 3 wt% – solid symbols; urea 6 %wt – open symbols)

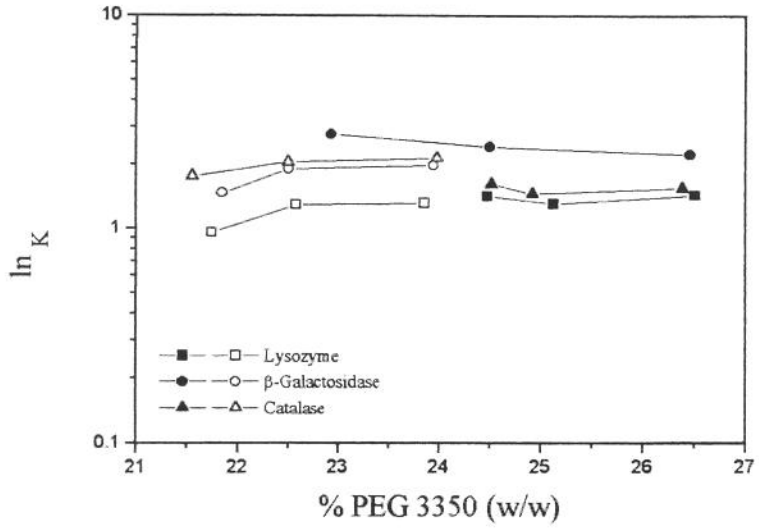

Figure 14 – Effect of urea on partition coefficient of proteins in PEG 3350 + potassium phosphate + urea at pH 7 (urea 3 wt% – solid symbols; urea 6 %wt – open symbols)

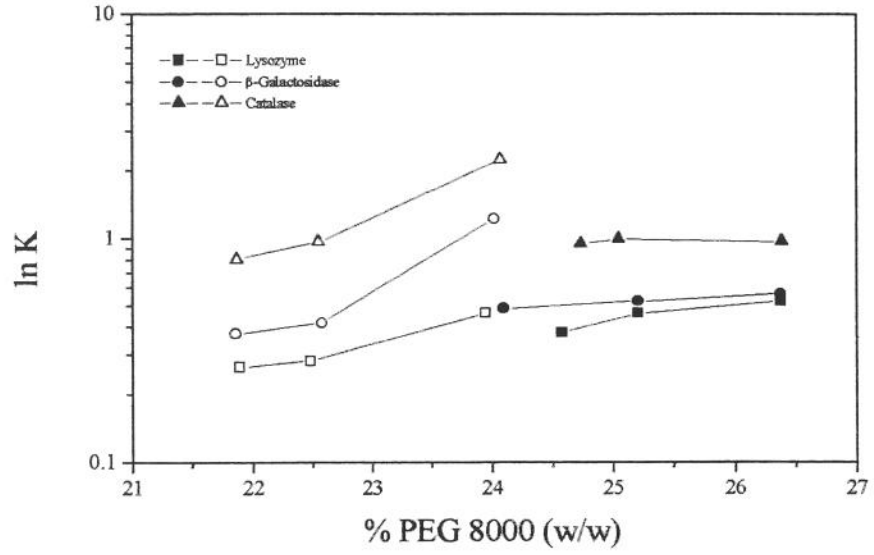

Figure 15 – Effect of urea on partition coefficient of proteins in PEG 8000 + potassium phosphate + urea at pH 7 (urea 3 wt% – solid symbols; urea 6 %wt – open symbols)

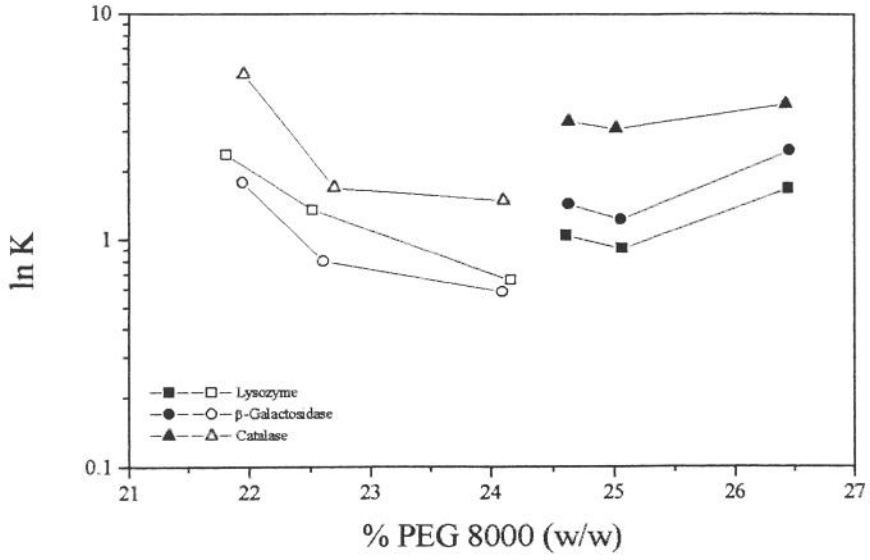

Figure 16 – Effect of urea on partition coefficient of proteins in PEG 8000 + potassium phosphate + urea at pH 9 (urea 3 wt% – solid symbols; urea 6 %wt – open symbols)

ANEXO 5.3 - PARTIÇÃO DE PROTEÍNAS: EFEITO DO pH

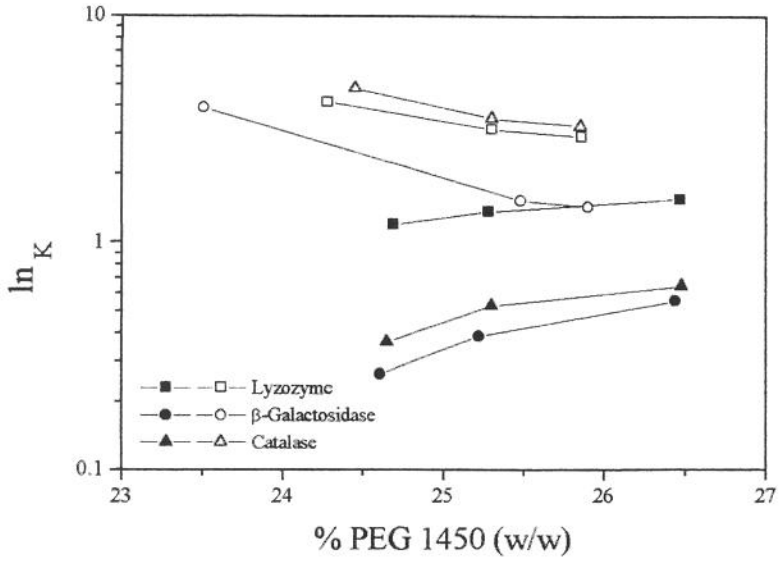

Figure 17 – Effect of pH on partition coefficient of proteins in PEG 1450 + potassium phosphate + urea 3 wt% (pH = 7 – solid symbols; pH = 9 – open symbols)

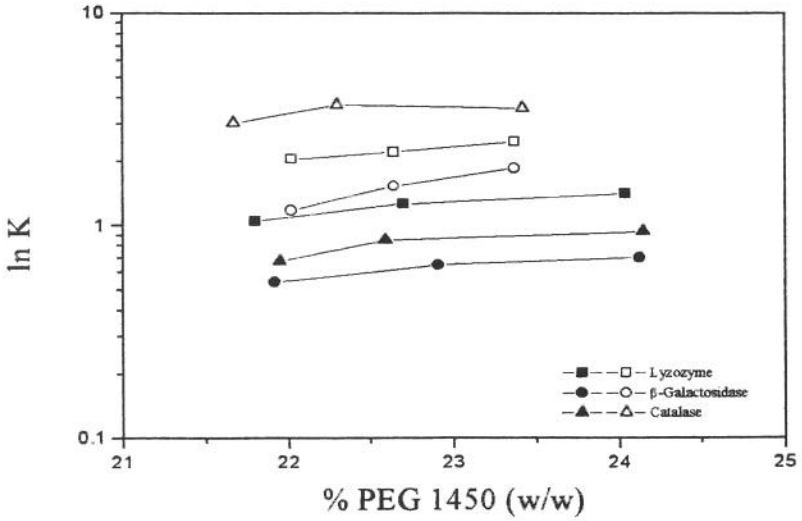

Figure 18 – Effect of pH on partition coefficient of proteins in PEG 1450 + potassium phosphate + urea 6 wt% (pH = 7 – solid symbols; pH = 9 – open symbols)

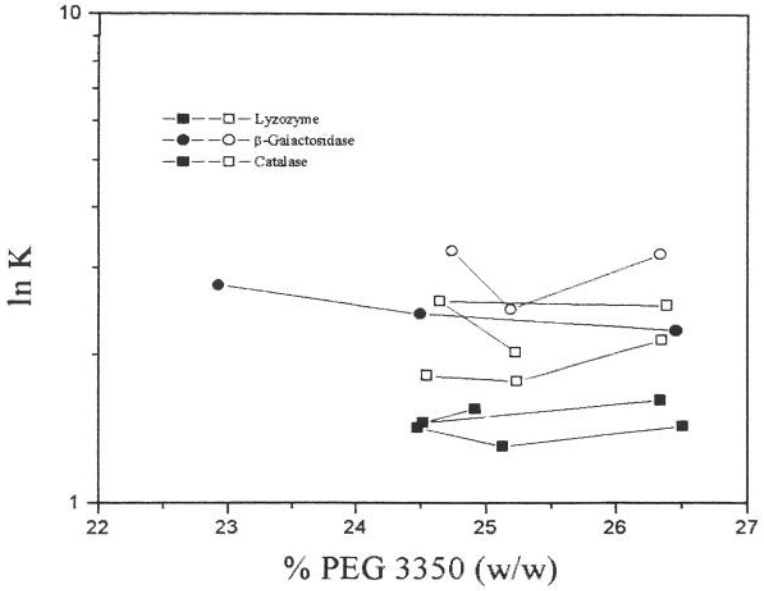

Figure 19 – Effect of pH on partition coefficient of proteins in PEG 3350 + potassium phosphate + urea 3 wt% (pH = 7 – solid symbols; pH = 9 – open symbols)

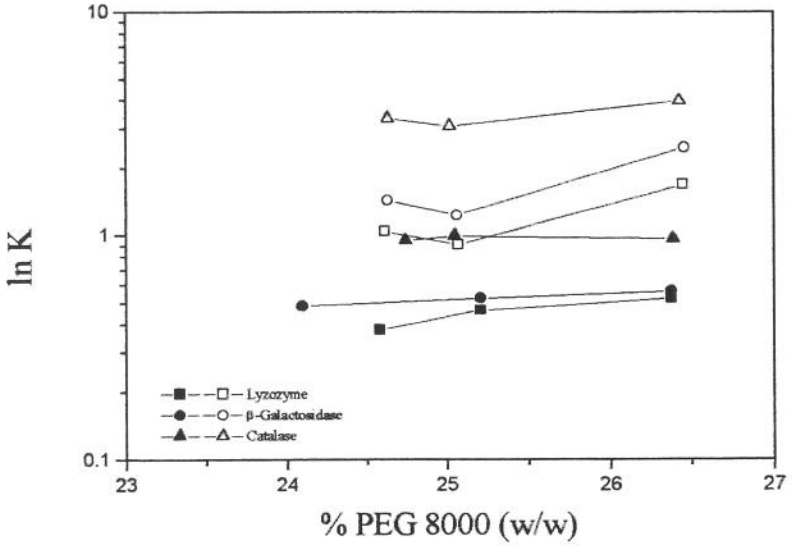

Figure 20 – Effect of pH on partition coefficient of proteins in PEG 8000 + potassium phosphate + urea 3 wt% (pH = 7 – solid symbols; pH = 9 – open symbols)

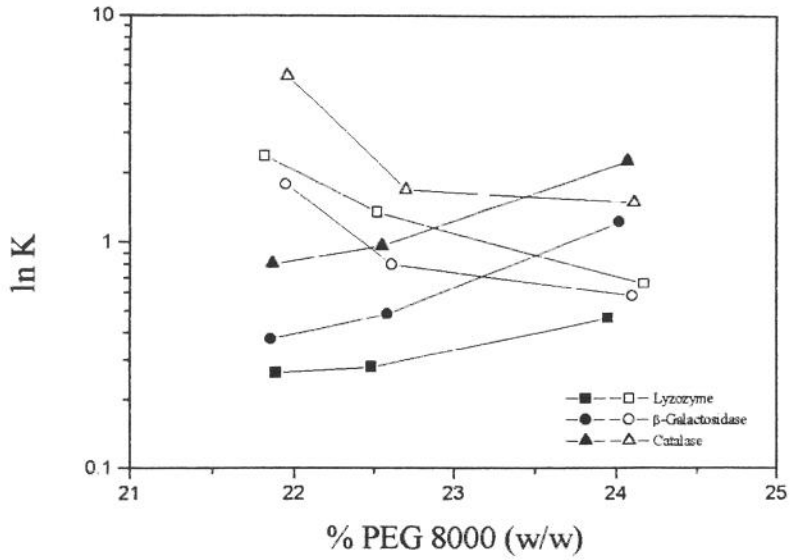

Figure 21 – Effect of pH on partition coefficient of proteins in PEG 8000 + potassium phosphate + urea 6 wt% (pH = 7 – solid symbols; pH = 9 – open symbols)

CAPÍTULO 6

CONCLUSÕES

CONCLUSÕES

Os resultados obtidos nesta pesquisa nos permitem concluir que:

1. As técnicas analíticas utilizadas para a obtenção das concentrações dos componentes, em todos os sistemas, apresentaram simplicidade de execução, boa reprodutibilidade e baixos valores de erro experimental.

2. A MD demonstrou ser uma boa alternativa à dextrana para uso em SABs, com características de separação muito semelhantes aos sistemas formados com dextrana. Apesar dos resultados indicarem que uma quantidade maior de MD nos sistemas PEG/MD é necessária para formar fases quando comparada à dextrana, o valor comercial é muitas vezes menor, justificando a substituição .

3. Nos sistemas PEG/MD o coeficiente de partição diminuiu a medida que aumentou a massa molar de PEG, os maiores valores de K foram encontrados para o sistema PEG 1450/MD 4000. Para os sistemas PEG/MD 2000 e PEG 1450/MD 4000 o coeficiente de partição aumentou a medida que a massa molar da proteína diminuiu (BSA < β-Lg < α-La). Nos sistemas PEG/MD 2000 as proteínas apresentaram valores de $K < 1$ mostrando a preferência pela fase de fundo rica em MD, já para os sistemas PEG/MD 4000 α-La no sistema PEG 1450/MD 4000 e β-Lg nos sistemas PEG 8000 e 10000/MD 4000 apresentaram $K > 1$, particionando na fase de topo rica em PEG.

4. Os sistemas PPG/MD apresentaram separação de fases com altos valores de comprimento das linhas de amarração, indicando que cada uma das fases (topo e fundo) contém uma concentração muito elevada de um dos componentes. Além disso, a partição de proteínas nestes sistemas apresentou valores de K maiores do que em sistemas PEG/MD, em geral $K > 1$, confirmando a preferência da partição pela fase de topo rica em PPG. Além de Ter menor massa molar, PPG é mais hidrofóbico que PEG.

5. O comprimento das linhas de amarração foi maior à medida que a diferença de hidrofobicidade entre os polímeros aumentou (sistemas PPG/MD), uma vez que as fases formadas são compostas por alta concentração de um dos componentes e praticamente nenhuma do outro. Além disso a concentração de água no sistema PPG/MD foi significativamente menor que o teor normalmente encontrado em SABs.

6. No caso do equilíbrio líquido-líquido, para se avaliar a qualidade dos dados experimentais não há um teste de consistência tal como para o equilíbrio líquido-vapor. Desta forma adotou-se a prática de comparar os desvios de balanço de massa obtidos, com aqueles calculados com dados de outros autores, levando em conta a escolha de pelo menos dois autores diferentes com dados julgados serem de boa qualidade. Visualmente, isto pode ser observado pela linearidade das três concentrações do sistema (topo, ponto de mistura e fundo). A partir do balanço de massa de cada componente em cada uma das fases, utilizando a água como referencial, já que ela é o componente que se apresenta em maior quantidade indicando um menor valor de erro, calculou-se o desvio experimental das linhas de amarração no capítulo 5. O mesmo procedimento foi adotado para os autores selecionados e os resultados obtidos apresentaram mesma ordem de grandeza.

7. Nos sistemas PEG/sal/uréia os maiores valores de K foram obtidos para os sistemas PEG 1450/uréia 3% e pH igual a 9. Nos sistemas PEG 1450 e pH 7, K aumentou quando a massa molar das proteínas diminuiu (lisozima < catalase < β-galactosidase)

8. O coeficiente de partição para a lisozima diminuiu com o aumento da massa molar de PEG. Em geral, K aumentou com o aumento da concentração de PEG.

9. Para a β-galactosidase e a catalase K aumentou quando a massa molar de PEG mudou de 1450 para 3350 e diminuiu quando a massa molar mudou de 3350 para 8000.

10. Nos sistemas PEG/sal na maioria dos casos para pH 9 as proteínas particionaram

preferencialmente na fase de topo rica em PEG (K > 1) e para pH 7 as proteínas particionaram na fase de fundo rica em sal (K < 1). Em geral K foi maior para pH 9 do que a pH 7.

11. .Para todos os sistemas estudados o aumento do peso molecular do polímero desloca a linha binodal para a esquerda, de forma que PEGs e PPGs com pesos moleculares maiores criam sistemas com regiões de separação maiores. A adição de uréia nos SABs PEG/sal diminuiu este efeito quando comparado aos mesmos sistemas sem uréia.

7.1.SUGESTÕES PARA TRABALHOS FUTUROS

Para dar continuidade ao trabalho desenvolvido novas idéias são sugeridas:

1. Testar novos sistemas aquosos bifásicos : Maltodextrina/Sal
   Polipropileno Glicol/Sal
   Polipropileno Glicol/Polietileno Glicol
   Polipropileno Glicol/Açúcares

2. Testar modelos termodinâmicos (UNIFAC, UNIQUAC, ASOG) para predição e modelagem dos dados de equilíbrio.

3. Utilizar redes neurais para a predição dos dados de equilíbrio.

4. Estudar o comportamento de partição de proteínas nestes sistemas com e sem adição de sais.

5. Testar modelos para a predição do coeficiente de partição.

APÊNDICES

APÊNDICE 8.1 - UMIDADE DOS POLÍMEROS

Tabela 1 - Umidade dos componentes dos SABs obtidas por Karl Fisher

Produto	Lote	Umidade (%)
Loremalt 2030	07/07/97	4,96
Loremalt 2001	07/07/97	5,87
Loremalt 2030	LQ 382	7,77
Loremalt 2001	LQ 274	7,72
PEG 1450	102H0550	0,75
PEG 3350	53H026	0,69
PEG 8000	26H0523	0,36
PEG 10000	121H2601	0,66
PPG 400	386716/1	0,49
PPG 3500	03112ER	0,43

APÊNDICE 8.2 - pH DAS FASES MEDIDOS EXPERIMENTALMENTE NOS SISTEMAS AQUOSOS BIFÁSICOS

Tabela 2 - pH das fases medidos nas amostras retiradas dos sistemas PEG/Sal.

Sistema	PEG 1450/7	PEG 3350/7	PEG 8000/7	PEG 1450/9	PEG 3350/9	PEG 8000/9
Tie line	pH das Fases nos sistemas					
T1	6,72	6,88	6,81	8,47	8,86	8,72
B1	6,66	6,96	6,92	8,89	8,95	9,02
T2	6,59	6,83	6,80	8,48	8,56	8,73
B2	6,94	6,91	6,92	8,86	9,00	8,95
T3	6,52	6,81	6,74	8,51	8,35	8,54
B3	6,90	6,92	6,91	8,66	9,01	8,98
T4	6,45	6,74	6,70	8,26	8,27	8,58
B4	6,71	6,92	6,90	8,83	8,99	9,17

T = topo; B = Fundo

Tabela 3 - pH das fases medidos nas amostras retiradas dos sistemas PEG/Maltodextrina.

Sistema	8000/2000	10000/2000	1450/4000	8000/4000	10000/4000
Tie line	pH das Fases nos sistemas				
T1	6,98	7,01	6,95	7,00	6,93
B1	7,00	7,01	6,99	7,00	6,95
T2	6,45	6,99	6,58	6,99	6,79
B2	6,96	7,03	6,87	7,08	6,94
T3	6,52	7,02	6,72	6,95	6,86
B3	6,54	7,05	6,90	6,98	6,90
T4	6,88	7,00	6,97	7,02	6,92
B4	6,90	7,01	7,00	7,06	6,98

T = topo; B = Fundo

APÊNDICE 8.3 – RESULTADOS DA ANÁLISE DE GPC

Tabela 4 – Distribuição de Pesos moleculares dos polímeros

Polímero	Peso molecular médio (Mw)	Polidispersidade (Mn/Mw)
Loremalt 2001	4004	1,987575
Loremalt 2030	2017	1,313645
Loremalt 2001	2374	1,744050
Loremalt 2030	1242	1,219054
PEG 1450	1468	1,03085
PEG 3350	2938	1,04340
PEG 8000	8768	1,09945
PEG 10000	11589	1,10638