



UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS
DEPARTAMENTO DE TECNOLOGIA DE ALIMENTOS

***RECUPERAÇÃO E ISOLAMENTO DE ENZIMAS POR
PARTIÇÃO DE BIOAFINIDADE EM
SISTEMAS DE DUAS FASES AQUOSAS***

PARECER

Este exemplar corresponde à redação final da tese defendida por Maria Estela da Silva aprovada pela Comissão Julgadora em 16 de agosto de 1999.

Campinas, 16 de agosto de 1999


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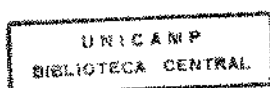
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*A Deus, aos meus pais
Paulo e Antonia, meus
irmãos Cesar, Carlos e
Érika, por me
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ABREVIACÕES

APGP	p-aminofenil- β -D-tiogalactopiranosídeo p-aminophenyl 1-thio- β -D-galactopyranoside
ATPS	Aqueous two-phase system
IDA	Ácido iminodiacético Iminodiacetic acid
K	Coeficiente de partição Partition coefficient
K _E	Coeficiente de partição da enzima
K _P	Coeficiente de partição das proteínas totais
MM	Massa Molecular
PEG	Polietilenoglicol Polyethylene glycol
PF	Purification Factor
R	Recuperação Recovery
S	Seletividade Selectivity
SA	Specific Activity
SDFA	Sistema de duas fases aquosas
U	Unidades Units

RESUMO

Este trabalho visou estudar o emprego de ligantes de afinidade para extração e purificação de diferentes enzimas por partição em sistemas de duas fases aquosas (SDFA). O emprego de ligantes específicos em um estágio inicial de um processo extrativo visa reduzir o número de etapas do mesmo, pois ao aumentar a seletividade e especificidade de uma operação, as próximas são diretamente beneficiadas pela redução de carga do material contaminante.

Inicialmente foram investigados métodos de ativação química do polietilenoglicol (PEG) com o intuito de torná-lo mais reativo para o acoplamento da molécula do ligante. Foram usados dois métodos de ativação deste polímero: com cloreto de tresila e com cloreto de tionila. Para ativação com cloreto de tresila, o rendimento obtido foi de 74%, determinado pela análise do enxofre elementar, enquanto que para a ativação com cloreto de tionila, o rendimento obtido foi de 86% (p/p) (capítulo 7).

Um estudo para extração e recuperação da β -galactosidase de diferentes origens microbianas: *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Aspergillus oryzae* e *Escherichia coli* foi então realizado (capítulo 3) e foi observado que somente a β -galactosidase de *Escherichia coli* era coletada na fase superior em sistema formado por PEG 4000 15% e fosfato 13,6% (coeficiente de partição, $K = 52$). As demais β -galactosidases eram sempre particionadas em direção à fase inferior salina juntamente com as principais proteínas contaminantes, impossibilitando a separação e purificação. Com o objetivo de separar a β -galactosidase das outras proteínas, um processo específico de partição por afinidade foi desenvolvido, o qual consistiu de duas etapas: acoplamento específico da enzima ao ligante e rompimento desta interação. Para eficiente separação entre a β -galactosidase de *Kluyveromyces fragilis* e os contaminantes protéicos foi desenvolvido um processo em duas etapas, aonde a primeira utiliza o ligante APGP (p-aminofenil- β -D-tiogalactopiranosídeo). Os sistemas usados foram formados, na primeira

etapa (acoplamento da enzima), por PEG 4000-APGP 6% e dextrana 505.000 8% e na segunda etapa (desacoplamento da enzima) por PEG-APGP 13% e fosfato 9%.

Na primeira etapa de purificação, o K da β -galactosidase teve um aumento de aproximadamente 2.800 vezes com o uso do PEG-APGP, apresentando recuperação de 55% da enzima na fase polimérica do sistema PEG-APGP/dextrana e a segunda etapa da purificação da enzima foi realizada em sistema formado por PEG-APGP 13% e fosfato 9%, mostrando-se altamente eficiente na reversão do K da enzima β -galactosidase para a fase oposta. O valor do coeficiente de partição foi de $2,2 \times 10^{-5}$ com 39% de recuperação da enzima na fase salina (capítulos 3 e 4).

Com o intuito de investigar a capacidade do polímero de afinidade PEG-IDA- Cu^{2+} em extrair proteínas nos SDFA, a partição da lisozima proveniente de clara de ovo e da peroxidase de soja foi investigada (capítulos 5 e 6). A lisozima de clara de ovo de galinha foi utilizada como enzima modelo nos estudos preliminares em sistemas de afinidade contendo o complexo PEG-IDA- Cu^{2+} . Esta enzima possui um resíduo de histidina disponível na sua superfície, tornando viável sua extração neste tipo de sistema, já que um resíduo de histidina é suficiente para formar o complexo enzima-metalo-ligante. As concentrações empregadas foram de PEG 4000 13% e fosfato de potássio 9% (p/p), pH 7,0 nos SDFA sem o ligante. Em sistemas de afinidade, as concentrações do PEG-IDA- Cu^{2+} foram de 1, 5 e 10% e as concentrações de PEG 4000 foram de 12, 8 e 3%, respectivamente, sendo que a concentração do fosfato permaneceu em 9%. O valor do coeficiente de partição, K, foi aumentado 9 vezes quando PEG-IDA- Cu^{2+} foi usado nos SDFA de afinidade, sendo possível recuperar 51% da lisozima.

Para recuperação da peroxidase de soja, *Glycine max*, o metalo-ligante cobre (Cu^{2+}) foi acoplado ao complexo PEG-IDA, formador da fase polimérica do sistema. Duas etapas foram suficientes para a recuperação da peroxidase usando sistemas de afinidade. Na primeira etapa da extração, o sistema foi composto por PEG-IDA- Cu^{2+} 14% e sulfato de sódio 8% para o acoplamento da enzima e na segunda etapa, um sistema constituído de

PEG-IDA-Cu²⁺ 14% e fosfato 10% foi usado para o desacoplamento da enzima, a qual foi recuperada na fase salina. Obteve-se um aumento de K de 24 vezes e recuperação maior que 100% da enzima, quando o sistema de afinidade foi usado. Na etapa de desacoplamento da enzima obteve-se 64% de recuperação. A fase polimérica foi reciclada e usada num sistema contendo PEG-IDA-Cu²⁺ 14% e sulfato de sódio 8%, obtendo-se um K = 23, com recuperação de 85% da peroxidase.

SUMMARY

In this work the extraction and purification of β -galactosidase from different species and soybean peroxidase (*Glycine max*) in aqueous two-phase systems (ATPS) by bioaffinity were studied. The synthesis of affinity polymers to recover this enzyme was also studied.

Initially the extraction of β -galactosidase from *Kluyveromyces lactis*, from *Kluyveromyces fragilis*, from *Aspergillus oryzae* and from *Escherichia coli* was compared. It was observed that only the β -galactosidase from *Escherichia coli* partitioned to the top phase in a system formed of 15% PEG 4000 and 13.6% phosphate (partition coefficient, $K = 52$). β -galactosidases from other sources partition into the bottom salt-rich phase, together with the main contaminant proteins, preventing separation and purification.

The specific ligand APGP (p-aminophenyl 1-thio- β -D-galactopyranoside) is an inhibitor of β -galactosidase, and it was attached to polyethylene glycol (PEG). Two methods of activation were used: activation with tresyl chloride and with tionyl chloride. The yield for activation of tresyl chloride was 74%, determined by elementary sulphur analysis, and the yield for activation with tionyl chloride was 86%.

A new two-step method for extraction and purification of the enzyme β -galactosidase from *Kluyveromyces fragilis* was then developed. In the first step, an affinity system composed of 6% PEG 4000-APGP and 8% dextran 505 was used, where β -galactosidase primarily partitioned toward the top phase ($K 2,330$). In the second step, a system formed of 13% PEG-APGP and 9% phosphate salt was used to revert the value of the partition coefficient, K , of β -galactosidase to 2×10^{-5} , thereby achieving the purification and recovery of 39% of the enzyme in the bottom salt-rich phase.

The extraction of the model protein lysozyme from chicken egg white was studied by affinity in systems with the complex PEG-IDA- Cu^{2+} . This enzyme has a histidine residue available on its surface, making possible its recovery in this system. Metal affinity

partitioning in ATPS is a useful tool to extract the proteins which have accessible histidine, cysteine or tryptophan on their surfaces.

Partitioning of lysozyme in a PEG 4000/phosphate system, pH 7.0, was studied in the presence and in the absence of PEG-IDA-Cu²⁺. The composition of systems without ligands was 13% PEG 4000 + 9% phosphate, and the affinity systems had correspondent amounts of PEG 4000 replaced by 1%, 5% and 10% of PEG-IDA-Cu²⁺. The partition coefficient, *K*, of the lysozyme increased ninefold when 5% PEG-IDA-Cu²⁺ was used, and 45% of the enzyme was recovered in the top ligand-rich phase of the system. This is a pioneer work on liquid-liquid extraction of lysozyme in ATPS with PEG-IDA-Cu²⁺.

Then the extraction of peroxidase from a crude extract of defatted soybean peroxidase (*Glycine max*) by metal affinity in an ATPS was studied. A two-step liquid-liquid extraction process using metal ligand was developed aiming to purify the peroxidase. In the first purification step, the system was composed of 14% PEG 4000-IDA-Cu²⁺ and 8% Na₂SO₄ and the peroxidase partitioned mainly to the top phase (*K* = 24). In the second step, a system formed of 14% PEG 4000 and 10% phosphate was used to revert the value of the partition coefficient of peroxidase to the bottom salt-rich phase (*K* = 0.05), providing the purification and recovery of 64% of the enzyme. The top PEG 4000-IDA-Cu²⁺-rich phase was washed by ultrafiltration in order to remove the sulphate salt and reused in another cycle of peroxidase purification.

Only two steps were enough to recover the enzymes β-galactosidase and peroxidase in the ATPS by bioaffinity.

INTRODUÇÃO

Os sistemas de duas fases aquosas (SDFA) são empregados para extração e purificação de biomoléculas, células e organelas, antibióticos, como também produtos de fermentação, como acetona, butanol e etanol.

Em 1956, Albertsson observou a partição de cloroplastos em SDFA composto por PEG/sal e de outras organelas celulares e macromoléculas em sistemas formados por PEG/dextrana. A partir da década de 1970, as características físico-químicas do sistema PEG/dextrana e as propriedades de partição de biomoléculas foram amplamente estudadas (ALBERTSSON, 1971; JOHANSSON, 1970; JOHANSSON *et al.*, 1973; JOHANSSON, 1974). Nos anos 80 diversos grupos de pesquisadores utilizaram os SDFA para recuperação em maior escala de biomoléculas (KRONER *et al.*, 1982a; KULA *et al.*, 1982; VEIDE *et al.*, 1983; KRONER *et al.*, 1984; CORDES & KULA, 1986; TJERNELD *et al.*, 1987; SZÖKE *et al.*, 1988; CORDES & KULA, 1994; LIN *et al.*, 1996), devido às dificuldades encontradas nos processos escalonados de purificação por cromatografia, por ser este último um processo descontínuo e devido à baixa taxa de transferência de massa (CARLSSON, 1988). Recentemente a termodinâmica da separação de fases foi desenvolvida (BROOKS *et al.*, 1985; BASKIR *et al.*, 1987; KANG & SANDLER, 1988; KING *et al.*, 1988; BASKIR *et al.*, 1989a; CABEZAS *et al.*, 1990; CABEZAS *et al.*, 1992; ROGERS & BAUER, 1996; WU *et al.*, 1998) e modelos matemáticos para partição molecular foram descritos (BROOKS *et al.*, 1985; BASKIR *et al.*, 1987; CORDES *et al.*, 1987; KANG & SANDLER, 1988; KING *et al.*, 1988; BASKIR *et al.*, 1989a; SUH & ARNOLD, 1990; LUTHER & GLATZ, 1994; EITEMAN *et al.*, 1994; WU & ZHU, 1999; WU *et al.*, 1999).

Os SDFA são geralmente formados por dois polímeros ou por um polímero e um sal liotrópico em água, que acima de determinadas concentrações ocorre a formação de duas fases. Um outro componente solúvel ao ser adicionado a este sistema distribui-se entre as fases após equilíbrio. A relação entre a concentração deste composto solúvel na fase superior (menor densidade) e fase inferior (maior densidade) define o coeficiente de partição, K .

O mecanismo de afinidade é comum a diversas biomoléculas, consistindo na capacidade que estas possuem em reconhecer e se ligar à outras moléculas especificamente. Essa característica é utilizada em diversos processos de separação e purificação por afinidade. O ligante é acoplado por ligação covalente à uma das fases ricas em polímero e portanto, sua partição ocorre de maneira extrema, em direção a fase superior ou inferior. Se a proteína "alvo" a ser isolada tiver afinidade pelo ligante, a formação do complexo ligante-proteína induzirá a alteração da partição da proteína para aquela fase enriquecida no ligante.

Segundo WALTER *et al.* (1985) o fenômeno de partição em SDFA abrange diversos temas como processos de separação, purificação de proteínas, biotecnologia, bioquímica vegetal, adesão bacteriana, diferenciação e desenvolvimento celular, imunologia, química orgânica sintética, etc., e os bons resultados obtidos por esta técnica nem sempre podem ser obtidos por outros métodos disponíveis de separação.

O objetivo deste trabalho é a aplicação conjunta de dois conceitos de purificação e extração de proteínas: utilização de ligantes específicos e partição em SDFA. Os sistemas de afinidade desenvolvidos foram empregados para extração e purificação da β -galactosidase microbiana e da peroxidase de soja, *Glycine max*.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1.1 Introdução

Partição de biomateriais em SDFA atua seletivamente na purificação de constituintes celulares de vários tamanhos, incluindo proteínas, ácidos nucleicos, membranas e organelas celulares. O uso dos SDFA é simples e pode ser realizado sem o uso de equipamentos sofisticados. Devido à sensibilidade e fragilidade das biomoléculas, torna-se necessário a escolha de processos não agressivos que mantenham suas propriedades físicas e químicas. Os SDFA contém elevada quantidade de água (80 a 90%) em ambas as fases e podem constituir um excelente ambiente para células, organelas ou proteínas biologicamente ativas (ALBERTSSON, 1971; ELLING *et al.*, 1990; ALBERTSSON & TJERNELD, 1994). Dos vários métodos existentes na literatura para extração de biomoléculas, a extração líquido-líquido vem ganhando destaque por atender estas exigências básicas e sua aplicação industrial ser viável.

Os SDFA formam-se a partir da mistura de soluções aquosas de dois polímeros hidrófilos incompatíveis, como PEG (polietilenoglicol) e dextrana, ou de um polímero e um sal liotrópico, como PEG e fosfato de potássio. A extração nestes sistemas pode substituir as etapas iniciais da purificação (precipitação, filtração) e da cromatografia preparativa de proteínas, podendo também ser escalonada, sem perda significativa na eficiência do processo.

O coeficiente de partição, K , de cada enzima é calculado a partir da relação entre as concentrações enzimáticas em cada fase (superior/inferior). O sucesso dos SDFA em biosseparações se deve ao fato de se explorar a máxima diferença do coeficiente de partição das biomoléculas, isto é, o valor de K pode ser alterado pela mudança das condições do sistema, portanto, deve-se elevar o coeficiente de partição de uma proteína “alvo” e manter o K das outras proteínas indesejáveis (contaminantes) para conseguir uma eficiente separação de biomoléculas. O valor do K está relacionado com a escolha dos polímeros, massa molecular, temperatura e outros fatores (BASKIR *et al.*, 1989). Além da afinidade, outras propriedades físicas da biomolécula a ser purificada, como carga, hidrofobicidade, tamanho e concentração, apresentam um papel importante no processo de partição em SDFA (ALBERTSSON, 1983).

Um caminho promissor para a efetiva extração seletiva tem sido através do acoplamento de ligantes às enzimas ou ao polímero usado no sistema, promovendo o aumento da seletividade da purificação (AGUIÑAGA-DIAZ & GUZMÁN, 1996).

O uso de ligantes bioespecíficos acoplados ao PEG tem sido empregado para a recuperação de enzimas por partição em SDFA. Estes polímeros são pouco reativos e necessitam ser quimicamente ativados para numa segunda etapa, reagirem com o ligante específico para a purificação da enzima.

1.2 β -galactosidase

A enzima lactase ou β -galactosidase (β -D-galactosídeo galactohidrolase E.C. 3.2.1.23) catalisa a hidrólise da lactose, o açúcar do leite, em glicose e galactose e possui inúmeras aplicações na área de tecnologia de produtos lácticos, ou seja, produtos industrializados que possuam lactose. Produtos hidrolisados por lactases são pouco produzidos no Brasil e a demanda por tal tipo de produto poderia ser atendida no caso desta enzima ser produzida a baixo custo e possuir elevada qualidade.

A enzima β -galactosidase é formada intracelularmente em leveduras e bactérias e excretada por fungos. As fontes mais importantes são as espécies de *Aspergillus niger*, *A. oryzae*, *Kluyveromyces fragilis*, *K. lactis*, sendo também produzida por *Neurospora* (LANDMAN, 1954), *Escherichia coli* (WALLENFELS *et al.*, 1960), *Saccharomyces lactis* (GUY & BINGHAM, 1978), *Bacillus circulans* (KITAHATA *et al.*, 1991), *Scopulariopsis* sp (PASTORE & PARK, 1980) e *Erwinia aroidea* (FLORES & ALEGRE, 1996).

A massa molecular (MM) desta enzima depende de sua origem. A enzima de *Kluyveromyces fragilis* possui uma MM de 201.000 Da de acordo com MAHONEY & WHITAKER (1978), no entanto, GONZALEZ *et al.* (1990) encontraram uma MM de 270.000 Da para esta mesma fonte. Para a enzima proveniente de *Aspergillus oryzae* a MM foi estimada em 90.000 Da (PARK *et al.*, 1979), de *Alternaria palmi* 160.000 Da

(AGRAWAL & DUTTA, 1987), de *Bacillus megaterium* 130.000 Da (POLLARD & STEERS, 1973).

WALLENFELS *et al.* (1960) estudaram a β -galactosidase produzida por *Escherichia coli* ML309. Verificaram a hidrólise de diversos substratos pela enzima, constatando que tiogalactosídeos possuem elevada afinidade pela mesma enzima, mas não são hidrolisados pela β -galactosidase. Observou-se também que a afinidade da enzima pelo substrato era aumentada por NaCl.

WOYCHICK & WONDOLOWSKI (1973) utilizaram a β -galactosidase de *Aspergillus niger* imobilizada com glutaraldeído em esferas porosas de vidro. Verificaram a aplicabilidade desta enzima na hidrólise de lactose de leite e derivados. A lactose de soro de leite "doce" e do leite integral foi hidrolisada em aproximadamente um terço em comparação com a taxa de hidrólise da lactose de soro ácido. A enzima imobilizada apresentou a mesma propriedade funcional e estabilidade que a enzima solúvel, restando aproximadamente 75% de sua atividade original. O pH de 4,5 e a temperatura ótima de 55°C oferecem a vantagem de operação industrial, diminuindo o risco de contaminação microbiana.

GUY & BINGHAM (1978) relataram as propriedades da β -galactosidase de *Saccharomyces lactis* em leite e derivados como agente de conversão de lactose em monossacarídeos e observaram que o pH ótimo para hidrólise era 6,5. O tratamento térmico da enzima por 1 minuto a 60°C resultou em 97% de inativação, sendo esta completa a 70°C. Foi verificado que os íons potássio, magnésio e manganês aceleram brandamente a atividade catalítica da enzima, enquanto que os íons sódio e cálcio inibem a reação significativamente.

KITAHATA *et al.* (1991) relataram a síntese de oligolactossacarídeos pela β -galactosidase de *Bacillus circulans*. Esta enzima apresentou uma intensa atividade de transgalactosilação. A síntese de galactoligossacarídeos é de interesse na atualidade pelo seu favorecimento ao crescimento dos *Lactobacillus bifidus*.

Esta enzima possui vários outros substratos como por exemplo, o-nitrofenil- β -D-galactopiranosídeo, p-nitrofenil- β -D-galactopiranosídeo, p-nitrofenil- α -D-galactopiranosídeo, p-nitrofenil-N-acetil- β -D-galactosamina, p-nitrofenil- α -D-manopiranosídeo, p-nitrofenil- α -L-fucopiranosídeo, o-nitrofenil- β -D-fucopiranosídeo, p-nitrofenil- β -D-glucopiranosídeo e o-nitrofenil- β -D-xilopiranosídeo (AGRAWAL & DUTTA, 1987). Estes autores determinaram a especificidade da enzima para este substratos, incluindo a lactose (substrato natural). A enzima foi observada ser altamente específica, hidrolisando somente ligações β -D-galactopiranosídeo.

1.2.1 Uso da β -galactosidase

A β -galactosidase já é usada em escala industrial na hidrólise de lactose em leite e soro de queijo (GEKAS & LOPEZ-LEIVA, 1985).

Esta enzima catalisa a hidrólise da lactose, o açúcar do leite, com formação de glicose e galactose, sendo largamente empregada na indústria de alimentos, evitando a cristalização da lactose nos tratamentos de leite e derivados que envolvam resfriamento, congelamento e/ou concentração. A presença da lactose confere ao produto final o defeito de textura relatado como "arenosidade". Além disso, a hidrólise da lactose em leite e derivados poderia tornar tais produtos aceitáveis para a parcela da população que apresenta intolerância a lactose (deficiência enzimática endógena da β -galactosidase) (AGRAWAL & DUTTA, 1987; SALOFF-COSTE, 1993).

A levedura *Kluyveromyces fragilis* é uma boa fonte de enzima e diferentemente de bactérias e fungos, é aprovada para uso em alimentos (MAHONEY & WHITAKER, 1978) e também é disponível comercialmente (AGRAWAL & DUTTA, 1987).

As lactases podem ser utilizadas para a transformação de soro de queijo, um produto de descarte da indústria queijeira, em xarope de sabor doce. Estes xaropes derivados de lactose podem servir como substitutos dos xaropes de sacarose e dos xaropes de glicose de milho para uso em panificação, refrigerantes, laticínios e sobremesas, apresentando

propriedades favoráveis ao processamento de alimentos como boa umectância e habilidade para promover reação de Maillard, com uso também na hidrólise de lactose, adoçantes, solubilizantes, remoção de cristais (produtos lácteos) e pasta de dente (GODFREY & REICHELT, 1983).

1.2.2 Extração e purificação da β -galactosidase

As propriedades físico-químicas da lactase microbiana que vão determinar os processos de extração e isolamento dependem da fonte da enzima, isto é, do tipo do microrganismo produtor.

Cromatografia de afinidade usando o ligante p-aminofenil- β -tiogalactopiransídeo (APGP) foi realizada por STEERS *et al.* (1971). Foi observado que a β -galactosidase de *Escherichia coli* não se ligou diretamente ao inibidor APGP acoplado a suporte sólido, sendo necessário usar um espaçador de 21 Å. A enzima foi recuperada em 95% em uma única etapa. A enzima foi eluída com tampão borato de sódio 0,1 M, pH 7,5.

A β -galactosidase de *Kluyveromyces fragilis* foi purificada pela primeira vez por UWAJIMA *et al.* (1972) por técnicas cromatográficas clássicas. MAHONEY & WHITAKER (1978) purificaram esta mesma enzima por cromatografia em três etapas: primeiramente usando APGP como ligante contendo um espaçador, obtendo purificação de 12,8 vezes, seguida por cromatografia usando hidroxapatita (purificação = 13,4 vezes) e então cromatografia de troca iônica (purificação = 14,9 vezes). Eles relataram que não foi possível recuperar a β -galactosidase em uma única etapa (afinidade), sendo necessária a utilização de outras etapas. STEERS *et al.* (1971) observaram que outras proteínas contaminantes podem competir com a β -galactosidase pelo ligante APGP.

PARK *et al.* (1979) produziram e caracterizaram as propriedades da β -galactosidase de *Aspergillus oryzae*. Verificaram que a temperatura ótima da enzima purificada era 50°C. Foi observado que os íons metálicos e reagentes sulfidrílicos não têm efeito na atividade

enzimática. Foi constatado também que a galactose inibia competitivamente a atividade enzimática, enquanto a glicose não.

O isolamento da β -galactosidase de *Scopulariopsis* sp tem sido feito convencionalmente, por cromatografia (PASTORE & PARK, 1980).

VEIDE *et al.* (1984) recuperaram β -galactosidase proveniente de *Escherichia coli* usando SDFA constituído de diferentes composições de PEG 4.000/fosfato de potássio, com extração contínua, obtendo rendimento (R) de 83,5% e fator de purificação (FP) de 13,6.

SDFA constituído de PEG 4000 5,3% e fosfato de potássio 12,5% foi utilizado em larga escala para uma purificação primária de β -galactosidase de *Escherichia coli* recombinante (VEIDE *et al.*, 1987) e conseguiram 105% de recuperação.

AGRAWAL & DUTTA (1987) purificaram a β -galactosidase de *Alternaria palmi* por cromatografia de troca iônica e obtiveram um aumento na purificação de 30 vezes e recuperação da enzima em 60%, utilizando 3 etapas: fracionamento com acetona, ultrafiltração e cromatografia.

KÖHLER & VEIDE (1994) através da tecnologia do DNA recombinante utilizando o microrganismo *Escherichia coli*, fundiram o plasmídio responsável pela produção da enzima β -galactosidase a um outro plasmídio contendo um resíduo hidrofóbico de fácil recuperação em SDFA (PEG/fosfato de potássio, pH 7), conseguindo 64% de recuperação desta enzima em uma única etapa.

β -galactosidase recombinante foi purificada em SDFA. LUTHER & GLATZ (1995) fundiram cadeias de peptídeos carregados à β -galactosidase, purificaram em SDFA compostos de PEG/dextrana contendo pequena quantidade de um polímero carregado DEAE ou dextrana-sulfato e obtiveram um aumento no coeficiente de partição da enzima acima de 37 vezes.

SDFA foi usado em fermentação extrativa de β -galactosidase de *Escherichia coli*. A enzima foi particionada para a fase superior, enquanto que os contaminantes e as células

foram particionados para a fase inferior (SILFWERBRAND *et al.*, 1991; KUBOI *et al.*, 1995).

A Tabela 1 apresenta uma comparação de métodos de purificação usados para β -galactosidase de *Kluyveromyces fragilis*.

Tabela 1. Comparação de métodos de purificação de β -galactosidase de *Kluyveromyces fragilis*.

Processos	Fator de Purificação	Recuperação (%)	Atividade Específica (U/mg)	Referência
1) APGP	12,8	66	194	MAHONEY &
2) Hidroxilapatita	13,4	63	204	WHITAKER
3) DEAE-Sephadex A-50 (gradiente linear)	14,7	61	223	(1978)
4) DEAE-Sephadex A-50 (gradiente de intervalos)	14,9	61	226	
SDFA convencional:				GONZALEZ <i>et</i>
1) PEG/fosfato	6,5	66	190	<i>al.</i> (1990)
SDFA convencional:				
1) PEG/dextrana	2,8	57	104	SILVA &
SDFA por afinidade				FRANCO
1) PEG-APGP/dextrana (acoplamento)	9,7	55	361	(1999a)
2) PEG-APGP/fosfato (desacoplamento)	19	39	708	

1.3 Peroxidase

Peroxidase (doador: hidrogênio-peróxido oxidoreductase EC 1.11.1.7) é a mais freqüente enzima usada na produção de kits de imunoanálises (MIRANDA *et al.*, 1995),

kits para diagnose médica e em medidas colorimétricas do peróxido de hidrogênio (MIRANDA & CASCONI, 1994). O esquema abaixo exemplifica a reação de óxido-redução catalisada pela peroxidase:



Peroxidases são heme-enzimas que possuem uma variedade de funções biossintéticas e degradativas usando peróxido de hidrogênio como aceptor de elétrons. Estas enzimas são encontradas em bactérias, fungos, plantas e vertebrados (DAWSON, 1988).

A peroxidase de nabo é a fonte mais abundante e comercialmente disponível (SHANNON *et al.*, 1966; LOBARZEWSKI & GINALSKA, 1996). A peroxidase de soja foi recentemente introduzida como substituta da peroxidase de nabo, devido a sua alta estabilidade em solvente, alto potencial redox e por possuir um custo menor (MIRANDA *et al.*, 1998), além da estabilidade similar à encontrada em enzimas microbianas termofílicas, pois é uma enzima altamente termoestável e sua temperatura de desnaturação é de 90,5°C, sendo atípico em outras peroxidases microbianas e de vegetais (McELDOON & DORDICK, 1996). O interesse científico em peroxidase de vegetais tem aumentado. Segundo LOBARZEWSKI & GINALSKA (1996), entre os anos 1970 e 1980 mais de 1.450 artigos sobre peroxidase de plantas foram publicados e entre 1980 e 1990, os números aumentaram para 3.293. Além das pesquisas focando o papel fisiológico e propriedades cinéticas das peroxidases de plantas, há também interesse desta enzima em aplicações industriais e analíticas.

A peroxidase de nabo é conhecida por ser uma glicoproteína (WELINDER, 1985), sendo que o conteúdo de carboidratos pode atingir 17% da massa molecular da peroxidase. Novas peroxidases de plantas têm sido isoladas e caracterizadas e geralmente são também glicosiladas (McMANUS & ASHFORD, 1997). Poucas peroxidases têm a estrutura da cadeia de seus carboidratos analisadas em detalhes.

Segundo SESSA & ANDERSON (1981) o pH ótimo da atividade da peroxidase de soja encontra-se entre 4 e 8, dependendo do tipo de tampão. Estudo comparativo verificou que o melhor pH desta enzima, quando incubada em tampão fosfato de potássio-ácido

cítrico 0.1 M, situava-se entre 4 e 6 e em tampão fosfato de potássio 0.1 M, o pH ótimo era entre 6,2 e 8. A peroxidase de soja tem uma massa molecular de 37.000 Da (McELDOON *et al.*, 1995).

1.3.1 Uso da peroxidase

Segundo EGOROV & GAZARYAN (1993) a peroxidase de soja é usada em processos para substituir íons cloreto no clareamento de papel, substituir oxidantes químicos para oxidar anilina no processo de produção de polianilina e quando microencapsulada, a peroxidase de soja é liberada e então produz um papel-carbono, com uma mudança de cor, sem as manchas deixadas pelo carbono. A aplicação da peroxidase se dá também em análises imunoquímicas, biossensores, degradação de compostos aromáticos e controle ambiental.

POKORA (1995) relata o uso desta enzima na produção de resinas fenólicas isentas de formaldeído.

Esta enzima também é usada em diagnose na determinação quantitativa de açúcares (LOBARZEWSKI & GINALSKA, 1996), especialmente para pessoas que apresentam diabetes.

MIRANDA *et al.* (1998) cita o uso de peroxidase em reagentes para diagnósticos clínicos, os quais requerem pequena quantidade de enzima altamente pura, na polimerização e precipitação de fenóis e aminas aromáticas no tratamento de água.

A peroxidase também pode ser aplicada na produção de aminas poliaromáticas e síntese de polifenóis (AKKARA *et al.*, 1999).

1.3.2 Extração e purificação da peroxidase

O processo de purificação de peroxidases com propósitos industriais requer elevadas quantidades de material vegetal, como por exemplo, no caso do nabo, e é usado somente por grandes indústrias como Merck, Boehringer, Sigma, Aldrich e Fluka (LOBARZEWSKI & GINALSKA, 1996), mas esse problema pode ser reduzido pelo uso de culturas líquidas de células de planta, descrito por CAIRNS *et al.* (1980) e MALDONALDO & VAN HUYSTEE (1980), os quais usaram células de amendoim. Este processo de purificação pode ser realizado em uma ou duas etapas.

Vários métodos têm sido descritos para a purificação da peroxidase. Eles envolvem precipitação por sulfato de amônio, exclusão por tamanho, cromatografia de troca iônica (SHANNON *et al.*, 1966; LASCU *et al.*, 1986; CHAVEZ & FLURKEY, 1984), por afinidade utilizando concavalina A (BRATTAIN *et al.*, 1976), metais (CHAGA *et al.*, 1992; CHAGA, 1994) e anticorpos monoclonais (ELLING *et al.*, 1991). Mas somente poucos métodos podem ser usados em larga escala. Cromatografia de interação hidrofóbica foi utilizada para a purificação da peroxidase de soja (FLURKEY *et al.*, 1978; SESSA & ANDERSON, 1981).

VILTER (1990) usou SDFA compostos de PEG/fosfato de potássio ou carbonato de potássio para purificar peroxidase de alga em pH 12 e relatou que a atividade da enzima era revertida pela troca de tampão a pH 7.

MIRANDA *et al.* (1998) desenvolveram um método para purificar peroxidase de nabo em SDFA em 2 etapas. Eles adicionaram microesferas contendo concavalina-A a sistemas compostos por PEG 1540 15% e fosfato 10%, pH 7,0 e recuperaram 60% da enzima adsorvida às mesmas.

A Tabela 2 apresenta uma comparação de processos de purificação para peroxidase de soja.

Tabela 2. Comparação de processos de purificação de peroxidase de soja.

Processos	Fator de Purificação	Recuperação (%)	Atividade Específica (U/mg)	Referência
1) Sulfato de amônio	1,44	96	9,80	SESSA & ANDERSSON (1981)
2) Bio-Gel P-60	2,54	76	17,30	
3) DEAE-Sephadex	8,20	51	56	
4) Concavalina A-Sepharose	115	14	782	
5) Fenil-Sepharose	199	7	1.352,90	
6) DEAE-Sepharose	628	4	4.272,70	
Cromatografia de afinidade:				MIRANDA <i>et al.</i>
1) Concavalina A	41	28	2.170	(1998)
SDFA:				
1) Triton X100/Triton X-45/ tampão acetato de sódio	2,80	99	310	
SDFA convencional:				SILVA & FRANCO (1999b)
1) PEG/sulfato de sódio	1,30	78	29	
SDFA por afinidade:				
1) PEG-IDA-Cu ²⁺ /sulfato de sódio (acoplamento)	1,50	106	35	
2) PEG-IDA-Cu ²⁺ /fosfato (desacoplamento)	145	64	3.333	

1.4 Sistema de Duas Fases Aquosas

Os SDFA são utilizados para extração e purificação de proteínas de elevado valor biológico, devido à redução do custo operacional em relação a processos convencionais e possibilidade de integração aos mesmos, operação contínua e controle facilitado. Outras

características desejáveis destes sistemas são a diferença de densidade entre as fases, o fato dos polímeros não serem tóxicos e serem biodegradáveis.

Os SDFA são empregados no isolamento e purificação de biomoléculas de importância comercial, células, proteínas, vírus, fragmentos de membranas e organelas celulares. A extração por SDFA oferece vantagens para o processamento em larga escala, tais como possibilidade de elevado rendimento e possibilidade de processamento contínuo (FLYGARE *et al.*, 1990; ENFORS *et al.*, 1992; ROSTAMI-JAFARABAD *et al.*, 1992a; ROSTAMI-JAFARABAD *et al.*, 1992b; COIMBRA *et al.*, 1994; TOMASKA *et al.*, 1995; PAWAR *et al.*, 1997; PORTO *et al.*, 1997; COIMBRA *et al.*, 1998), e redução do custo operacional em relação à processos convencionais (KULA, 1990).

Vários polímeros hidrófilos (naturais ou sintéticos), são capazes de produzir sistemas bifásicos ao se misturarem com um segundo polímero ou com um sal liotrópico de baixa massa molecular, como por exemplo, um sal de cátions bivalentes.

Este processo se fundamenta na formação de duas fases distintas de uma solução contendo dois polímeros incompatíveis solúveis em água (Dextrana, Ficoll), ou um polímero e uma alta concentração de um sal liotrópico (fosfatos, sulfatos). Após equilíbrio, a fase superior estará rica em PEG (menor densidade) e outra rica no segundo componente (dextrana, fosfato). Os sistemas mais empregados na produção de enzimas ou outras proteínas são compostos de um polímero de elevada massa molecular e de uma solução salina concentrada, ou de duas soluções concentradas de diferentes polímeros capazes de promover a separação de fases onde moléculas biológicas possam ser particionadas. As propriedades físico-químicas destes componentes vão modular as características dos SDFA na obtenção de melhor seletividade para a separação das moléculas biológicas. A separação de fases é resultante de interações energeticamente desfavoráveis entre as moléculas polímero-polímero ou polímero-sal (ALBERTSSON, 1986; ANDREWS & ASENJO, 1989).

Alterando a composição dos sistemas, a massa molecular e concentração dos polímeros, escolha e concentração do sal, carga, configuração molecular, hidrofobicidade e

a presença de ligantes específicos, pode-se manipular a partição de uma biomolécula (WALTER *et al.*, 1985).

1.5 Coeficiente de partição

Para substâncias solúveis (enzimas e proteínas) o coeficiente de partição, K , representa a distribuição de suas moléculas entre as duas fases líquidas do SDFA. Para proteínas, K_P , ele é expresso pela relação entre a concentração de moléculas da proteína P na fase superior sobre a concentração da mesma na fase inferior:

$$K_P = \frac{[P]_{\text{fase superior}}}{[P]_{\text{fase inferior}}}$$

e para enzimas, K_E , ele é expresso pela relação entre a atividade enzimática encontrada em cada fase:

$$K_E = \frac{\text{Atividade enzimática}_{\text{fase superior}}}{\text{Atividade enzimática}_{\text{fase inferior}}}$$

Para alcançar elevada recuperação e concentração de uma proteína desejada em SDFA, é necessário maximizar a diferença entre os valores de K da proteína a ser purificada e o K dos contaminantes. A influência de diversos fatores sobre a partição de proteínas é ilustrada pelo logaritmo do coeficiente de partição (K). Ela pode ser considerada a soma do logaritmo dos seguintes termos:

$$\ln K = \ln K_{\text{elet}} + \ln K_{\text{hidrof}} + \ln K_{\text{afin}} + \ln K_{\text{tamanho}} + \ln K_{\text{conform}}$$

Esta equação inclui um termo eletrostático (determinado pela carga livre e pH, $\ln K_{\text{elet}}$), um termo hidrofóbico ($\ln K_{\text{hidrof}}$), um termo de especificidade-afinidade ($\ln K_{\text{afin}}$), termo do tamanho molecular ($\ln K_{\text{tamanho}}$) e de conformação espacial ($\ln K_{\text{conform}}$).

O efeito de cargas eletrostáticas sobre o coeficiente de partição de proteínas foi extensivamente investigado por diversos autores (JOHANSSON, 1985; ALBERTSSON, 1986). Sais podem alterar as propriedades físicas dos sistemas, tais como o comprimento das linhas de amarração, a diferença de hidrofobicidade entre as fases, as quais afetam a partição de proteínas. A hidrofobicidade da superfície de proteínas e a interação com os polímeros e sais dos SDFA foi relatado pela literatura. Foi evidenciado que sistemas formados por PEG/fosfato, PEG/citrato e PEG/sulfato, possuem melhor resolução para detectar pequenas diferenças de hidrofobicidade protéica do que sistemas formados por dois polímeros, tais como PEG e dextrana (FRANCO *et al.*, 1996a). Foi observado que proteínas e oligopeptídeos com superfícies hidrofóbicas maiores tendem a se particionarem em direção a fase superior quando os demais parâmetros são mantidos (EITEMAN & GAINER, 1990; FRANCO *et al.*, 1996b). A partição de proteínas para uma das fases pode ser realizada pelo acoplamento de um ligante específico acoplado ao polímero formador do sistema (JOHANSSON, 1985; ANDREWS *et al.*, 1990; BIRKENMEIER, 1994). Tamanho e conformação das biomoléculas atuam significativamente na partição em SDFA. Como o processo de purificação de proteínas em SDFA depende das propriedades da superfície das moléculas (proteínas, contaminantes e outros componentes do sistema), pode-se dizer que este é um processo superfície-dependente (ALBERTSSON, 1971).

1.6 Tipos de sistemas

Os SDFA são agrupados em diferentes classes (Tabela 3) de acordo com as propriedades e características de seus componentes.

Tabela 3. SDFA descritos por ALBERTSSON (1986).

Classe 1: Sistemas polímero-polímero-água		
Polietilenoglicol	com	Alcool polivinílico Dextrana Ficoll
Polipropilenoglicol	com	Alcool polivinílico Dextrana Hidroxipropil-dextrana Metoxi-polietilenoglicol Polietilenoglicol
Alcool polivinílico	com	Metil-celulose Hidroxipropil-dextrana Dextrana
Dextrana	com	Etil hidroxietil celulose Hidroxipropil-dextrana Ficoll
Classe 2: Sistemas polímero/compostos de baixa massa molecular-água		
Polipropilenoglicol	com	Fosfato de sódio/potássio
Polietilenoglicol		Fosfato de sódio/potássio
Polipropilenoglicol	com	Glicose/glicerol
Dextrana	com	Alcool propílico

Os SDFA têm sido utilizados para recuperação e isolamento de proteínas e outros materiais biológicos nos últimos trinta anos. A Tabela 4 descreve a purificação de algumas biomoléculas por partição em SDFA.

Tabela 4. Biomoléculas purificadas em SDFA, descritas pela literatura.

Sistema	Biomolécula	Recup. (%)	Fator Purific.	Referência
PEG/fosfato	xilanase	41	57	DUARTE <i>et al.</i> (1999)
PEG/Dext.	β -glicosidase	85 - 95	2-3	JOHANSSON & RECZEY (1998)
PEG/fosfato	polifenol oxidase	50	5	SOJO <i>et al.</i> (1998)
PEG/Na ₂ SO ₄	β -galactosidase	90	-	CHANG & KOO (1998)
PEG/fosfato	glicoamilase	96	3	MINAMI & KILIKIAN (1998)
Polivinilpirrolidona/Reppal	peroxidase de nabo	75	7,3	MIRANDA & CASONE (1997)
PEG/fosfato/NaCl	Taumatina	90 - 95	20	CASONE <i>et al.</i> (1991)
PEG/fosfato de potássio	β -galactosidase	85 - 100	-	KÖHLER & VEIDE (1994)
	Fumarato hidratase (<i>Brevibacterium ammoniagenes</i>)	83	7,5	
	Fumarato hidratase <i>E. coli</i>	93	3,4	
	Aspartato-amônia-liase <i>E. coli</i>	96	6,6	
PEG/sal	Isoleucil-tRNA-sintetase <i>E. coli</i>	93	2,3	BOLAND <i>et al.</i> (1989)
	Penicilina amidase <i>E. coli</i>	90	8,2	
	β -galactosidase <i>E. coli</i>	87	9,3	
	Glucose-6-fosfato-desidrogenase <i>Leuconostoc</i> sp	94	1,3	
	Desidrogenase alcoólica <i>Saccharomyces cerevisiae</i>	96	2,5	
	L-2-hidroxiisocoproato desidrogenase <i>Lactobacillus casei</i>	93	17	
PEG/Dext.	Formaldeído desidrogenase <i>Candida boidinii</i>	94	-	
	Leucina desidrogenase <i>Bacillus sphaericus</i>	98	2,4	

A maioria das proteínas intracelulares de leveduras são mais eficientemente recuperadas se PEG de baixa massa molecular for usado na extração primária (HUDDLESTON *et al.*, 1991). Muitos autores usam PEG com massa molecular entre 4.000

e 6.000, sendo que polímeros maiores do que estes não são vantajosos, devido à viscosidade das soluções ser maior (INGHAM, 1984).

1.7 Diagrama de fase (curva binodal)

Os diagramas de fase dos SDFA representam a concentração dos seus componentes nas fases. Podem ser construídos de diferentes maneiras sendo mais utilizada a titulação de uma solução polimérica, X (primeiro componente), por uma solução polimérica ou salina Y (segundo componente). A concentração de X está representada na abcissa (x) e a concentração de Y na ordenada (y), sendo ambas expressas em porcentagem massa/massa. A obtenção de duas fases se caracterizará pelo turvamento da solução. Ao se adicionar uma concentração Z de água a esta mistura, a solução se tornará límpida novamente e monofásica. Portanto, a representação das concentrações do polímero X e Y nas duas situações (bi ou monofásica) inicia a construção de uma curva denominada curva binodal. A composição das concentrações relativas a cada ponto dos sistemas será representada por esta curva, sendo expressa em porcentagem de massa/massa. A Figura 1 representa um diagrama de fase de um sistema composto de PEG 6000, Dextrana 500 e água. As misturas formadas por concentrações dos componentes X e Y com pontos localizados na área acima da curva binodal (direita) produzem sistemas bifásicos e as localizadas abaixo da curva (esquerda) produzem sistemas monofásicos.

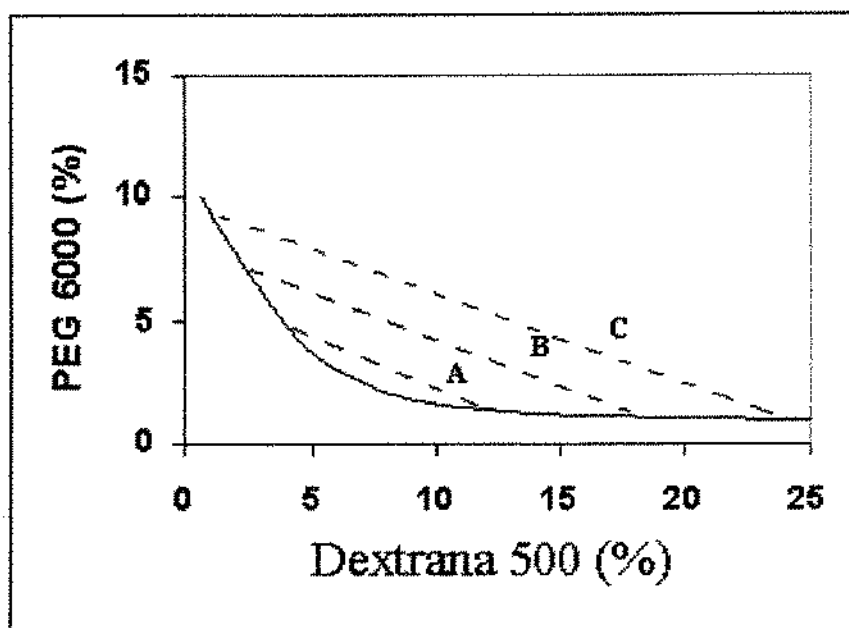


Figura 1. Curva binodal (—) de um sistema de duas fases aquosas composto de PEG 6000-Dextrana. Linhas de amarração A, B e C (- - -).

Ao longo de cada linha de amarração todos os sistemas bifásicos ali representados possuem fase superior com idêntica composição, o mesmo ocorrendo com a composição da fase inferior. No entanto, os sistemas possuem composição total e razão de volumes diferentes.

1.8 Partição por afinidade em SDFA

Nos últimos 30 anos, vários grupos estudaram métodos para aumentar a partição pelo uso de interações bioespecíficas em SDFA (FLANAGAN & BARONDES, 1975; JOHANSSON *et al.*, 1983; JOHANSSON *et al.*, 1984; LUONG & NGUYEN, 1990).

Os primeiros trabalhos com afinidade em SDFA relataram a purificação de tripsina usando o ligante p-aminobenzamidina (TAKERKAR *et al.*, 1974) e a proteína mieloma S-23 usando dinitrofenol como ligante (FLANAGAN & BARONDES, 1975).

O aumento da partição pela introdução de um ligante acoplado ao polímero é descrito pelo parâmetro K_{afin} , sendo a razão entre o coeficiente de partição de uma proteína em sistemas com (K) e sem PEG-ligante (K_0) (JOHANSSON, 1985):

$$K_{\text{afin}} = \frac{K}{K_0}$$

Partição por afinidade resulta em extração específica de proteínas, ácidos nucleicos, membranas, organelas e até células, principalmente quando ligantes bioespecíficos são usados (WALTER *et al.*, 1985).

A partição por afinidade é um processo pelo qual o ligante bioespecífico é usado para manipular o comportamento de partição da biomolécula (FLANAGAN & BARONDES, 1975), ou seja, acoplado-se um ligante à uma das fases do sistema, a partição da biomolécula ocorrerá na fase contendo o ligante (CARLSSON, 1988) enquanto que os contaminantes (moléculas não desejáveis) estarão presentes na fase oposta.

O ligante pode ser uma substância natural ou uma molécula sintetizada. Existem ligantes que interagem seletivamente com proteínas devido aos sítios de ligação específica. Por exemplo, enzimas nucleotídeo-dependente possuem um elemento estrutural comum conhecido como nucleotídeo-ligante (KOPPERSCHLÄGER, 1994). Outro grupo de ligantes específicos são os hidrofóbicos e os tiofilicos (PORATH *et al.*, 1975), os metais (WUENSCHHELL *et al.*, 1990; ARNOLD, 1991; BIRKENMEIER *et al.*, 1991; ZUTAUTAS *et al.*, 1992; CHUNG *et al.*, 1994), os compostos quaternários de amônio (JOHANSSON *et al.*, 1981), os corantes (KOPPERSCHLÄGER & JOHANSSON, 1982; KRONER *et al.*, 1982; JOHANSSON *et al.*, 1983; JOHANSSON & ANDERSSON, 1984a; JOHANSSON & ANDERSSON 1984b; JOHANSSON *et al.*, 1984; SCHIEMANN & KOPPERSCHLÄGER, 1984; BIRKENMEIER *et al.*, 1984; JOHANSSON & JOELSSON, 1985a; JOHANSSON & JOELSSON, 1985b; CORDES *et al.*, 1987; GIULIANO, 1991; WANG *et al.*, 1992; ZUTAUTAS *et al.*, 1992; BIRKENMEIER, 1994; KOPPERCHLÄGER, 1994; LIN *et al.*, 1996), os ácidos graxos (JOHANSSON &

SHAMBHAG, 1984; JOHANSSON *et al.*, 1985), os inibidores (SILVA *et al.*, 1997) e anticorpos monoclonais (ELLING *et al.*, 1990; ELLING & KULA, 1991; ELLING *et al.*, 1991; ANDREWS *et al.*, 1996).

Os ligantes biológicos naturais são geralmente caros devido ao elevado custo de sua purificação e labilidade química e biológica (KOPPERSCHLÄGER, 1994).

A Figura 2 mostra o comportamento das moléculas num sistema composto por PEG-ligante/sal. A Tabela 5 apresenta uma série de biomoléculas purificadas em SDFA por afinidade nos últimos 10 anos.

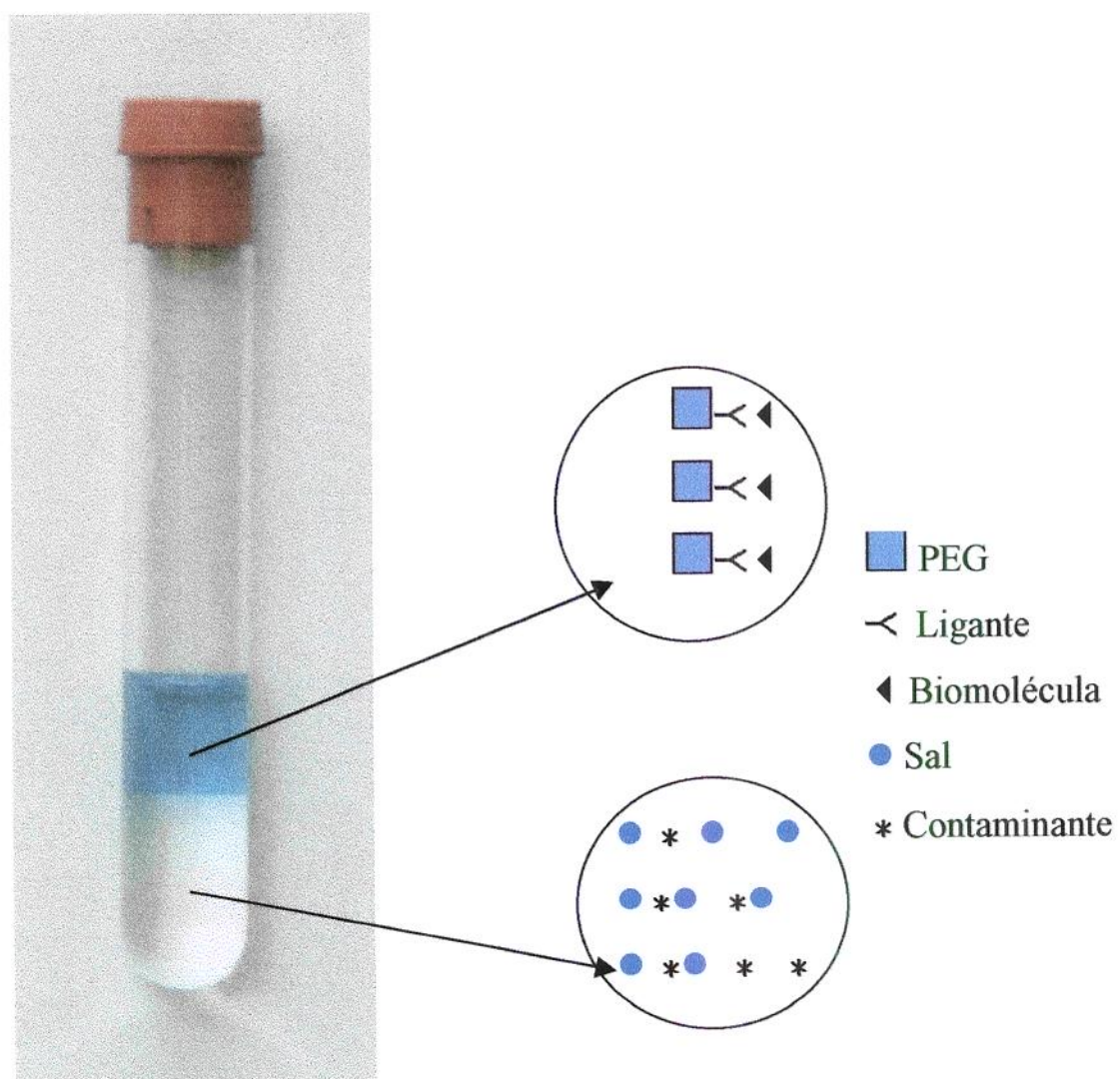


Figura 2. Comportamento das moléculas em SDFA composto de PEG-ligante/sal.

Tabela 5. Biomoléculas purificadas em SDFA por afinidade.

Sistema	Biomolécula	Ligante acoplado ao PEG	R (%)	FP	Referência
PEG/dext.	Tripsina	Inibidor de tripsina	82	-	LUONG & NGUYEN (1990)
PEG/fosf.	Penicilina acilase	Trimetilamina	97	25,70	GUAN <i>et al.</i> (1992)
PEG/fosf. potássio	Lactato desidrogenase	Eudrogit-Cibacron Blue	54	11,70	GUOQIANG <i>et al.</i> (1994)
PEG/fosf.	Proteína A	IgG humana	87	-	SUZUKI <i>et al.</i> (1995)
PEG/dext.	β -galactosidase	APGP	83	6	SILVA <i>et al.</i> (1997)
PEG/fosfato	Lactato desidrogenase	Corante triazina/Cibacron Blue F3G-A	81	7,40	LIN <i>et al.</i> (1998)
Ligante acoplado à Dextrana					
Dext./hidro xipropildext.	Quimosina	pepistatina	83	6,20	CHEN & JEN (1993)
PEG/dext.	Lipossomos	avidina	95	-	EKBLAD <i>et al.</i> (1998)

A purificação de proteínas foi consideravelmente desenvolvida com o advento dos processos de separação por afinidade, como pode ser evidenciado pelo elevado número de publicações encontradas atualmente nessa área de estudos.

As técnicas de separação de proteínas por afinidade são usualmente reservadas para as etapas finais do processo de purificação. O exemplo mais destacado dessa estratégia é o uso da cromatografia por afinidade, empregado em diversas etapas de purificação de bio-produtos. Sob condições ótimas de laboratório os resultados de purificação por cromatografia de afinidade podem ser espetaculares. O sucesso da cromatografia por

afinidade em purificações de pequena escala tem sido em muitos casos difícil de ser repetido ao ser escalonado (ASENJO & PATRICK, 1995). Esse fato é parcialmente devido às dificuldades técnicas e econômicas envolvidas com a derivatização de partículas sólidas macroporosas com ligantes de afinidade e também dos problemas oriundos da obtenção de vazões elevadas em leitos fixos. Por essas razões existe um interesse contínuo no desenvolvimento de novos processos alternativos de concentração e por afinidade.

Aplicações das interações por afinidade nas fases iniciais do processo de extração foram descritas por SADA (1990). Embora, em alguns casos, possam ser obtidos graus de purificação menos elevados do que nas etapas finais, a extração inicial da molécula desejada pode ser conveniente e recompensadora se for levado em conta que o extrato bruto é usualmente hostil à biomoléculas e contém material particulado que reduz a aplicabilidade de técnicas em leito fixo. O conceito do uso de interações por afinidade para isolamento inicial é atrativo por ser uma técnica de alta resolução, que ao ser aplicada inicialmente no processo de purificação possibilita a redução do volume de material a ser manipulado posteriormente, reduzindo assim o consumo de produtos químicos utilizados no processo, além da diminuição da perda da atividade biológica da biomolécula.

1.9 Escolha de um ligante

Segundo LUONG & NGUYEN (1992), a escolha de ligantes deve obedecer os seguintes critérios:

- A constante de dissociação enzima-ligante deve ser menor que 10^{-3} M.
- O ligante deve ser bifuncional, oferecer um sítio de ligação para afinidade específica da biomolécula e um sítio para imobilização.
- O ligante deve ser estável durante imobilização e sob condições de operação.
- O ligante não deve conter grupos hidrofóbicos ou carregados, que poderiam causar adsorção não específica da proteína.
- O ligante deve ser específico para a molécula desejada.

- O ligante não deve ser tóxico, deve ser barato e disponível em grandes quantidades. Isto pode ser uma desvantagem para ligantes, tais como anticorpos, proteínas e cofatores, pois são caros e desnaturam facilmente e rapidamente.

A escolha de um ligante deve estar também relacionada com o custo e qualidade do produto purificado, a natureza de extratos brutos, o método usado para purificação e o pretendido uso do produto purificado.

1.10 Reciclagem do polímero de afinidade

Existem várias técnicas para recuperação do polímero, algumas delas são descritas por CARLSSON (1988): o PEG pode ser reciclado por extração com clorofórmio e reutilizado em novos sistemas de fases. Este método pode ser usado em partição por afinidade, onde o polímero-derivado possui alto custo.

Outra alternativa citada por este mesmo autor é a transferência da fase polimérica para um novo sistema. Muitas proteínas podem ser extraídas na fase contendo o PEG em sistemas PEG/dextrana quando altas concentrações de sal são adicionadas (ALBERTSSON, 1986), então a fase do PEG poderá ser extraída em uma nova fase contendo fosfato, desde que um novo sistema de duas fases seja formado. A Figura 3 apresenta o esquema de transferência de PEG para uma nova fase.

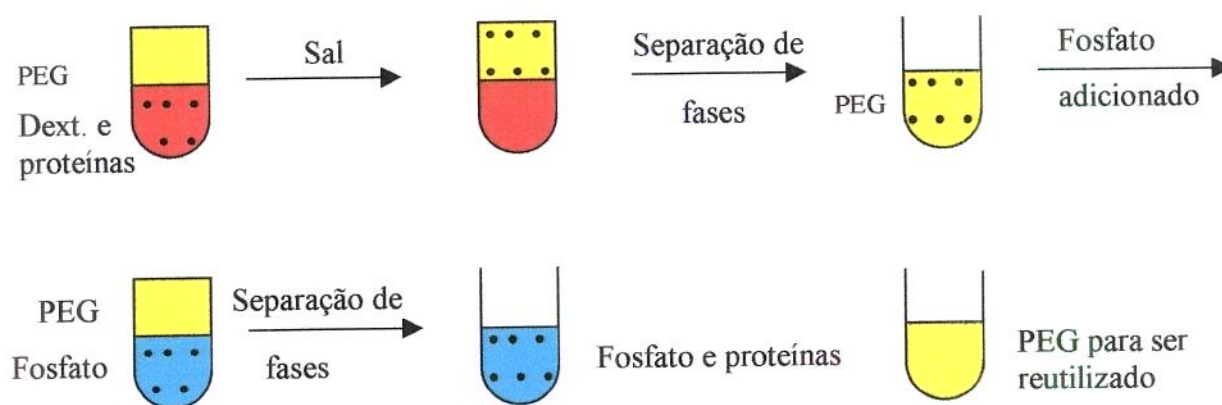


Figura 3. Processo para recuperar o PEG isento de proteínas.

Ultrafiltração também pode ser usada para recuperar o PEG e reter as proteínas, como demonstrado por KULA *et al.* (1982), porém este método não é muito usado em SDFA devido aos custos de diluição e reconcentração (HUSTEDT *et al.*, 1985).

Eletroforese e cromatografia também foi relatado para recuperação de polímeros (CARLSSON, 1988).

A recuperação dos polímeros e proteínas após a extração é uma etapa importante, tanto para processos laboratoriais como em escala industrial. Vários métodos podem ser usados para remover o polímero da proteína ou vice-versa. A capacidade de se reciclar os polímeros faria com que os SDFA fossem mais utilizados em processos de larga escala, sendo economicamente viável (CARLSSON, 1988).

1.11 Síntese dos polímeros de afinidade

Muitos métodos usados para imobilizar enzimas à matrizes cromatográficas são baseados no acoplamento de grupos amino (NILSSON & MOSBACH, 1984). Pouco se conhece a respeito de ativadores acoplados à fases líquidas. O método de ativação do PEG com cloreto de sulfonila pode ser utilizado para ativação de polímeros hidrossolúveis, os quais são muito usados em separações bioquímicas. Cloreto de tresila pode ser usado para ativação de PEG e para subsequente acoplamento do PEG a proteínas, ligantes de afinidade e células (NILSSON & MOSBACH, 1984).

Para o acoplamento covalente de um ligante bioespecífico ao polímero PEG, é necessário que os grupos hidroxilas do polímero sejam substituídos por grupos mais reativos numa primeira etapa denominada de “ativação” do PEG. Numa segunda etapa, o PEG ativado por resíduos químicos mais reativos, é acoplado por ligação covalente às moléculas do ligante e este será reconhecido posteriormente (pela proteína a ser extraída) no processo de purificação. A Tabela 6 apresenta os principais ativadores de PEG descritos pela literatura.

Tabela 6. Métodos de ativação do PEG.

Ativadores	Tempo de reação (horas)	Ligantes/Proteínas extraídas	Referências
Epoxioxirano, Periodato e Epícloroidrina	17	Glutathiona/taumatina anti-BSA/BSA inibidor de tripsina/ tripsina proteína A/IgG	ANDREWS <i>et al.</i> (1990)
Cloreto de Tresila	1,50	BSA	DELGADO <i>et al.</i> (1990) NILSSON & MOSBACH (1984)
Cloreto de Tresila	1,50	APGP/ β -galactosidase	SILVA <i>et al.</i> (1997)
Carboimidazol	2	Lactoferrina Superóxido dismutase α_2 macroglobulina	BEAUCHAMP <i>et al.</i> (1983)
Cloreto cianúrico	12-40	BSA	ABUCHOWSKI <i>et al.</i> (1977)
Cloreto de Tionila	5	Cobre/hemoglobina humana Ferro/fosvitina Ferro/hemoglobina bovina	CHUNG <i>et al.</i> (1994)

1.12 APGP (p-aminofenil- β -D-tiogalactopiranosídeo)

A enzima β -galactosidase possui um inibidor sintético específico que é o APGP, molécula que pode ser ligada covalentemente a compostos como tensoativos, polímeros e partículas sólidas. O APGP pode ser usado como ligante para recuperação desta enzima

tanto em cromatografia (STEERS *et al.*, 1971; POLLARD & STEERS, 1973; ROBINSON *et al.*, 1974) como também em SDFA (SILVA *et al.*, 1997), possuindo o mesmo mecanismo já utilizado na cromatografia por afinidade. Métodos cromatográficos exigem “espaçadores” de ligação entre as moléculas da matriz cromatográfica e os ligantes de afinidade para garantir espaço e melhor exposição do ligante para se unir a biomolécula a ser purificada. Entretanto, a partição em SDFA não necessariamente requer espaçadores entre o PEG e a molécula do ligante devido a flexibilidade do polímero.

Um sistema de duas fases aquosas de afinidade composto por dois polímeros é mais comumente usado do que um sistema constituído por um polímero e um sal, pois a elevada concentração do sal poderá dissociar a interação entre a enzima e o ligante (JOHANSSON & TIERNELD, 1989).

Devido a baixa reatividade dos grupos hidroxilas do PEG, é necessário que eles sejam quimicamente ativados. A ativação do PEG-4000 com cloreto de tresila se dá por uma reação livre de água para evitar a hidrólise do cloreto de sulfonila. Piridina é adicionada para neutralizar os prótons liberados. Altos rendimentos são obtidos quando os solventes usados são desidratados (NILSSON & MOSBACH, 1987). Cloreto de tresila pode ser usado com a vantagem de se acoplar a ligantes ou proteínas sensíveis a pH extremos, com alto rendimento, quando em pH neutro (NILSSON & MOSBACH, 1981).

DELGADO *et al.* (1990) ativaram PEG 4000 com cloreto de tresila, obtendo 80% de ativação, o mesmo conseguido por NILSSON & MOSBACH (1984). Esses autores observaram o alto nível de ativação obtido em 1,5 hora de reação mostrando a alta reatividade do cloreto de tresila. Este curto tempo de reação é vantajoso comparado com o tempo de reação longo requerido para obtenção de níveis similares de ativação com cloreto cianúrico, variando de 12 horas (ABUSCHOWSKI *et al.*, 1977) a 40 horas (SUZUKI *et al.*, 1984) e 24 horas com fenilcloroformatos (VERONESE *et al.*, 1985).

NILSSON & MOSBACH (1980, 1981 e 1987) introduziram o cloreto de sulfonila orgânico como reagente ativador para ativar agarose e outras matrizes hidroxílicas. Cloretos de sulfonila orgânicos, tais como p-toluenosulfonila (cloreto de tosila) e 2,2,2-trifluoroetanosulfonila (cloreto de tresila) convertem grupos hidroxilas de matrizes em

sulfonados ativos. Estes sulfonados são grupos abandonadores que, após a reação com nucleófilos, formam ligações estáveis entre o nucleófilo e o carbono hidroxila terminal.

SILVA *et al.* (1996) e PIZA *et al.* (1996) compararam o poder de ativação do PEG-cloreto de tresila e PEG-cloreto de tionila e observaram 74% e 87% de ativação, respectivamente.

A Figura 4 apresenta a síntese do polímero de afinidade PEG-Cl-APGP.

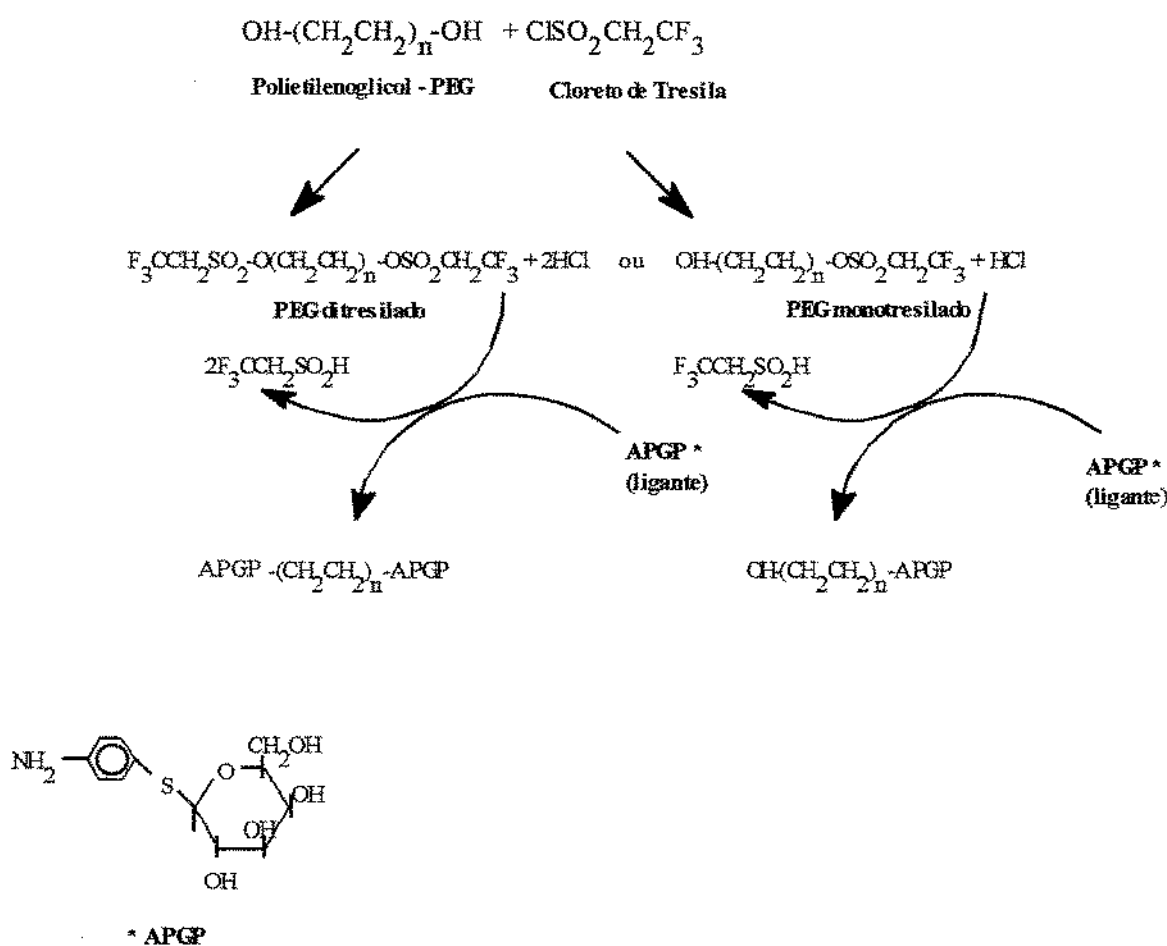


Figura 4. Ativação do PEG com cloreto de tresila e acoplamento subsequente do ligante APGP.

1.13 Metalo-ligantes

Afinidade usando metais como ligantes é fundamentada no sítio de ligação entre metais de transição quelados pelo ácido iminodiacético (IDA) e grupos doadores de elétrons presentes na superfície da proteína (PESLIAKAS *et al.*, 1994).

A cromatografia de afinidade usando metais como ligantes foi introduzida por PORATH *et al.* (1975) e tem sido empregada para purificação de proteínas. Através da adaptação da metodologia foi possível desenvolver métodos de ativação para polímeros hidrossolúveis usando um quelante, ácido iminodiacético, que é capaz de quelar metais de transição como Cu^{2+} , Ni^{2+} , Zn^{2+} e Co^{2+} (LABOUREAU *et al.*, 1996).

Diversos autores usaram PEG-iminodiacetato (PEG-IDA) contendo íons Cu^{2+} em sistemas de duas fases aquosas constituídos de PEG/sal e PEG/dextrana (SUH & ARNOLD, 1990; PLUNKETT & ARNOLD, 1990; BIRKENMEIER *et al.*, 1991). A Figura 5 apresenta o sítio de ligação de proteínas contendo histidina que se acoplam ao complexo PEG-IDA- Cu^{2+} .

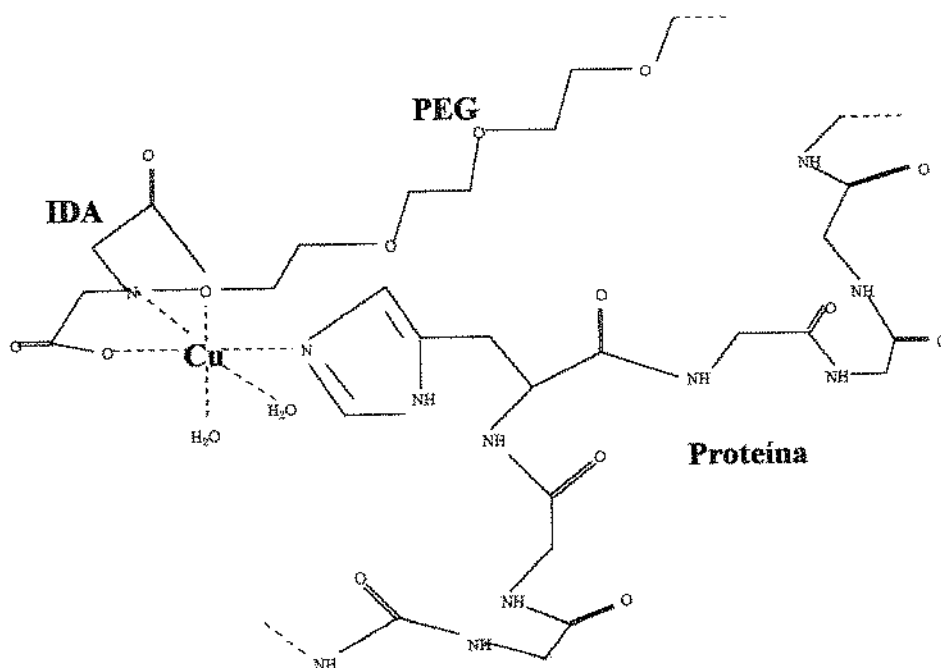


Figura 5. Sítio de ligação entre a proteína contendo histidina e o PEG-IDA- Cu^{2+} .

O ácido iminodiacético é um quelante tridentado, ocupando 3 sítios de coordenação do metal. Quando o complexo IDA-Cu²⁺ se acopla covalentemente ao PEG, moléculas com sítios acessíveis para se ligar ao PEG-IDA-Cu²⁺ serão extraídas na fase polimérica (CHUNG *et al.*, 1994).

De acordo com os autores PORATH *et al.* (1975), PORATH & OLIN (1983), PORATH (1985), PORATH (1988) e SUH & ARNOLD (1990), somente os resíduos de aminoácidos presentes na superfície da proteína, como histidina, cisteína e triptofano são os responsáveis pelos sítios primários da interação proteína-metal imobilizado. Estudos mais recentes mostraram que destes aminoácidos os resíduos histidil disponíveis na superfície da biomolécula parecem ser o fator que irá formar o complexo metal-biomolécula (PORATH, 1988; SULKOWSKI, 1985; HEMDAN *et al.*, 1989; BELEW & PORATH, 1990; ZHAO *et al.*, 1991).

De acordo com WUENSCHHELL *et al.* (1990), o uso de PEG-IDA-Cu²⁺ em sistemas PEG/dextrana aumenta seletivamente a partição de proteínas para a fase de PEG, dependendo apenas do número de resíduos de histidinas acessíveis presentes na superfície da proteína. De acordo com WOKER (1992) os principais aminoácidos que se ligam a metais são histidina, metionina, cisteína, selenocisteína, tirosina, aspartato e glutamato.

Foram estudados em sistemas PEG/sal e PEG/dextrana a partição de proteínas recombinantes contendo sítios His-X₃-His acoplados à sua superfície (ARNOLD, 1991; SUH *et al.*, 1991; TODD *et al.*, 1991; ARNOLD & HAYMORE, 1991), eritrócitos (BOTROS *et al.*, 1991) e isoenzima de lactato desidrogenase (OTTO & BIRKENMEIER, 1993).

As principais vantagens do uso de metais como ligantes são: 1) podem ser reciclados diversas vezes com insignificante perda do comportamento, 2) elevada quantidade de metal imobilizado e conseqüente elevada capacidade para a proteína ser “atraída” 3) relativa facilidade da eluição da proteína e regeneração do ligante, 4) baixo custo dos metais (ARNOLD, 1991), 5) são estáveis na presença de certos solventes e temperatura e 6) sua interação com biomoléculas é específica e reversível (CHUNG *et al.*, 1994).

A Tabela 7 descreve a purificação de proteínas em SDFA de afinidade usando metais, de acordo com a literatura.

Tabela 7. Uso de metais como ligantes em SDFA, descritos pela literatura.

Proteína	Metal	Referência
<i>Candida krusei</i> citocromo c	Cobre	WUENSCHHELL <i>et al.</i> (1990)
Hemoglobina bovina	Cobre	WUENSCHHELL <i>et al.</i> (1990)
	Ferro	CHUNG <i>et al.</i> (1994)
Eritrócitos	Cobre	BOTROS <i>et al.</i> (1991)
	Zinco	
α_2 macroglobulina	Cobre	BIRKENMEIER <i>et al.</i> (1991)
	Zinco	
Hemoglobina humana	Cobre	CHUNG <i>et al.</i> (1994)
Fosvitina	Ferro	CHUNG <i>et al.</i> (1994)
Oxinitrilase	Cobre	WOKER <i>et al.</i> (1994)
Desidrogenase alcoólica	Cobre	PESLIAKAS <i>et al.</i> (1994)
Lactato desidrogenase	Níquel	
Malato desidrogenase	Zinco	
	Cádmio	
Linfócitos	Cobre	LABOUREAU <i>et al.</i> (1996)
	Níquel	
	Zinco	
Lactato desidrogenase	Cobre	FRANCO <i>et al.</i> (1997)
Lisozima	Cobre	SILVA & FRANCO (1999c)

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CAPÍTULO 2

LIQUID-LIQUID EXTRACTION OF BIOMOLECULES IN DOWNSTREAM PROCESSING - A REVIEW PAPER

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SUMMARY

Economic analysis shows that protein separation and purification are a very important aspect of the biomolecules production and processing. This is particularly true for protein processing which, because of the complexity of the starting material, often requires many steps to reach the levels of purity required for medical and food applications. The separation specialists' task is to develop safe and simple processes to achieve products with a high level of purity. On a large scale, chromatography of proteins is not an easily applied method, although on a laboratory scale it is very effective and relatively simple. When it is scaled up, shortcomings such as discontinuity in the process, slow protein diffusion and large pressure drops in the system are seen. For these reasons a substantial research effort has been directed toward the use of aqueous two-phase systems (ATPS) to replace the initial steps in protein purification and chromatography. ATPS can easily be scaled up without an appreciable change in the nature or efficiency of the process. In addition, since there is no solid phase, thorough mixing of the two phases is possible and hence interphase transport is rapid. Only seconds are required to bring most two-phase systems into equilibrium. Another benefit is that the phases are compatible with almost all known proteins. They are an attractive alternative procedure for the separation and purification of proteins on a large scale. The question of selectivity in protein partitioning still needs to be better understood. An increased knowledge of protein behaviour in aqueous-two phase systems will also lead to the ability to predict the partitioning of target proteins, often found in a heterogeneous and complex mixture of proteins.

Partitioning in aqueous two-phase systems is mainly a process in which the exposed groups of molecules come into contact with the phase components, and it is therefore a surface-dependent phenomenon. The influence of different factors upon the partitioning of proteins is illustrated by the logarithm of the partition coefficient. This can be considered to be the sum of the logarithms of several terms:

$$\ln K = \ln K_{\text{elec}} + \ln K_{\text{hphob}} + \ln K_{\text{biosp}} + \ln K_{\text{size}} + \ln K_{\text{conf}}$$

This equation includes a hydrophobic term (K_{hphob}), a term that includes electrostatic effects (K_{elec}) mainly determined by the protein net charge and hence pH, ion distribution and also the charge of the polymers involved. Protein and polymer size (K_{size}), conformation (K_{conf}) and affinity ligands (K_{biosp}) can also be important.

INTRODUCTION

1. AQUEOUS TWO-PHASE SYSTEMS

The tendency for two different aqueous polymers (e.g., gelatine and agar, gelatin and soluble starch) to separate into two distinct phases in a common solvent has been recognized since the end of last century, and it has been shown to be the general rule for most water-soluble polymer-polymer systems. For example, a mixture of dextran and polyethylene glycol dissolved in water is turbid above certain polymer concentrations, and the two phases are in equilibrium. The lighter phase is enriched in polyethylene glycol while the heavier is enriched in dextran (Figure 1). Both polymers are fully water soluble, yet the two polymers are incompatible and separate into two phases (ALBERTSSON, 1986). ALBERTSSON (1971) compared the two-phase aqueous polymer with conventional solvents, according to their hydrophobic/hydrophilic nature. At the bottom of the scale (lowest hydrophobicity) is the aqueous salt solution. Acetone comes above most aqueous two-phase systems, with hydrophobicity increasing up to heptane. It is possible to have an extremely selective separation of substances using aqueous polymer systems. Aqueous two-phase systems provide a gentle and protective environment for biological material, since both phases are composed primarily of water (ABOTT *et al.*, 1990; BASKIR *et al.*, 1987; BASKIR *et al.*, 1989).

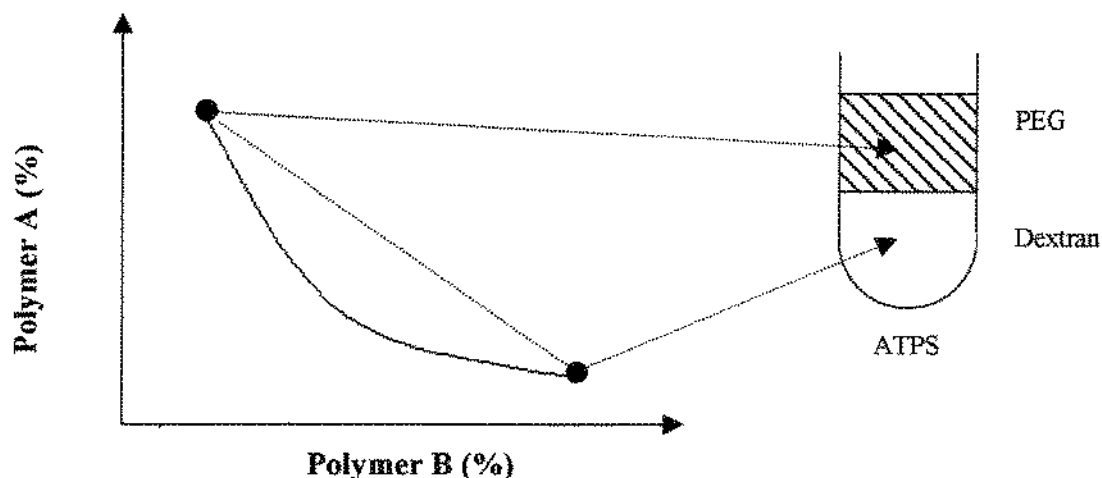


Figure 1. Binodial curve and tie line of an aqueous two-phase system composed of polymer-polymer.

Polyethylene glycol is one of the most useful polymers in ATPS. Its solubilization in water is attributed to the attachment of water molecules to many or all of the ether oxygen sites along the polyethylene oxide chain. This attachment occurs by a hydrogen-bonding mechanism. It was found that the addition of monovalent cations to polyethylene-oxide products decreases their solubility; this decrease in the cloud point happens when the competition of salt ions for water effectively reduces the amount of free water available to solubilize the polyethylene. Some inorganic salts are more able to promote this effect (e.g., CaCl_2 , MgCl_2 , AlCl_3) when the ions form association complexes with the ether groups.

According to CLELAND *et al.* (1992), polyethylene glycol can significantly enhance the refolding of recombinant proteins when accumulated in the form of inclusion bodies that need to be solubilized and refolded to recover activity.

Amongst the variety of aqueous two-phase systems, PEG-dextran is the best-studied system for liquid-liquid partitioning of proteins, in contrast to PEG-salt systems. The effects of polymer molecular mass, concentration, phase density and the presence of salts in aqueous two-phase systems have been studied by many authors: ALBERTSSON (1971), ALBERTSSON (1986), JOHANSSON (1985a; 1985b), BROOKS *et al.* (1985), BAMBERGER *et al.* (1984), ZASLAVSKY *et al.* (1978; 1982a; 1982b; 1983; 1992a;

1992b), CARLSSON (1988), HUDDLESTON *et al.* (1991a), HUDDLESTON *et al.* (1991b), ASENJO *et al.* (1991) and FRANCO *et al.* (1996a).

Separation of other molecules, including proteins in aqueous two-phase systems, is generally considered to depend on the molecular surface characteristics of the compounds to be partitioned, such as charge, size and hydrophobic properties (ALBERTSSON, 1971; LaMARCA *et al.*, 1990). Table 1 presents a list of some biomolecules purified in the last ten years.

Table 1. Biomolecules purified in ATPS.

Biomolecule	Recovery (%)	Purific. Factor	References
Xylanase	41	57	DUARTE <i>et al.</i> (1999)
β -glucosidase	85 - 95	2-3	JOHANSSON & RECZEY (1998)
Polyphenol oxidase	50	5	SOJO <i>et al.</i> (1998)
Lipase	68	41	BOMPENSIERI <i>et al.</i> (1998)
β -galactosidase	90	-	CHANG & KOO (1998)
Glucoamylase	96	3	MINAMI & KILIKIAN (1998)
Horseradish peroxidase	75	7.3	MIRANDA & CASONE (1997)
Thaumatococcus	90 - 95	20	CASONE <i>et al.</i> (1991)
β -galactosidase	85 - 100	-	KÖHLER <i>et al.</i> (1991)
Fumarate hydratase (<i>Brevibacterium ammoniagenes</i>)	83	7.5	BOLAND <i>et al.</i> (1989)
Fumarate hydratase (<i>E. coli</i>)	93	3.4	
Aspartate-ammonia-lyase (<i>E. coli</i>)	96	6.6	
Isoleucyl-tRNA-synthetase (<i>E. coli</i>)	93	2.3	
Penicillin amidase (<i>E. coli</i>)	90	8.2	
β -galactosidase (<i>E. coli</i>)	87	9.3	
Glucose-6-phosphate- dehydrogenase (<i>Leuconostoc</i> sp)	94	1.3	
Alcohol dehydrogenase (<i>Saccharomyces cerevisiae</i>)	96	2.5	
L-2-hydroxyisocaproate- dehydrogenase (<i>Lactobacillus casei</i>)	93	17	
Formaldehyde dehydrogenase (<i>Candida boidinii</i>)	94	-	
Leucine dehydrogenase (<i>Bacillus sphaericus</i>)	98	2.4	

Extraction by an ATPS offers advantages for processing on a large scale, such as the possibility of obtaining a high yield, the possibility of continuous processing (FLYGARE *et al.*, 1990; ENFORS *et al.*, 1992; ROSTAMI-JAFARABAD *et al.*, 1992a; ROSTAMI-JAFARABAD *et al.*, 1992b; COIMBRA *et al.*, 1994; TOMASKA *et al.*, 1995; PAWAR *et al.*, 1997; PORTO *et al.*, 1997; COIMBRA *et al.*, 1998) and a reduction in operational cost in relation to the costs of conventional processes (KULA, 1990).

1.1 PEG-DEXTRAN SYSTEMS

1.1.1 Effect of Polymer Molecular Mass (MM)

An increase in the molecular mass of dextran or of PEG will lower the concentration required for phase separation. The polymer molecular mass influences protein partitioning as a direct result of interactions between the two polymers. It has been found that the partitioned protein behaves as if it were more attracted by smaller polymer sizes and more repelled by larger polymers, provided all other factors such as polymer concentrations, salt composition, temperature and pH are kept constant. It was observed that smaller protein molecules and amino acids were not affected as much as larger ones. For some proteins (ALBERTSSON *et al.*, 1987) the partition coefficients increased as the MM of dextran increased if all other conditions were kept constant, but little effect was found for low MM proteins (Cytochrome C, 16,000). When the same proteins were partitioned in systems with different PEG MM, their partition coefficients decreased as the PEG MM increased, and for cytochrome C the effect was the smallest. This was attributed to the fact that when the PEG MM is increased, a weaker repulsion energy is required to cause phase separation. Repulsive interactions between the polymer and the protein become stronger as the polymer MM is increased, resulting in a distribution of the protein towards the phase containing the polymer with an unchanged MM. A weak net repulsion between the proteins and the polymer is sufficient to change the distribution when the polymer MM is changed.

1.1.2 Effect of Polymer Concentration

An increase in polymer concentration seems to increase the density of the bottom dextran-rich phase. This difference in density is linearly correlated to tie-line length (BAMBERGER *et al.*, 1984).

DIAMOND & HSU (1989a; 1989b) found that proteins with MM less than 20,000 showed a linear relationship between the $\ln K$ in PEG-dextran systems and a difference in PEG concentration between the phases, for any particular system. They found that it was possible to predict the partitioning of a protein at any concentration in that particular system if one partition coefficient in the system were known.

NIVEN *et al.* (1990), however, found that for some proteins the partition coefficient was inversely correlated to phase concentration in a PEG-dextran system, showing that better separation could be achieved at high polymer concentrations. This, however, may also affect the concentration of proteins that can be manipulated in the system as polymer concentration has a directly inverse effect on protein solubility.

GUAN *et al.* (1992) showed that the effect of increasing the tie-line length of a PEG-phosphate system, upon the partitioning of penicillin acylase, is considerably higher at low phosphate concentrations (a higher water content), becoming smaller at high concentrations of phosphate salts. Gradual increases in the tie-line lengths lead to enhanced partition coefficients.

1.1.3 Effect of Salts

Salts can affect protein partitioning in different ways in PEG-dextran systems: one is by altering the physical properties of the systems (tie-line lengths) (KULA, 1979; KULA, 1985; CHEN, 1992), the hydrophobic difference between the phases (ZASLAVSKY *et al.*, 1982b) and the other is by the partitioning of ions between the phases, which affects the partitioning of proteins according to their molecular charge (ALBERTSSON, 1971;

WALTER *et al.*, 1972).

Salts have been added to PEG-dextran systems to increase the selectivity of protein partitioning in the aqueous two-phase methodology application for biological separations (JOHANSSON, 1970; JOHANSSON *et al.*, 1973; JOHANSSON, 1974; JOHANSSON, 1985a; ALBERTSSON, 1971; HUSTEDT *et al.*, 1978; HUSTEDT *et al.*, 1985; SCHMIDT *et al.*, 1994; FRANCO *et al.*, 1996a).

1.1.3.1 Difference in Salt Partitioning and Electrical Potential Between the Two Phases

It was observed that salt ions partition differently between the phases, causing an uneven distribution in the system (ALBERTSSON, 1971; JOHANSSON, 1970; JOHANSSON, 1974; JOHANSSON, 1985a; JOHANSSON, 1985b; SASAKAWA & WALTER, 1972; REITHERMAN *et al.*, 1973) that generates a difference in electrical potential between the phases. This difference in electrical potential would be independent of salt concentration, but linearly dependent on the partition behaviour of the ions.

It was also observed by JOHANSSON (1974) that polyvalent anions such as phosphate, sulphate and citrate partitioned preferentially into dextran-rich phases, while halides partitioned nearly equally. ALBERTSSON (1971) showed that for various inorganic salts the partitioning of all negatively charged materials followed the same order as the Hofmeister series. WALTER *et al.* (1972) demonstrated that this effect was reversed when positively charged materials were partitioned. As an example, negatively charged materials have higher partition coefficients in phases containing sodium sulphate rather than sodium chloride, while the reverse holds for positively charged materials. Partition coefficients of negatively charged materials decrease when the cationic series is changed from lithium to sodium to potassium (JOHANSSON, 1974). The ratio between the phosphate ions, rather than the concentration, was decisive for the difference in electrical potential. This applies to multivalent ions, which show a series of pH-dependent dissociations and was clearly the reason for the potential difference found between the two phases (KULA *et al.*, 1982).

1.1.3.2 Effect of Salts on the Hydrophobicity of the ATPS

KULA *et al.* (1982) observed that in the presence of PEG and dextran phosphate ions and sulphate ions accelerated a gel formation, which was pH dependent. It was attributed to a complex formation of spaced hydroxyl groups in the polyglucan backbone of dextran. This association of dextran chains increased the exclusion volume, which in turn might increase the partition coefficient of any protein of a given size. LEE and SANDLER (1990) observed that the partition coefficient of the antibiotic vancomycin (MM 1,448, pI 8.1) in PEG-dextran systems at pH 7.0 increased exponentially with either NaCl or sodium sulphate concentrations, rather than decrease with sodium sulphate, as would be expected if electrostatic effects were dominant. This effect was attributed to possible hydrophobic interactions between vancomycin and PEG and electrostatic interactions.

ZASLAVSKY *et al.* (1982a) found that the partition coefficient of ions was dependent on their own concentration, in the range of 0 to 285 mM in PEG-dextran systems, and also on the type of ion present. This followed the order $\text{NaH}_2\text{PO}_4 < \text{sodium phosphate pH 6.8} < \text{Na}_2\text{HPO}_4$. An electrical potential of 3.0 mV, created by 0.01 M of HPO_4^- - $\text{H}_2\text{PO}_4^=$, was almost neutralized by a NaCl concentration of 0.15 M. Their conclusion was that in a PEG-dextran system containing 0.01 M sodium phosphate buffer pH 6.8, small amounts of NaCl reduced the difference between the relative hydrophobicity of the phases and this difference remained the same up to 0.12 moles/kg. A further increase in NaCl concentration to 0.15 increases this difference, which appears to be invariable in the range of 0.15 to 0.5 moles/kg. A slight increase in relative difference occurs at higher concentrations at higher salt concentrations (e.g., 1.0 M). The phase hydrophobicity measurements were carried out by calculating the free energy of transfer of CH_2 groups from one phase to the other and from one phase to a reference solution (ZASLAVSKY *et al.*, 1982a; ZASLAVSKY *et al.*, 1982b; ZASLAVSKY *et al.*, 1983).

BAMBERGER *et al.* (1984) and BROOKS *et al.* (1984) observed that some anions (e.g., $\text{SO}_4^=$ and $\text{PO}_4^=$) had a greater tendency to leave the PEG phase than others (F^- , Cl^- ,

Br^- , I^- , ClO^-) and that some others correlations (difference in electrical potential between the phases and tie-line lengths) were direct functions of these effects. NO_3^- and Cl^- did not leave any phase.

1.1.3.3 Combined Effects of Salts upon Protein Partitioning in PEG-Dextran Systems

Most of the problems with testing the predicted charge dependence of K are difficult to recognize due to the accompanying changes in phase composition, such as variation in pH and salt concentration.

Depending on the distribution of charged ions, the phases will be attractive or repulsive to proteins (poly-ions) so the proteins will tend to move towards the phases with the opposite charge. The electrostatic interactions (attraction/repulsion) between the phases and the proteins will only be detected if other important factors, such as hydrophobicity, bioaffinity, etc., do not overwhelm them. The electrostatic effects in aqueous two-phase systems for protein partitioning can be observed at low salt concentrations up to 0.10 mol/kg of the phase. This is like the "salting in" effect, which typically involves low salt concentrations and is based on electrostatic interactions (MELANDER & HORVATH, 1977; SCOPES, 1994).

The difference in electrical potential is a rather controversial subject (ZASLAVSKY *et al.*, 1982b). According to these authors, the electrical potential represents the difference in hydration of the ions taking part in the distribution equilibrium, and hence, seems to represent (in a rather limited way) the difference in the relative hydration abilities of the two phases of the system. According to BROOKS *et al.* (1984), absolute potential measurements cannot be made in a strict thermodynamic sense due to the unknown effect of the liquid junction potentials at the tips of the salt bridges in the electrode measuring it. According to BROOKS *et al.* (1984) and ZASLAVSKY *et al.* (1982b), it is therefore necessary to demonstrate that the measured potentials are consistent with the partition coefficients observed in the ATPS. PFENNIG *et al.* (1998) have observed that the electrostatic potential

difference between coexisting phases is a common property at interfaces even though the phases are strictly electroneutral and can not be measured, however it can be quantified under controlled conditions.

1.2 PEG-SALT SYSTEMS

The formation of PEG-salt systems was first observed by ALBERTSSON in the 1950s, but the theoretical fundamentals have not been well explained. BOUCHER and HINES (1976) found that for PEG solutions the addition of some inorganic salts (sulphates and carbonates) is more effective than the addition of others in reducing the critical concentration of cloud point curves. ANANTHAPADMANBHAN and GODDARD (1987) found that inorganic salts dramatically reduced the PEG cloud point at high temperatures.

PEG-salts systems have been introduced for the practical application of large-scale protein separation because of the larger droplet size, greater difference in density between the phases, lower viscosity and lower costs, leading to a much faster separation than in PEG-dextran systems. Industrial application of PEG-salt systems was improved by the availability of commercial separators, which allowed faster continuous protein separations (KRONER & KULA, 1978; KRONER *et al.*, 1982; KULA *et al.*, 1982; KULA, 1990).

For PEG-salt systems, salting-out effects appear to operate with increasing tie-line length, shifting proteins from the salt phase into the PEG-rich phase, or if protein solubility in the PEG phase is not sufficient, they tend to precipitate at the interface. Solubility and salting-out limits are dependent on the properties of individual proteins; therefore, a differential response is expected when a mixture of proteins is handled (KULA *et al.*, 1982; ANDREWS & ASENJO, 1989).

KÖHLER *et al.* (1991) studied the precipitation curves for β -galactosidase and four others samples of β -galactosidase fused to small proteins (SpA β gal) in PEG and in phosphate solution and found they were strongly related to protein partitioning in PEG-phosphate two-phase systems. It was suggested that the considerable increases in the partition coefficient of SpA β gal at longer tie-line lengths were due to the loss of solubility in

the phosphate phase followed by adsorption at the interface, rather than an increase in the partition coefficient as such.

Initially PEG-phosphate systems were widely used (KULA, 1979; KULA, 1985; KULA, 1990; KULA *et al.*, 1982; HUSTEDT & KULA, 1977; HUSTEDT *et al.*, 1978; VEIDE *et al.*, 1983). GREVE and KULA (1991) have studied ways of recycling the phosphate phase of the systems to minimize environmental pollution. The recycling of the phosphate phase was achieved by its separation from the solids by the use of alcohols. PEG from the top PEG-rich phase can also be successfully recycled (KRONER & KULA, 1978; KRONER *et al.*, 1982; HUSTEDT *et al.*, 1985; HUSTEDT *et al.*, 1988; HUSTEDT, 1986).

More recently PEG-sulphate systems have begun to be used. CHIANG and WANG (1988) used them for recuperation of L-aspartase from fumarase, produced by *E.coli*. The best separation was achieved with PEG 4000 and $(\text{NH}_4)_2\text{SO}_4$ at pH 7-7.5. The presence of 2% NaCl (0.17 M) made the separation much worse. With 4% NaCl (0.34 M), a poor separation was obtained (a tenfold decrease in K for aspartase). Since a pH or phase ratio change was not observed, the dramatic change in K was considered to be due to a change in hydrophobicity between the phases. LEE and SANDLER (1990) found that the partitioning of antibiotic vancomycin in PEG 8000-phosphate systems was exponentially increased by addition of NaCl (0 to 1.5 moles/kg) or sodium sulphate (0 to 0.7 moles/kg), but not significantly changed by thiocyanate. It was suggested that the increased partitioning of vancomycin was due to hydrophobic interactions promoted by water-structure-making salts in combination with potassium phosphate (from the phase components), which would reduce the water activity and alter the phase composition.

EITEMAN and GAINER (1990) studied the influence of the difference in composition between the phases on the partitioning of small alcohols and peptides. A model for protein partitioning was suggested, taking into account the difference in PEG concentration between the phases and the solute hydrophobicity relative to the hydrophobic difference between the two phases.

Citrate systems were developed in order to make ATPS less harmful to the

environment. Citrate is biodegradable and non-toxic, and it could be discharged into a wastewater treatment plant (VERNAU & KULA, 1990).

1.3 MODELS FOR PARTITIONING BETWEEN THE PHASES

1.3.1 Bronsted Theory

The basis for separation by a two-phase system is the selective distribution of substances between the phases. For soluble substances, distribution takes place mainly between the two bulk phases. Distribution is characterized by the partition coefficient, K , defined as the concentration in the top lighter phase, C_{top} , divided by the concentration in the heavier bottom phase, C_{bottom} :

$$K = C_{top} / C_{bottom} \quad (1.1)$$

Obviously the choice of a suitable phase system is the key step in all partitioning work.

If the energy needed to move a protein molecule from one phase to another is ΔE , one would expect that at equilibrium a relationship between the partition coefficient and ΔE would be expressed as follows (BASKIR *et al.*, 1987):

$$C_1/C_2 = e^{-\Delta E/k T} \quad (1.2)$$

where k is the Boltzman constant and T the absolute temperature, and C_1 and C_2 are the concentration of protein molecules in phases 1 and 2.

ΔE must depend on the size of the particle or molecule, since the larger the size, the greater the number of exposed atoms which can interact with the surrounding phase, and the following formula was suggested:

$$C_1/C_2 = e^{\lambda M/k.T} \quad (1.3)$$

where M is MM and λ in this case is a factor that depends on properties other than MM . For a spherical molecule, M could be replaced by A , the surface area of the molecule:

$$C_1/C_2 = e^{\lambda A/k.T} \quad (1.4)$$

and λ in this case is a factor that depends on properties other than surface area, for example surface properties as expressed by the surface free energy per unit area.

This is known as the Bronsted partition theory, and its main point is the exponential relationship between the partition coefficient and properties that enter into the λ factor, for example hydrophobicity or affinity and charge. Small changes in such factors will cause relatively large changes in the partition coefficient. The theory therefore predicts a high degree of selectivity. Although the Bronsted model demonstrated why protein partitioning may be sensitive to particle MM , it is at best a qualitative model, since it combines all the other system variables into a single parameter.

1.3.2 Albertsson's Model

ALBERTSSON (1971) derived an expression for the dependence of the distribution potential difference, ψ , between the two phases on the buffering salt:

$$\psi = \frac{RT}{(Z^+ + Z^-)F} \ln \frac{K^-}{K^+} \quad (1.5)$$

R represents the gas constant

T represents absolute temperature

K^+ represents the cation salt partition

K^- represents the anion salt partition

Z^+ and Z^- represent the number of charges on the salt ions (e.g., valence)

ψ represents the electrical distribution potential

This is also a qualitative model, and it demonstrates the high exponential dependence of protein partitioning on its charge, surface area, surface energy and difference in distribution potential between the phases.

In practice, however, there has been no consistent and generalized effort to use these theories to predict partitioning behaviour, so they have been used only as descriptive tools to explain observed phenomena.

These models are limited to examining only the effect of protein molecule characteristics without attempting to analyze in detail the contribution of phase environment. These theories also give no indication of how molecular mass, concentration or the choice of phase polymer will influence partitioning in aqueous two-phase systems.

1.3.3 Lattice Model Proposed by Brooks *et al.* (1985) (Model for PEG-Dextran Systems)

The increased interest in ATPS for a wide range of applications, combined with the lack of a useful predictive model for protein partitioning, has led to an interest in the development of models. BROOKS *et al.* (1985) developed a lattice model by extending the theory of polymer-polymer solvent mixing to multicomponent systems. In this case, the system is considered to be a four-component system, containing water (component 1) and three polymeric solutes (components 2, 3, and 4), one of which is biomaterial (component 4). It is assumed that there is a very low concentration of biomaterial relative to the other components, and all the polymeric components are assumed to be equally soluble in the solvent. The Brooks model qualitatively demonstrates several of the trends found empirically. Less partitioning occurs between the phases, even for large biomaterials. Also

the value of K is higher for greater differences in polymer concentration between the phases. All other factors being equal, the protein will partition in favour of the phase containing the polymer with a lower MM. Their model provides some basis for understanding biomaterial partitioning, but Brooks *et al.* pointed out that the physical picture of the biomaterial as a random coiling polymer is unrealistic. It is well known that like most biological molecules, proteins are generally tightly folded, compact globular structures which contain a large proportion of the polypeptide shielded internally from contact with the surrounding solution. One should expect that real native proteins would have much lower surface contact areas than random coiling polymers. In addition, the nature of steric interactions between the phases and the biomaterial will be different if a compact hard body model, rather than a random coil, is assumed.

1.3.4 The Virial Expansion Model

KING *et al.* (1988) developed a model for polymer-polymer systems that assumes a general form for the chemical potential of the components, and various terms are determined by independent measurement, allowing the calculation of the phase diagram and the protein partitioning coefficient with it. The model states that protein partitioning is a function of polymer concentration and the interactions between the polymers and the protein. The virial expansion model can be modified and used to predict the effect of moderate concentrations of salts on protein partitioning. It gives a qualitative relationship between K and polymer concentrations and information about the volume "excluded" by each component in solution, which is dependent on the molecular mass of the component. Measurement of the interaction coefficient by low-angle light scattering indicates that protein-polymer interactions are generally only slightly affected by the presence of different salts. The protein-protein interaction coefficient for many proteins has been found to be dependent upon the choice of salt. The dependence of protein-protein interactions on salt type could be due to electrostatic interactions, stabilization of the protein, and the interaction of the salt solution with the hydrophobic portions of the protein (MELANDER & HORVATH, 1977). This model, however, is not fully understood and is only applicable to polymer-polymer systems and not

to polymer-salt systems.

HAYNES *et al.* (1989a; 1989b) improved King's model, developing a molecular thermodynamic model to predict properties of aqueous two-phase systems containing polymers, electrolytes and proteins. According to the Debye-Hückel theory, at concentrations of electrolyte in solution of less than 0.1 M, electrostatic interactions can be well explained. Haynes' model uses this theory and independent measurements of coefficients of polymer-polymer, ion-ion and ion-polymer interactions. The model can predict salt ion partitioning, which is inversely correlated with tie-line length (TLL) and ion size and is also in agreement with the Hofmeister series. Electrical potential differences were predicted as a function of TLL, and binodial curves of phase diagrams were in good agreement with experimental data. The shortcomings of their model were the unreal ratio between the size of protein-ion interactions, which are in disagreement with the Debye-Hückel theory, and the uneven distribution of charge over the protein surface. It also had to be improved for higher polymer concentrations.

CABEZAS *et al.* (1989; 1990; 1991) developed a statistical mechanism model for the prediction of phase diagrams of aqueous two-phase systems. It incorporated the dependence of the phase diagram on polymer molecular mass and polydispersity. The model was in agreement with independent thermodynamic calculations from experimental data.

1.3.5 Model for Polymer-Salt Systems

A model for polymer-salt systems was developed, indicating that the polymer concentration of the salt-rich phase of the ATPS can be neglected, since it is generally small compared to the salt concentration. The protein solubility term is expressed by a relationship between protein solubility in the salt-rich phase, the molar salt concentration in the salt-rich phase, the solubility of the protein in pure water and the salting-out constant of the salt for the protein. The salting-out constant depends on the protein and the salt and is related to the lyotropic series (BASKIR *et al.*, 1987; BASKIR *et al.*, 1989). In the polymer-rich phase,

however, the concentration of both polymer and salt are significant. Randomly coiled polymer chains are known to contain a large amount of solvent. The polymer in the polymer- rich phase "excludes" the protein and the salt from a portion of the water present in the phase. The formation of hydrated PEG removes some of the water, which would otherwise be available for dissolving the salt or the protein. Consequently the protein and the salt are concentrated in the portion of available water in the phase.

1.3.6 Eiteman and Gainer Model

A mathematical model was developed by EITEMAN and GAINER (1990, 1991) to predict partition coefficients for solutes in PEG-sulphate systems. Their expression has been validated for peptides, showing that increasing the difference in concentration of PEG (Δw) between the phases or increasing solute hydrophobicity also increases the partition coefficient.

$$\log K_{\text{hydrophob}} = D \Delta w \log (P/P_0)$$

An increase in Δw can be considered to be equivalent to an increase in tie-line length. The model still has to be improved for proteins. K values for papain and trypsin increased as Δw increased. K for lysozyme first decreased, and after reaching a minimum, then increased as Δw increased (FRANCO *et al.*, 1996b).

1.4 AFFINITY PARTITIONING

In the last 30 years, several groups have studied methods to increase partitioning by the use of biospecific interactions in ATPS (FLANAGAN & BARONDES, 1975; JOHANSSON *et al.*, 1983; JOHANSSON *et al.*, 1984; LUONG & NGUYEN, 1990).

The initial works on affinity partitioning in ATPS were to purify trypsin by using

PEG-bound ligand p-aminobenzamidine (TAKERKAR *et al.*, 1974) and S-23 myeloma protein by using dinitrophenol as ligand (FLANAGAN & BARONDES, 1975).

The degree of affinity partitioning, K_{aff} , can be described by the ratio between the partition coefficients of proteins with and without a ligand (JOHANSSON, 1985b):

$$K_{\text{aff}} = \frac{K(\text{PEG-ligand})}{K(\text{PEG-without ligand})}$$

This equation describes the increase in the partition coefficient of a protein by the binding of a specific ligand to the PEG-rich phase.

Affinity partitioning results in specific extractions of proteins, nucleic acids, membranes, organelles and even cells, mainly when biospecific ligands are used (WALTER *et al.*, 1985).

The ligand can be a natural substance or a synthesized molecule. There are ligands that interact selectively with proteins due to the specific sites. There are many groups of ligands, such as hydrophobic and thiophilic ligands (PORATH *et al.*, 1975), metal chelates (WUENSCHHELL *et al.*, 1990; ARNOLD, 1991; BIRKENMEIER *et al.*, 1991; ZUTAUTAS *et al.*, 1992; CHUNG *et al.*, 1994), quaternary ammonium compounds (JOHANSSON *et al.*, 1981), dyes (KOPPERSCHLÄGER & JOHANSSON, 1982; KRONER *et al.*, 1982; JOHANSSON *et al.*, 1983; JOHANSSON & ANDERSSON, 1984a; JOHANSSON & ANDERSSON, 1984b; JOHANSSON *et al.*, 1984; BIRKENMEIER *et al.*, 1984; JOHANSSON & JOELSSON, 1985a; JOHANSSON & JOELSSON, 1985b; CORDES *et al.*, 1987; GIULIANO, 1991; WANG *et al.*, 1992; ZUTAUTAS *et al.*, 1992; BIRKENMEIER, 1994; KOPPERCHLÄGER, 1994; LIN *et al.*, 1996), fatty acids (JOHANSSON & SHAMBHAG, 1984; JOHANSSON *et al.*, 1985), inhibitors (SILVA *et al.*, 1997) and monoclonal antibodies (ELLING *et al.*, 1990; ELLING & KULA, 1991; ELLING *et al.*, 1991; ANDREWS *et al.*, 1996). Natural ligands are generally expensive due to the high cost of purification, chemical and biological lability (KOPPERSCHLÄGER, 1994).

Table 2 reports the purification of different biomolecules by affinity partitioning described in the literature.

Table 2. Biomolecules purified in ATPS by affinity.

Biomolecule	Ligands attached to PEG	Recovery (%)	Purific. factor	Reference
Lactate dehydrogenase	Tryazine dye-Cibacron Blue F3G-A	81.3	7.4	LIN <i>et al.</i> (1998)
β -galactosidase	p-aminophenyl- β -D-thiogalactopiranoside – (APGP)	83	6	SILVA <i>et al.</i> (1997)
Protein A	IgG human	87	-	SUZUKI <i>et al.</i> (1995)
Lactate dehydrogenase	Eudrogit-Cibacron Blue	54	11.7	GUOQIANG <i>et al.</i> (1994)
Penicillin acylase	Trimethylamina	97	25.7	GUAN <i>et al.</i> (1992)
Trypsin	Trypsin inhibitor	82	-	LUONG & NGUYEN (1990)
Ligands attached to Dextran				
Liposome	Avidin	95	-	EKBLAD <i>et al.</i> (1998)
Chymosin	Pepstatin	83	6.2	CHEN & JEN (1993)

Downstream processing of proteins has been considerably advanced with the use of affinity separation methods, as can be evidenced by the extensive publications in this field.

Application of affinity separation methods in the initial steps of the extraction process was initially described by SADA (1990). The concept of the use of affinity interactions for initial isolation is attractive as a technique of high resolution applied early in the purification process, reducing the volume of material to be manipulated later on, the consumption of chemical products used in the process and biological activity loss of biomolecule (ASENJO & PATRICK, 1995).

According to KOPPERSCHLÄGER (1994), this technique can operate continuously and be scaled up. Also, the target biomolecule can be purified by a single or multistep

extraction, and affinity partitioning of biomolecules is a simple and adequate approach to the qualitative and quantitative study of protein-ligand interaction.

BLENNOW (1994) showed the following advantages of using affinity partitioning in ATPS: 1) enzyme recovery is high, 2) the conditions are mild, 3) the procedure can be scaled up, 4) the method is reasonably inexpensive and 5) standard laboratory devices can be used.

ARNOLD (1991) reports the following advantages of metal-affinity partitioning in ATPS over affinity chromatography for protein purification: 1) metals can be recycled a large number of times, 2) high metal loadings and high protein capacities then can be attained, 3) product elution is achieved relatively easily, 4) ligands are regenerated and 5) metal chelate ligands are cheap.

1.5 CONTINUOUS EXTRACTION IN ATPS AND RECYCLING OF PHASE COMPONENTS

Recent advances in ATPS have enhanced the choices of commercial applicability for large-scale operation (WINTER *et al.*, 1999; LORCH, 1999). However, as the main properties of ATPS are quite extreme when compared with non-polar solvents (Table 3), detailed studies had to be conducted in order to adapt the extraction equipment to aqueous two-phase systems. Very fine droplets are formed in a continuous extraction column, allowing a very high interfacial area for the rapid transfer of enzyme or protein (BHAWSAR *et al.*, 1994).

Table 3. Comparison between conventional liquid-liquid extraction and ATPS according to BHAWSAR *et al.* (1994).

Properties	Conventional liquid-liquid extraction	ATPS
Viscosity	< 1 mPa s	14 mPa s
Difference in density	twice x	x
Tension	13 mM m ⁻¹	10 ⁻⁴ to 10 ⁻¹ mM m ⁻¹

Enzyme mass transfer was investigated in a sieve-plate extraction column (BHAWSAR *et al.*, 1994), in a packed extraction column (PATIL *et al.*, 1991), in a modified spray column (PAWAR *et al.*, 1997) and in a conventional spray column (SAWANT *et al.*, 1990).

The effect of design parameters (number of plates or plate spacing) and operating parameters (dispersed phase velocity and physical properties) on the overall volumetric mass transfer coefficient and hold-up have been studied by some authors (SAWANT *et al.*, 1990; PAWAR *et al.*, 1997; BHAWSAR *et al.*, 1994; PATIL *et al.*, 1991). It was observed that the viscosity of the dispersed phase was usually more than 14 mPa s, which is much higher than that in conventional liquid-liquid extraction. Also, the difference in density of a non-polar system is at least twice that in the case of ATPS. In addition, the diffusivity of enzymes and proteins in ATPS is about one order of magnitude lower than that in conventional systems. BHAWSAR *et al.* (1994) studied the enzyme mass transfer in a sieve-plate extraction column and observed that the total hold-up, the fractional dispersed hold-up and the volumetric mass transfer coefficient increased as the orifice diameter and the number of orifices on plates in the sieve-plate extraction column increased. The values of the mass transfer coefficient and total hold-up also increased as the number of plates in the column increased together with a simultaneous decrease in the plate spacing. It was also found that these three parameters decreased as the tie-line length (higher concentrations) of the ATPSs increased and that the PEG-rich dispersed phase controlled the resistance to mass transfer. It was observed that the major contributions to mass transfer of amyloglucosidase were

attributed to the drop rise and the coalescent stages. The drop formation contribution was found to be negligible.

The recovery of polymers after extraction of a biomolecule is a procedure as important which is in laboratorial processes as it is on an industrial scale. The ability to easily recycle the polymers would make large-scale ATPS more economically attractive (CARLSSON, 1988).

Methods of recycling the phase-forming components in ATPS have been used on small scale, as described by different authors (CARLSSON, 1988; KULA *et al.*, 1982). CARLSSON (1988) reports that PEG is recycled by extraction with chloroform and reused in a new phase system. Another alternative mentioned by this author is the transfer of a PEG-rich phase of a system where the biomolecules had partitioned in a first system to a second fresh saline phase with a different composition. The biomolecules would then partition into the bottom-rich phase of this second system, releasing a protein-free PEG-rich phase that would be reused in another extraction process.

KULA *et al.* (1982) described some methods to recycle PEG, such as ultrafiltration or dialysis. The other way to obtain PEG-free enzymes is the adsorption of the enzyme by suitable adsorbents, thus recovering the PEG phase.

GREVE and KULA (1991) studied three ways to recycle salt and the best was by using an aqueous ethanol solution to extract salt. The alcohol and salt-rich phase flow to an evaporator where the alcohol is removed and recycled. The salt solution is concentrated, if necessary, in a second evaporator, from which most of the water may also be recycled. The concentrated salt solution is then reintroduced into the protein extraction process.

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CAPÍTULO 3

PURIFICATION OF THREE DIFFERENT MICROBIAL β -GALACTOSIDASES BY PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS

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PURIFICATION OF THREE DIFFERENT MICROBIAL β -GALACTOSIDASES BY PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS¹

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SUMMARY

This work investigated the effect of the molecular weight of polyethyleneglycol (PEG) upon the partition coefficient of β -galactosidases from three different microorganisms: *Escherichia coli*, *Klueveromyces lactis* and *Aspergillus oryzae*. It was found that PEG 6,000 and PEG 8,000/phosphate were the best systems for achieving the highest purification factors of *E. coli* β -galactosidase. However, the other two yeast β -galactosidases were not efficiently separated from their contaminants in any of the PEG/salt systems. In order to improve the separation of *Klueveromyces lactis* β -galactosidase from the main protein contaminants, the biospecific ligand p-aminophenyl 1-thio- β -D-galactopyranoside (APGP) was attached to activated PEG 4000. The affinity PEG having APGP bound to its backbone was synthesized in two steps. The partitioning of *Klueveromyces lactis* β -galactosidase in aqueous two-phase systems prepared with 6% APGP-PEG4000 + 12% dextran T505,000 increased 1.6-fold the purification factor of the target enzyme, allowing the recovery of 83% of the enzyme in the top PEG-rich phase.

Key-words: β -galactosidase, protein recovery, aqueous two-phase systems, downstream processing.

RESUMO

PURIFICAÇÃO DE TRÊS DIFERENTES β -GALACTOSIDASES MICROBIANAS POR PARTIÇÃO EM SISTEMAS DE DUAS FASES AQUOSAS. Este trabalho tratou da investigação do efeito do peso molecular de polietilenoglicol (PEG) sobre a partição de enzimas β -galactosidases de diferentes origens microbianas: *Escherichia coli*, *Klueveromyces lactis* e *Aspergillus oryzae* em sistemas de duas fases aquosas (SDFA). Foi observado que os melhores sistemas para purificação da enzima de *E. coli* foram os formados por PEG 4000, 6000 e 8000/fosfato, fornecendo os mais elevados fatores de purificação da enzima. As enzimas de *Klueveromyces lactis* e *Aspergillus oryzae* não foram eficientemente purificadas nestes sistemas sendo insensíveis a alterações do peso molecular do PEG. Portanto, um outro sistema de duas fases aquosas foi desenvolvido contendo um ligante específico, p-aminofenil 1-tio- β -D-galactopiranosídeo (APGP), acoplado ao PEG para purificar a enzima de *Klueveromyces lactis*. Uma etapa simples de partição no SDFA formado por 6% APGP-PEG4000 + 12% dextrana T505,000 foi capaz de recuperar 83% da enzima na fase superior do sistema e de aumentar 1.6 vezes o fator de purificação.

Palavras-chave: β -galactosidase, recuperação de proteína, sistemas aquosos bifásicos.

1 — INTRODUCTION

The enzymatic hydrolysis of lactose to glucose and galactose with β -galactosidase is one of the most ancient

biotechnological processes known to humanity. The microorganisms usually associated with β -galactosidase high production are the *Escherichia coli*, *Bacillus megaterium*, *Bacillus stearothermophilus* bacteria and the *Klueveromyces lactis* and *Aspergillus oryzae* yeasts, in liquid (16) and in solid-state fermentation (3). The improvement of β -galactosidase downstream processing from its microbial sources would be desirable to reduce the cost of enzyme preparation, since extraction, purification and concentration steps are commonly responsible for up to 40% of total production cost (9).

Purification of target proteins requires their separation from the media or from the raw extract used for the maintenance of the biomolecules. The product is usually present at low levels and also needs to be concentrated. Protein extraction in aqueous two-phase systems (ATPS) is a rapid procedure which avoids most of problems of denaturing fragile molecules in chromatographic beads. ATPS provide a gentle environment for biologically active proteins and may be employed on a large scale (1, 2, 9).

In order to have a high yield, recovery and also a good purification factor for a target protein, a composition of the ATPS has to be selected. Compositions of desirable systems are those which promote high values for the partition coefficients (K), where the target protein is primarily extracted in one of the phases and the main contaminants are extracted in the other phase. The partition coefficient of a particle, K, is defined as the ratio of the concentration of the partitioned substance in the top phase to the concentration in the bottom phase. For extreme K values of the target protein, most of the contaminants should partition to the bottom (higher density) phase and for very low K values of the target protein, the majority of the contaminants would have to partition towards the top (lower density) phase for a good separation to occur. By following the above approach for the designing of compositions of the ATPS, it is possible to quantitatively extract the desirable protein from one of the phases with minimal concentration of contaminant molecules. A single-extraction step is usually sufficient for removal of noncritical contaminants, such as albumin in albumin-free pharmaceutical products. However, critical contaminants can be eliminated by countercurrent extraction, using a fresh pure phase from identical systems without the sample. They may also be removed by polishing the material collected from the enriched phase by using another process, usually a chromatographic step (2, 6, 7, 9).

The main factors which usually affect the partitioning of the target protein and its contaminants, are the molecular weight and the concentration of the polymers, the pH, the type of salt used for phase separation, e.g., phosphate, citrate or sulfate, and the addition of extra salt.

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The composition of the required ATPS can be found by investigating the effects of a single parameter on the partition coefficients (one by one) or by applying an experimental factorial design.

Liquid-liquid partitioning of proteins in aqueous two-phase systems (ATPS) can be an efficient and versatile method for the separation and purification of proteins. The systems are usually composed of aqueous solutions of two polymers, polyethyleneglycol (PEG) and dextran or of PEG and a lyotropic salt, usually phosphate, citrate or sulfate (4, 6).

The advantages of two-phase partitioning, when compared with other purification processes, include rapid and continuous operation leading to high yields, ease of scale-up, low material costs and polymer and salt recycling (9), minimal protein denaturation and the possibility of having particulate material in the process.

This work was organized in two parts, the first investigated the partitioning of three different β -galactosidases in PEG/phosphate systems in a simple single-step procedure and aimed to achieve a high purification factor. The second part investigated the use of an affinity ATPS for the purification of the enzyme.

2 — MATERIAL AND METHODS

The β -galactosidases investigated were from three microorganisms: *Escherichia coli*, *Clueveromyces lactis* and *Aspergillus oryzae*.

2.1 — Production of β -galactosidases

For *Escherichia coli* β -galactosidase production, *E. coli* was grown at 37°C for 24 h in the following culture medium: 20.0 (g/L) bacto tryptone, 5.0 (g/L) lactose, 4.5 (g/L) KH_2PO_4 , 4.0 (g/L) K_2HPO_4 and 5.0 (g/L) NaCl with pH adjusted to 6.9. The fermented culture broth was centrifuged at 2,000 g for 20 min. The cells were broken with chloroform + phosphate buffer and magnetically stirred for an hour (twice) in order to release the intracellular target enzyme. The aqueous supernatant was used as the source of crude β -galactosidase. The enzyme from *Clueveromyces lactis* (Lactomax 50) was a gift from Solvay (Buenos Aires, Argentina) and the *Aspergillus oryzae* enzyme was purchased from Sigma (St. Louis, USA). All the other reagents used were of an analytical grade.

2.2 — Aqueous two-phase systems preparation

The aqueous two-phases phosphate systems were prepared according to Franco *et al.* (6) from stock solutions of 40% polyethyleneglycol (w/w) of four different molecular weights (PEG 1,500, 4,000, 6,000 and 8,000), 40% phosphate (w/w) and 25% dextran T505,000 (w/w). Phosphate stock solutions were formulated with different ratios of K_2HPO_4 to KH_2PO_4 to give the desired pH values.

2.3 — Determination of β -galactosidase activity

The β -galactosidase activities of the three microorganisms were determined by using o-nitrophenyl- β -galactopyranoside (ONPG) as substrate and by reading the formed colored complex at 420 nm. The enzyme from *E. coli* was

assayed at 37°C, the enzyme from *K. lactis* at 45°C and the one from *A. oryzae* at 30°C. One β -galactosidase unit corresponds to 1.0 micromol of orthophenol released per minute under each given condition.

2.4 — Partitioning of β -galactosidase

One hundred μl of enzyme were mixed with the ATPS, using a Vortex for 1 minute. Phase separation was achieved by centrifugation for 3 min. at 3,000 g and the phases were carefully separated and the interface of each tube discharged. A known volume of each phase was transferred and the activity was determined. The partition coefficient (K) was calculated from the ratio of the β -galactosidase activity found in the top phase and the activity found in the bottom phase.

2.5 — BSA standard curve

The protein concentration was measured using the dye-binding technique of Sedmark and Grossberg (14). Fifty to 100 μl of each BSA solution were transferred (in triplicate) to a cuvette containing 2.4 ml of water and 1.0 ml of Coomassie blue solution and mixed well, and the OD₅₉₅ was read with a spectrophotometer, against a blank which has 50 μl of water instead of BSA. BSA concentrations were plotted against the OD₅₉₅.

2.6 — Partitioning of total proteins

Fifty to 100 μl of the top phase were transferred from each prepared system to a cuvette containing 2.4 ml of water and 1.0 ml of Coomassie blue solution and mixed well, and the OD₅₉₅ was read with a spectrophotometer, against a blank which has 50 μl of a top phase of a system which has been equally prepared without any sample, instead of BSA. The blanks were done to correct the interference of the phase components. The procedure was repeated for the bottom phase of each system. A BSA standard curve was used to calculate protein concentration. The partition coefficient, K, was calculated as the ratio of protein in the top phase to that in the bottom phase at room temperature.

2.7 — Synthesis of modified PEG

The modified PEG was synthesized according to Delgado *et al.* (5) and Nilson and Mosbach (10). The affinity ligand p-aminophenyl 1-thio- β -D-galactopyranoside (APGP) was bound to PEG 4000 in two steps. Firstly PEG 4000 was dried by azeotropic distillation in toluene and then activated with 2,2,2-trifluoroethanesulphonyl (tresyl chloride from Sigma) to form the precursor PEG-tresylated (TPEG). TPEG was washed twice with HCl-ethanol (250:1, v/v), precipitated at 2°C and kept in a desiccator. In the second reaction step, TPEG reacted with APGP in phosphate buffer (pH 7.5) for five hours. Samples were taken each hour for measuring the effect of the ligand binding upon the partition coefficient of the enzyme. The reaction was stopped with 0.18 g tris.

2.8 — Specific β -galactosidase activity ($\text{SA}_{\beta\text{-gal}}$)

It is defined as the ratio of enzyme activity (U/ml) to the total protein concentration (mg/ml) and is expressed in U/mg of protein (equation 1).

$$SA_{\beta\text{-gal}} = \frac{\text{enzyme activity}}{\text{protein concentration}} \quad (1)$$

2.9 - Purification factor (PF)

The PF concept has been used in this work as a measurement to follow the purification operations, according to several authors (8, 13), and is defined as the ratio of the specific β -galactosidase activity after a purification step to the initial specific β -galactosidase activity (from the aqueous enzyme extract, or from a previous purification step) (equation 2).

$$PF = \frac{SA_{\beta\text{-gal}} \text{ in the collected phase}}{\text{Initial } SA_{\beta\text{-gal}}} \quad (2)$$

The recovery of the enzyme, R, is defined as the ratio of the enzyme activity collected into an aqueous phase after partitioning to the total enzyme activity added to the system (equation 3).

$$R (\%) = \frac{\text{enzyme activity collected in a phase}}{\text{total enzyme activity added to the system}} \quad (3)$$

3 - RESULTS AND DISCUSSION

3.1 - Partitioning in PEG/phosphate systems

β -galactosidases of different origins, from *E. coli*, *K. lactis* and *A. oryzae*, were partitioned in ATPS composed of PEG and phosphate. Several different parameters were selected for influencing the partitioning of proteins in ATPS, the most effective usually being the molecular weight (MW) of PEG, PEG and salt concentration, pH and ionic strength and the presence of a specific ligand in the system. The effect of PEG molecular weight (MW) upon the partition coefficient of the β -galactosidases was firstly investigated in this work (Table 1). In order to achieve a good separation of the β -galactosidase and its main contaminants, it would be desirable to find an ATPS where they are mostly extracted in opposite phases. The activity in the aqueous *E. coli* β -galactosidase extract was 630 U/ml and protein concentration was 20 mg/ml; therefore, the $SA_{\beta\text{-gal}}$ was 31 U/mg of protein. The volume ratio of the phases was approximately 1:0 for all the systems. The volume of enzyme added to each ATPS was 100 μ l (63 U). Therefore, PF was calculated as the ratio of the specific β -galactosidase activity in the aqueous phase (after a partitioning purification step) to the initial specific β -galactosidase activity.

Figure 1 represents the *E. coli* β -galactosidase partitioning in PEG/phosphate systems. It can be observed that the molecular weight of PEG has a significant effect upon the partitioning of *E. coli* β -galactosidase, showing that $K_{\beta\text{-gal}}$ was highest in PEG 1,500 and PEG 6,000/phosphate. However, as K_{prot} was also highest in PEG 1,500/phosphate, the PF achieved was the lowest. The purification factors increased with PEG MW. PF were 2.5, 6.4, 8.0 and 9.7 in systems composed of PEG 1,500, 4,000, 6,000 and 8,000/phosphate, respectively. Very high recoveries were achieved for the enzyme in the top phase of the systems: 96%, 90%, 95% and 72% of β -galactosidase were collected in PEG 1,500,

TABLE 1. Effect of PEG molecular weight (MW) on the partition coefficient, purification factor (PF $_{\beta\text{-gal}}$) and recovery (R) of *E. coli*, *Klueveromyces lactis* and *Aspergillus oryzae* β -galactosidases.

Microorganism	<i>E. coli</i>				<i>Klueveromyces lactis</i>		<i>Aspergillus oryzae</i>	
PEG MW	$K_{\beta\text{-gal}}$	K_{prot}	PF $_{\beta\text{-gal}}$	R (%)	$K_{\beta\text{-gal}}$	K_{prot}	$K_{\beta\text{-gal}}$	K_{prot}
PEG 1500	101	0.85	2.5	96	0.01	0.10	0.20	0.10
PEG 4000	39	0.18	6.4	90	0.02	0.16	0.12	0.13
PEG 6000	81	0.15	8.0	95	0.05	0.16	0.20	0.14
PEG 8000	19	0.11	9.7	72	0.05	0.22	0.21	0.16

systems a and b: 15.0 % PEG + 13.6% phosphate (pH 7.0)

system c: 15.0% PEG + 16.0 % phosphate (pH 5.0)

PEG 4,000, PEG 6,000 and PEG 8,000 top phases, respectively. As total protein partitioning is also affected by PEG molecular weight, remaining mostly at the bottom phosphate-rich phase. PEG 8,000/phosphate was the system with the highest PF, 9.7, despite its lowest enzyme partitioning coefficient ($K=19.0$). *K. lactis* and *A. oryzae* β -galactosidases did not seem to partition towards the top phase, independently of the PEG MW. The PEG/phosphate systems investigated were not able to separate β -galactosidase from the main contaminants. The addition of sodium chloride to PEG/salt and to PEG/dextran systems is commonly utilized to improve protein extraction and separation. However, concentrations as high as 12% NaCl (w/w) were added to the systems without any improvement in the purification of *K. lactis* and *A. oryzae* β -galactosidases. It seems that *K. lactis* β -galactosidase is the least hydrophobic enzyme of the three investigated, since according to Franco *et al.* (6,7) only very hydrophilic proteins do not have their partition coefficients raised by the addition of NaCl to PEG/phosphate systems. The very low K (0.01 to 0.05) of *K. lactis* β -galactosidase can also be considered an indication of low hydrophobicity.

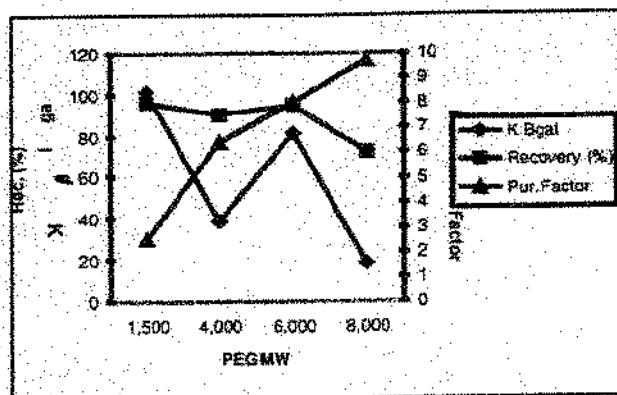


FIGURE 1. Effect of PEG MW upon the partition coefficient, purification factor and recovery of *E. coli* β -galactosidase in PEG/phosphate system.

3.2 - Affinity partitioning

In order to improve the extraction and separation of the *K. lactis* β -galactosidase, an affinity ATPS was developed. The system consisted of the APGP biospecific ligand chemically attached to PEG (two-step reaction) and dextran

T505,000. In the first step PEG hydroxyls had to be activated with tresyl chloride to become more reactive. In the second step the ligand APGP was finally bound to PEG.

The elemental analysis for sulfur indicated that 74% of total hydroxyl groups in PEG had been transformed into tresyl esters in the first activation step. This activation rate is slightly lower than the rates achieved by Nilson and Mosbach (10) and by Delgado *et al.* (5), indicating that some of the tresyl chloride molecules might have been hydrolyzed by water before reacting with PEG hydroxyls despite laboratory precautions taken to achieve water-free solvents and conditions. Nilson and Mosbach (10) have activated 80% of PEG hydroxyl groups with tresyl chloride and Delgado *et al.* (5) have achieved approximately 83% activation.

The amount of APGP bound to PEG was determined indirectly by preparing an ATPS composed of the reacted polymer and dextran T500 (6% PEG4000 + 12% dextran) for the partitioning of β -galactosidase from *Klueveromyces lactis*. A direct method to control the chemical binding of APGP to TPEG using HPLC was previously investigated. It was not possible to detect the decrease of APGP concentration in the supernatant and the elution times of TPEG and APGP-PEG were found to be very close (gel permeation chromatography, Bio-Gel SEC 30XL column). The results from Table 2 show that the partition coefficient of β -galactosidase increased more than 250% for the highest ratio of APGP attached to TPEG. Approximately 49 units of β -galactosidase were recovered (83%) in the top phase of the system with the highest APGP content (polymer which reacted for 5 hours with APGP) from 50 units which had been previously added to the system. It was also found that more than 80% of the contaminant proteins had partitioned towards the bottom phase. Therefore, a 1.6-fold increase in the purification factor was achieved in just a single step of partitioning in this system.

TABLE 2: Effect of APGP binding to TPEG on the partition coefficient of *Klueveromyces lactis* β -galactosidase, proteins and PF (6% PEG 4000 + 12% dextran T505,000 systems, pH=6.5).

Reaction time (h)	K _{0-gal}	K _{prot}	PF _{β-gal}
blank (without APGP)	0.9	0.20	2.4
0.25	1.0	0.21	2.5
1.00	1.1	0.21	2.7
2.00	1.2	0.22	2.8
3.00	1.7	0.21	3.2
4.00	2.4	0.21	3.6
5.00	3.3	0.20	3.9

Park *et al.* (11) have extracted and purified a β -galactosidase from *Aspergillus oryzae* by using three different chromatographic steps, achieving very low yields despite a high specific activity. Pastore and Park (12) have purified a β -galactosidase from *Scopulariopsis sp* by precipitation with ammonium sulfate and two chromatographic steps leading to a 4% yield of the desired pure enzyme. Stred'ansky *et al.* (15) have optimized the enzyme extraction from *Klueveromyces lactis* by choosing the ideal pH and temperature values, as well as the concentrations of phosphate and chloroform, in

order to release 95% of the intracellular β -galactosidase. Veide *et al.* (16) have developed an isolation and purification industrial process for *E. coli* β -galactosidase by partitioning in PEG4000/potassium phosphate systems, followed by an ultrafiltration step for the recycling of the salt-rich phase. They were able to recover 95% of the β -galactosidase. The only enzyme which was efficiently purified in simple ATPS was from *E. coli*, following the same trend as in the previous authors, probably due to its higher surface hydrophobicity, since the other two hydrophilic yeast β -galactosidases do not partition towards the top PEG-rich phase almost independently of the polymer molecular weight or the addition of extra salt.

It seems clear that partitioning in ATPS can be a very effective way of purifying and concentrating enzymes and other biomolecules. It can be highly improved by designing efficient and specific bioligands which should not be expensive and should need to be recycled (i.e. ultrafiltration). Research on affinity techniques, binding chemistry and the designing of specific equipments for extraction in ATPS will be of great help in the improvement of downstream processing of enzymes.

4 — CONCLUSIONS

A significative effect of the molecular weight of PEG upon the partitioning of *E. coli* β -galactosidase was observed. PEG 6,000 and PEG 8,000/phosphate systems gave better separations of the *E. coli* enzyme from the main contaminants and higher purification factor values. Recoveries of 95 and 72% of the enzyme and purification factors of 8.0 to 9.7 were achieved in systems composed of PEG 6,000/phosphate and PEG 8,000/phosphate, respectively, in a single-partitioning step.

However, *K. lactis* and *A. oryzae* β -galactosidases were not purified in PEG/salt systems independently of the PEG MW, and an affinity ATPS was developed for their purification. It was possible to recover 83% of *K. lactis* β -galactosidase in the top APGP-PEG 4000 phase, with a 1.6-fold increase in the purification factor, by using an affinity ligand attached to PEG in the PEG/dextran T505,000 system. More than 80% of the contaminant proteins were collected in the dextran-rich bottom phase.

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CAPÍTULO 4

PURIFICATION OF MICROBIAL β -GALACTOSIDASE FROM *Kluyveromyces fragilis* BY BIOAFFINITY PARTITIONING

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Trabalho submetido para publicação na revista **Journal of the Brazilian Society for
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SUMMARY

This work investigated the partitioning of β -galactosidase from *Kluyveromyces fragilis* in aqueous two-phase systems (ATPS) by bioaffinity. PEG 4000 was chemically activated with thresyl chloride, and the biospecific ligand p-aminophenyl 1-thio- β -D-galactopyranoside (APGP) was attached to the activated PEG 4000. A new two-step method for extraction and purification of the enzyme β -galactosidase from *Kluyveromyces fragilis* was developed. In the first step, a system composed of 6% PEG 4000-APGP and 8% dextran 505 was used, where β -galactosidase was strongly partitioned to the top phase ($K = 2,330$). In the second step, a system formed of 13% PEG-APGP and 9% phosphate salt was used to revert the value of the partition coefficient of β -galactosidase ($K = 2 \times 10^{-5}$) in order to provide the purification and recovery of 39% of the enzyme in the bottom salt-rich phase.

INTRODUCTION

Partitioning of biomaterials in aqueous two-phase systems (ATPS) is a selective method for purification and for analytical studies of cellular components of several sizes, including proteins, nucleic acids, membranes and cellular organelles. An ATPS is formed by the addition of aqueous solutions of two polymers, such as PEG and dextran, or a polymer and a lyotropic salt, such as PEG and potassium phosphate. The extraction and separation process in ATPS can be used as a substitute for the initial steps of purification and preparative chromatography of biomaterials, can be scaled-up without a significant efficiency loss and can be accomplished in the absence of sophisticated equipment.

The choice of a non-aggressive method such as ATPS partitioning maintains the biological properties of biomolecules. An ATPS contains a large amount of water in both phases, constituting an excellent mild biological method to recover cells, organelles or active proteins (ALBERTSSON, 1971). Methods described in the literature, such as liquid-liquid extraction, are gaining prominence in meeting the basic demand due to the viability of their industrial applications.

Dextran and polyethylene glycol are not toxic chemicals, are included in the pharmacopoeias of many countries and have applications in the food industry. Utilization of these polymers can be considered an advantage in the development of new technologies because a lot of applications of enzymes and biologically active proteins are in the food and pharmaceutical industries.

In order to achieve a high recovery and the concentration of a target protein in one phase of the ATPS, it is necessary to increase the difference between the value of the partition coefficient (K = concentration of the protein in the top phase/bottom phase) of the target protein and the K value of the contaminant material (K_P). Figure 1 shows a diagram of an ATPS.

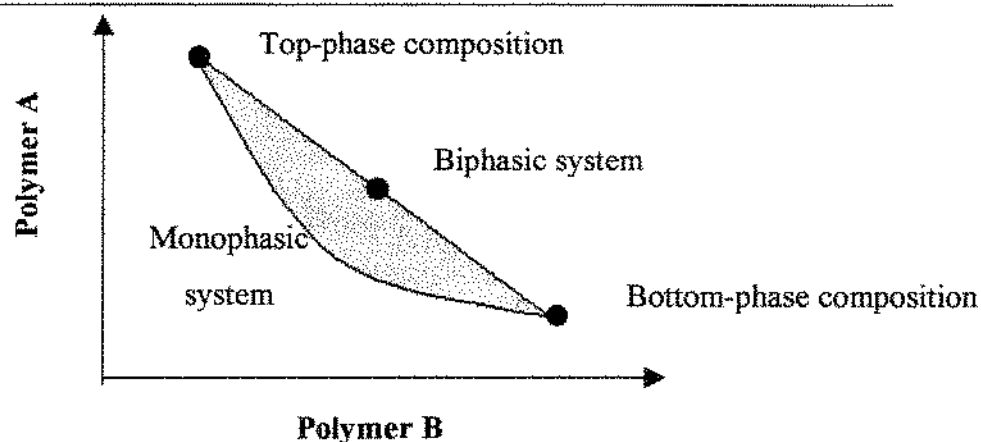


Figure 1. Binodial curve of an aqueous two-phase system.

Parameters such as polymer molecular mass and concentration, type and concentration of salt, pH and temperature can affect K .

The process of purification in ATPS by bioaffinity combines the property of biological recognition and partitioning in a liquid environment. The ligand is coupled by covalent bonds to the polymer phase; therefore it will have a high partition coefficient towards the phase enriched with the polymer. If the target protein to be isolated has a specific affinity for the ligand, the formation of a ligand-biomolecule complex will induce an increase in the partition coefficient of the protein towards the phase enriched in the ligand (KOPPERSCHLÄGER, 1994; BIRKENMEIER, 1994), while the presence of contaminant molecules will be predominant in the opposite phase (DIAMOND & HSU, 1992).

The enzyme lactase or β -galactosidase has many applications in dairy technology, such as for industrialized products containing lactose. β -galactosidase can also be applied in crystal removal, production of sweeteners, solubilisers and toothpaste and research and analytic activities (GODFREY & REICHELT, 1983). The transglycosylation activity of β -galactosidase, leading to the synthesis of oligosaccharides, reported by PRENOSIL *et al.* (1987) and BRENA *et al.* (1994). Lactase-hydrolyzed products are currently being manufactured in Brazil, and the demand for such products would increase if this enzyme were produced at a lower cost and a higher quality.

This enzyme is intracellularly formed in yeasts and bacteria and secreted by fungi. The most important microorganisms which produce this enzyme are species of *Aspergillus niger*, *A. oryzae*, *Kluyveromyces fragilis* and *K. lactis*. Its production by *Neurospora* (LANDMAN, 1954), *Escherichia coli* (WALLENFELS *et al.*, 1960), *Saccharomyces lactis* (GUY & BINGHAM, 1978), *Bacillus circulans* (KITAHATA *et al.*, 1991), *Scopulariopsis* sp (PASTORE & PARK, 1980) and *Erwinia aroidea* (FLORES & ALEGRE, 1996) has also been reported.

MATERIALS AND METHODS

PEG 4000 was purchased from Fluka (Switzerland) and tresyl chloride from Sigma (St. Louis, MO, USA). The *Kluyveromyces fragilis* employed was obtained from the American Type Culture Collection (ATCC 46537). A commercial β -galactosidase (Lactozym) was the kind gift of Novo Nordisk.

Enzyme production

For the production of β -galactosidase, *K. fragilis* was grown at 37°C for 24 h in the following culture medium: 30 ml of commercial milk, 0.15 g of $(\text{NH}_4)_2\text{SO}_4$ and 0.06 g of KH_2PO_4 , with pH adjusted to 6.5. The fermented culture broth was centrifuged at 3,000 g for 20 min. The mass of cells was weighed and chloroform was added in the proportion of 1:1 (w/w). This suspension was kept under magnetic stirring for 1 hour, and then 20 ml of 0.05 M phosphate buffer, pH 7, was added and centrifuged. This procedure was repeated three times. The supernatant containing intracellular material of *K. fragilis* was used in the experiments.

Determination of β -galactosidase activity

The enzyme activity was determined by using *o*-nitrophenyl- β -galactopyranoside (ONPG) as the substrate and by spectrophotometric measurement at 420 nm. The enzyme was assayed at 37°C. One β -galactosidase unit corresponds to 1.0 μ mol of orthophenol released per minute under the given condition.

Partitioning in the ATPS

One hundred μ l of enzyme sample was mixed into the ATPS, using a Vortex for 30 sec. Phase separation was achieved by centrifugation for 5 min. at 3,000 g and the interface of each system discharged. A known volume of each phase was collected and the activity was determined. The partition coefficient (K_E) of the enzyme was calculated from the ratio of β -galactosidase activity found in the top phase and activity found in the bottom phase.

Protein assay

The main contaminant protein concentration was determined by the dye-binding technique of SEDMAK and GROSSBERG (1977). Fifty to 100 μ l of the top phase was transferred from each prepared system to a cuvette containing 2.4 ml of water and 1.0 ml of Coomassie blue solution and mixed well, and the OD₅₉₅ was measured in a spectrophotometer, versus a blank which had 50 μ l of a top phase of a system which had been equally prepared without any sample. The blanks were done to correct the interference of the phase components. The procedure was repeated for the bottom phase of each system. A BSA standard curve was used to calculate protein concentration. The partitioning of the main contaminant protein, K_P , was calculated as the ratio of protein in the top phase to that in the bottom phase at room temperature.

Synthesis of PEG-APGP

The PEG-APGP was synthesized according to DELGADO *et al.* (1990) and NILSSON & MOSBACH (1984).

Activation of PEG 4000 with tresyl chloride

(I) Solid PEG 4000 (20 g) was dried by azeotropic distillation in toluene and then dried in vacuum. The white solid was dissolved in 45 ml of dry dichloromethane at room temperature. The mixture was cooled at 0°C and stirred magnetically, and 1.125 ml of pyridine and 1 g of tresyl chloride at 0°C were added drop by drop. The reaction was continued at room temperature with constant stirring for 1.5 h, and the dichloromethane was removed by evaporation under reduced pressure. The white solid was dried in vacuum overnight at room temperature and formed the PEG-tresylated precursor (TPEG). TPEG was washed twice with HCl-ethanol (1:250, v/v), precipitated at 4°C and kept in a desiccator. This procedure was repeated six times and the white solid was collected and dried in vacuum. In a second step, TPEG (I) reacted with Tris-HCl 0.2 M buffer, pH 8.0 for 12 hours at 4°C. It was dialyzed against water to remove the excess Tris-HCl and then concentrated by ultrafiltration (50 ml) (II).

Attachment of APGP to TPEG

(III) - APGP (0.60 g) was added to the TPEG (II) and it reacted for 12 hours, ultrafiltered with water and then dried in vacuum.

Aqueous two-phase system preparation

Aqueous two-phase systems were prepared according to FRANCO *et al.* (1996). They were prepared from stock solutions of PEG 4000 (50%, w/w), dextran T505,000 (30%, w/w) and potassium phosphate solution with K_2HPO_4 to KH_2PO_4 (40%, w/w) with a molar

ratio of 0.6, pH 6.5. In the first step, the compounds were mixed to form 8.0 g of a system having a final concentration of 6% PEG 4000-APGP (compound III) and 8% dextran T505, pH 6.5. In the second step, the bottom phase of the PEG 4000-APGP/dextran system was discarded and replaced with a fresh phosphate phase, pH 6.5. The composition of the system (6.0 g total weight) was 13% PEG 4000-APGP and 9% K_2HPO_4/KH_2PO_4 , pH 6.5. The system was mixed and centrifuged.

Electrophoresis

SDS electrophoresis (SDS-PAGE) was carried out in 12% homogeneous gel (LAEMMLI, 1970). The gels were stained with Bio-Rad silver. The molecular mass markers consisted of thyroglobulin (330 kDa), ferritin (220 kDa – half unit), albumin (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa) and ferritin (18.5 kDa), available as a standard kit (Pharmacia Biotech).

Specific β -galactosidase activity ($SA_{\beta-gal}$)

It is defined as the ratio of enzyme activity (U/ml) to the total protein concentration (mg/ml) and is expressed in U/mg of protein (equation 1).

$$SA_{\beta-gal} = \frac{\text{Enzyme activity}}{\text{Protein concentration}} \quad (1)$$

Purification factor (PF)

The PF concept has been used in this work as a measurement to follow the purification operations (PRICE & STEVENS, 1989; SILVA *et al.*, 1997) and is defined as the ratio of the specific β -galactosidase activity after a purification step to the initial specific β -galactosidase activity (from the aqueous enzyme extract or from a previous purification step) (equation 2).

$$PF = \frac{SA_{\beta\text{-gal}} \text{ in the collected phase}}{\text{Initial } SA_{\beta\text{-gal}}} \quad (2)$$

Recovery (R)

It is defined as the ratio of the enzyme activity collected from an aqueous phase after partitioning to the total enzyme activity added to the system (equation 3).

$$R (\%) = \frac{\text{enzyme activity of the phase}}{\text{total enzyme activity added to the system}} \quad (3)$$

Selectivity (S)

It is defined as the ratio of the partition coefficient of the enzyme, K_E , to the partition coefficient of the protein, K_P .

$$S = \frac{K_E}{K_P} \quad (4)$$

RESULTS AND DISCUSSION

Our earlier experiments showed that β -galactosidase from *Kluyveromyces lactis* was not separated from the main contaminant proteins of the broth (SILVA *et al.*, 1997) in conventional aqueous two-phase systems. The pool of total proteins was mainly partitioned towards the bottom salt-rich phase of PEG/phosphate systems independently of PEG molecular mass. In order to achieve a good and efficient separation of the β -galactosidase and its main contaminants, it would be desirable to find an ATPS composition where they are mostly extracted in opposite phases. Therefore an affinity system was developed in

which the APGP biospecific ligand chemically attached to PEG 4000 and dextran T505, which was accomplished in two chemical reactions. In the first reaction PEG hydroxyls were activated with chloride to become more reactive. In the second reaction the APGP ligand was finally bound to PEG. In this present work, the amount of activated and reacted PEG was not measured because our earlier results had shown that approximately 74% of the total number of hydroxyl groups in PEG had been transformed into tresyl esters in the first activation reaction. Also, the amount of APGP bound to PEG in the second reaction had been indirectly observed to be higher than 80% of the activated hydroxyls after five hours of reaction (SILVA *et al.*, 1997). Therefore in order to assure at least the same amount of APGP bound to PEG in this work, the second reaction lasted 12 hours. Another modification of the synthesis of the PEG-APGP was the excess of free APGP molecules removal and Tris-HCl buffer excess, which was done by extensively washing by ultrafiltration.

Partitioning of the *Kluyveromyces fragilis* β -galactosidase

In order to extract and separate β -galactosidase from the contaminant proteins, a strain of *K. fragilis* was fermented and the cells were disrupted with chloroform. The enzyme extract was partitioned in the aqueous two-phase systems described in Table 1. The observed results show a 3,280-fold increase in the K_E value, when PEG-APGP replaced the plain PEG in the 6% PEG 4000 and 8% dextran system, indicating the strong affinity of β -galactosidase for the phase containing the APGP ligand. The purification factor rose from 2.8 to 9.7 and the selectivity factor of the system rose from 1.6 to 1,650. The high selectivity value indicates the potentiality of the system for a selective extraction of β -galactosidase by liquid extraction. Enzyme yield in the first-step partitioning in the affinity system was 55%, and the 45% β -galactosidase loss at the interface would possibly be due to the strong interaction between the APGP and the enzyme, which was not totally disrupted by the condition of the β -galactosidase assay with the ONPG substrate. STEERS *et al.* (1971) observed that in affinity chromatography the interaction between APGP and β -galactosidase was stronger than the interaction between β -galactosidase and other substrates such as

lactose, o-nitrophenyl- β -D-galactopyranoside and isopropyl- β -D-galactopyranoside. They found that the APGP-enzyme binding could only be disrupted when a low degree of ligand substitution was used or when an alkaline buffer of borate, pH 10, was employed to elute the enzyme. A second-step purification was developed by separating the top PEG-APGP-rich phase where the enzyme was collected and mixing it with a new fresh phosphate phase.

ALBERTSSON (1971) reports on the binodial curve of the PEG 4000/dextran system, where the top phase contained 8% PEG 4000 and 3% dextran and the bottom phase contained 5% PEG 4000 and 9% dextran. As a new PEG 4000/phosphate system, pH 6.5, had to be used in order to invert the partition coefficient of the β -galactosidase by disrupting the interaction between the APGP and the enzyme, the top PEG-APGP phase was mixed with a stock phosphate solution to give a final composition of 13% PEG-APGP and 9% phosphate system. It was assumed that the binodial curve of the PEG-APGP/dextran system was similar to the PEG 4000/dextran system.

Table 1. Liquid-liquid extraction process of β -galactosidase from *Kluyveromyces fragilis*, pH 6.5.

System	K_E	K_P	R (%)	SA (U/mg)	PF	S
6% PEG 4000 8% Dextran	0.71	1.2	57 (bottom phase)	104	2.8	1.6
6% PEG 4000-APGP 8% Dextran	2,330	1.4	55 (top phase)	361	9.7	1,650
13% PEG 4000-APGP 9% phosphate	2.2×10^{-5}	0.8	39 (bottom phase)	708	19	2.7×10^{-5}

β -galactosidase activity = 295 U/ml

Protein concentration = 7.92 mg/ml

Specific activity = 37 U/mg

The results from Table 1 show that the partition coefficient of β -galactosidase decreased from 2,330 to 2.2×10^{-5} in the second partitioning procedure in the PEG 4000/phosphate system. The purification factor, specific activity and selectivity were 19, 708 and 2.7×10^{-5} , respectively. Due to the extremely low enzyme concentration in the dextran-

rich phase from the PEG-APGP systems (first step) and in the PEG-APGP-rich phase (second step), the partition coefficients given are lower limit estimates based on the limit of detection of the spectrophotometer.

A commercial enzyme (Lactozym) produced by the same specie of microorganism was also extracted and purified by the procedure developed in this work (Table 2).

Table 2. Liquid-liquid extraction process of β -galactosidase from Lactozym.

Systems	K_E	R (%)
6% PEG 4000 8% Dextran	0.40	87 (bottom phase)
6% PEG 4000-APGP 8% Dextran	2,380	50 (top phase)
13% PEG 4000-APGP 9% phosphate	5.3×10^{-4}	26 (bottom phase)

The K_E increased 5,950-fold and recovery was only 26% of the enzyme activity. The K_E observed for the Lactozym enzyme was 2,330, similar to the K_E observed for the β -galactosidase from fermentation, which was 2,380 for *K. fragilis*. The system composed of 13% PEG 4000-APGP and 9% phosphate favoured the disruption of the complex enzyme ligand, and in the same way, the commercial β -galactosidase was concentrated in the bottom phosphate-rich phase.

In systems without a ligand, contaminant proteins were evenly distributed in both phases ($K = 1.2$). When the ligand was added, an insignificant increase in the partition coefficient of the contaminant proteins was found. Figure 2 shows an electrophoretic gel of the β -galactosidase purification.

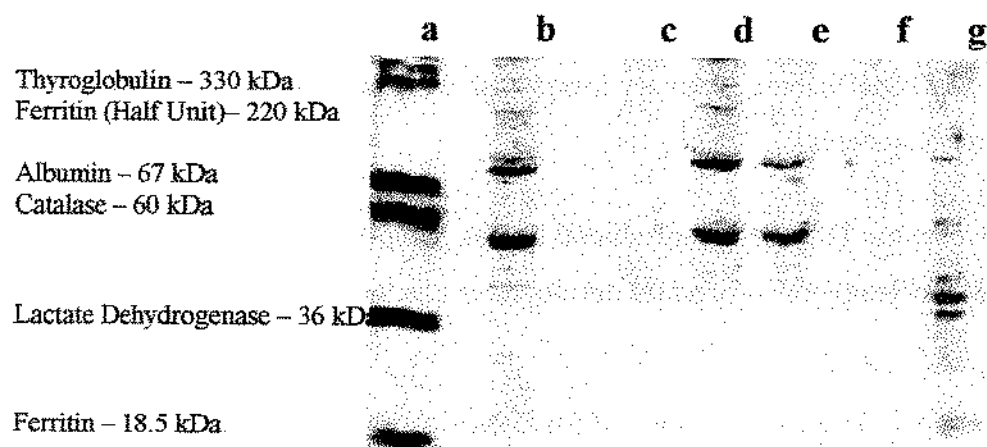


Figure 2. SDS-PAGE gel electrophoresis of β -galactosidase from Lactozym: a) marker proteins; b) Lactozym β -galactosidase; c) β -galactosidase extracted without ligand, top phase of ATPS; d) β -galactosidase extracted without ligand, bottom phase of ATPS; e) β -galactosidase extracted with ligand, top phase of ATPS; f) β -galactosidase extracted with ligand, bottom phase of ATPS; g) β -galactosidase (fermented).

The gel shows two major bands of corresponding molecular mass, 117 kDa and 70 kDa, on the purified material. According to the literature, the molecular mass of β -galactosidase from *Kluyveromyces fragilis* is 201 kDa (MAHONEY & WHITAKER, 1978), calculated by size exclusion chromatography. They also found by SDS-PAGE that this enzyme was composed of two protein chains of MM of 120 kDa and 90 kDa, which in native conditions, would behave as a dimer of approximately 200 kDa. As the enzyme purified in our work is globular, a molecular mass of 187 kDa is calculated, which is only 5% below the β -galactosidase described by MAHONEY and WHITAKER (1978).

The K/K_0 value determines the efficiency of an affinity partitioning procedure. It is described by the ratio of K , the partition coefficient in system with ligands and K_0 , the partition coefficient of the enzyme in a system without ligands under otherwise identical conditions. When a ligand is coupled to the top polymeric phase, K_E increases until some saturating value is reached. The value K/K_0 is proportional to the number of available

ligands in the polymeric phase (CHUNG *et al.*, 1994; AGUIÑAGA-DIAZ & GUZMÁN, 1996).

Figure 3 shows the effect of the enzyme concentration added to the system on the $\log K/K_0$ values.

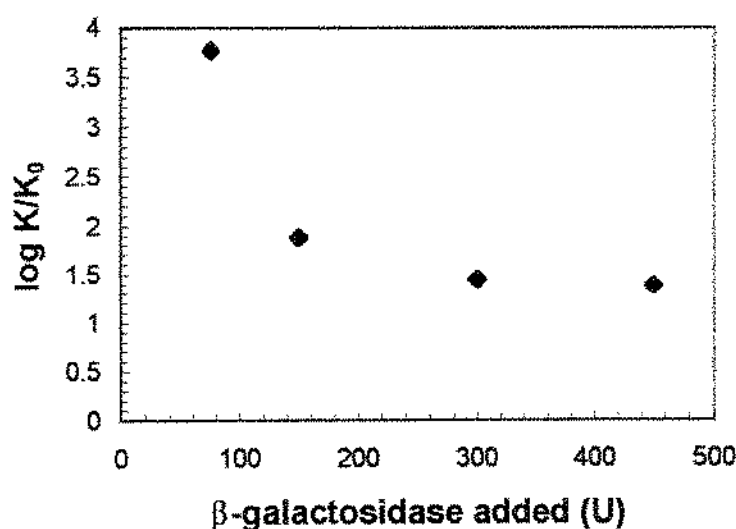


Figure 3. Effect of the enzyme concentration added to the system on the K/K_0 values.

It is observed that the highest K/K_0 value 5,950, was obtained when 75 U of commercial β -galactosidase was added to the 8.0 g system.

PASTORE and PARK (1980) purified β -galactosidase from *Scopulariopsis sp* by precipitation with ammonium sulphate and two chromatographic steps leading to a 4% yield of the pure enzyme desired. VEIDE *et al.* (1987) developed an process of industrial isolation and purification by PEG 4000/potassium phosphate partitioning systems for *E. coli* β -galactosidase, which is followed by an ultrafiltration step to recycle the salt-rich phase. They were able to recover 95% of the β -galactosidase. SILVA *et al.* (1997) observed that the partition coefficient of β -galactosidase from *Kluyveromyces lactis* increased 3.7-fold in a 6% PEG 4000-APGP and 12% dextran system. The purification factor increased 1.6-fold and the recovery of the target enzyme was 83%.

It seems clear that partitioning in ATPS can be an effective way of purifying and concentrating enzymes and other biomolecules. It can be greatly improved by designing efficient and specific bioligands which are not expensive and can be recycled (i.e., ultrafiltrated). Research on affinity techniques, binding chemistry and the designing of specific equipments for extraction in ATPS will be of great help in the improvement of downstream enzyme processing.

CONCLUSIONS

An extractive liquid-liquid system was developed with the objective of purifying the microbial enzyme β -galactosidase. As the enzyme from *Kluyveromyces fragilis* is very hydrophilic, being partitioned with the main contaminant proteins to the saline phase in conventional PEG/phosphate systems, a new two-step process was developed for the extraction and purification of β -galactosidase. In the first step, the system involves a biospecific ligand composed of 6% PEG4000-APGP and 8% dextran. In this system the partition coefficient of β -galactosidase increased 3,280-fold compared to the initial value in a system without ligands. In the second step, a system formed of PEG 4000-APGP and phosphate was used to revert the value of the partition coefficient of the β -galactosidase ($K = 2.2 \times 10^{-5}$), providing the purification and recovery of 39% of the enzyme. The main benefit of this work was the development of a feasible process of chemical activation of polyethylene glycol and the subsequent binding of a ligand to PEG (a hydrosoluble polymer), capable of completely altering the value of the partition coefficient of the enzyme. In the affinity step, a great number of contaminant materials were removed to the bottom phase, as the enzyme was partitioned to the top phase. In the second step, the enzyme-ligand bond was broken in the presence of a high concentration of phosphate, providing a 19-fold purification of the β -galactosidase in the saline phase in a procedure of just two steps.

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CAPÍTULO 5

EXTRACTION AND PURIFICATION OF SOYBEAN PEROXIDASE (*Glycine max*) BY AFFINITY PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS

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Trabalho submetido para publicação na revista **Journal of Chromatography**

SUMMARY

Combining two concepts in downstream processing this work investigated the partitioning of soybean peroxidase (*Glycine max*) in ATPS by bioaffinity. A liquid-liquid extraction process using metal ligands was developed in two steps aiming to purify the enzyme peroxidase. The phase diagrams (binodial curves) of conventional systems composed of polyethylene glycol (PEG 4000) and sodium sulphate and of PEG 4000 and potassium phosphate were studied. PEG 4000 was activated using thionyl chloride, covalently linked to iminodiacetic acid (IDA), and the specific metal ligand Cu^{2+} was attached to the PEG 4000-IDA. In the first step, the system was composed of 14% (w/w) PEG 4000-IDA- Cu^{2+} and 8% (w/w) Na_2SO_4 , and the peroxidase partitioned mainly to the top phase ($K = 24$). In the second step, a system formed by 14% PEG 4000 and 10% phosphate was used to revert the value of the partition coefficient of peroxidase to the bottom salt-rich phase ($K = 0.05$), thereby achieving the purification and recovery of 60% of the enzyme.

INTRODUCTION

Partitioning in an ATPS (aqueous two-phase system) is a selective method used for biomolecule purification (ALBERTSSON, 1986; KULA *et al.*, 1982). The tendency for two different aqueous polymers (polyethylene glycol and dextran) or a polymer and a lyotropic salt to separate into two distinct phases in a common solvent has been recognized since the end of the last century. For example, a mixture of sodium sulphate and polyethylene glycol dissolved in water is turbid above certain concentrations and the two phases are in equilibrium. The lighter phase is enriched in PEG while the heavier is enriched in salt (Figure 1). A potentially useful method for protein extraction in downstream processing is the use of metals as ligands in ATPS (BIRKENMEIER *et al.*, 1991; SUH & ARNOLD, 1990). Partitioning using metals increases the selectivity of protein extraction in ATPS, including proteins of clinical and industrial interest (WUENSCHHELL *et al.*, 1990), and is a simple and rapid procedure (FRANCO *et al.*, 1997). The iminodiacetic chelator is coupled by covalent binding to one of the phase-forming polymers; therefore its partitioning occurs either into the top or into the bottom phase and the metal ions are loaded into the chelate polymer. If the target protein to be isolated has an affinity for the metal ligand, the formation of the metal-protein complex will alter the partitioning of a protein (KOPPERSCHLÄGER, 1994; BIRKENMEIER, 1994).

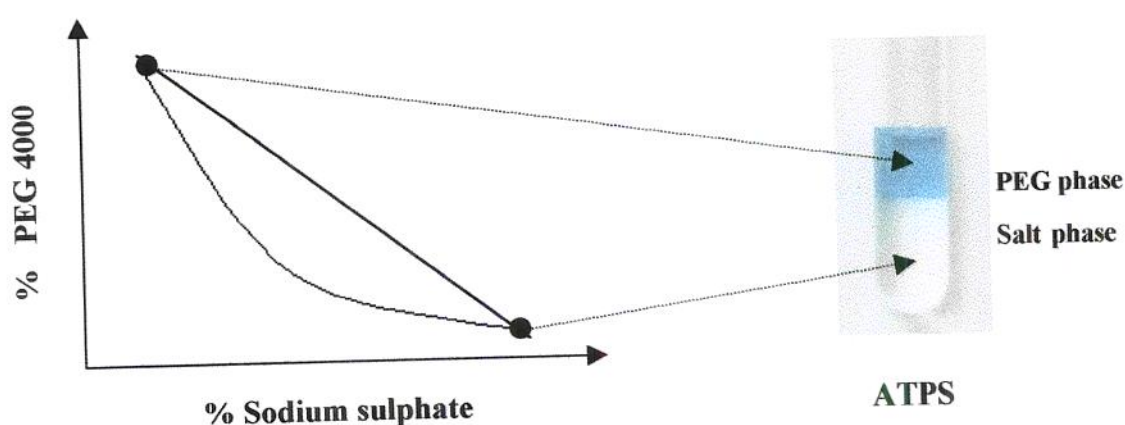


Figure 1. Binodial curve and tie-line of an aqueous two-phase composed of polymer/salt.

Proteins with histidine, cysteine and triptophane residues on the surface are favoured for this technique (PORATH *et al.*, 1975; SULKOWSKI, 1985; HEMDAN *et al.*, 1989; CHUNG *et al.*, 1994). The principle is based on the interactions between these protein surface-accessible amino acids and the metal ions attached to a metal-chelating derivative of polyethyleneglycol (PEG) (CHUNG & ARNOLD, 1991).

The advantages of using metals as ligands are: 1) they can be recycled many times with insignificant loss in performance, 2) high metal loadings and therefore high protein capacities can be attained, 3) product elution and ligand regeneration are achieved with relative ease, 4) the cost of the metals used is low (ARNOLD, 1991), 5) they are stable under a wide range of solvent conditions and temperatures, 6) their interactions with target molecules are reasonably specific and 7) these interactions are reversible under mild conditions (CHUNG *et al.*, 1994).

Peroxidase (EC 1.11.1.7) is the enzyme most frequently used in the manufacture of enzyme immunoassay kits (MIRANDA *et al.*, 1995) and medical diagnosis kits (MIRANDA & CASONE, 1994). It is used in the enzymatic determination of glucose content, especially for people suffering from diabetes (LOBARZEWSKI & GINALSKA, 1995). According to EGOROV and GAZARYAN (1993), peroxidase can also be employed in biosensors, the transformation of drugs, the production of chemicals, the degradation of aromatic compounds and environmental control. POKORA (1995) reported the use of peroxidase in processes for making a wide variety of chemical intermediates, as well as formaldehyde-free phenolic resins. Another important class of materials produced by peroxidase-catalysed reactions in organic solvents are the polyaromatic amines and polyphenol synthesis (AKKARA *et al.*, 1999).

Several methods for peroxidase extraction and purification, mainly involving ion-exchange chromatography (SHANNON *et al.*, 1966; LASCU *et al.*, 1986), hydrophobic interaction chromatography (CHAVEZ & FLURKEY, 1984), affinity chromatography using concavalin A as the ligand (BRATTAIN *et al.*, 1976), monoclonal antibodies (ELLING *et al.*, 1991) and partitioning (MIRANDA *et al.*, 1998), have been described.

Affinity partitioning in ATPS offers a fast and selective alternative for extracting enzymes with good applicability to scale up and can replace some of the chromatography steps in downstream processing.

This article reports on the feasibility of the extraction and purification of soybean peroxidase, *Glycine max*, by metal affinity partitioning in ATPS.

MATERIALS AND METHODS

Material

PEG 4000 was purchased from Fluka (Switzerland), thionyl chloride from Aldrich (Steinheim, Germany), iminodiacetic acid (IDA) and copper sulfate from Sigma (St. Louis, MO, USA), and sodium sulphate, sodium bicarbonate and hydrogen peroxide from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Crude enzyme extract

Crude extract was prepared by crushing and drying the seeds of *Glycine max* PL1-IAC. One g of defatted soybean flakes was mixed in 10 mM phosphate buffer, pH 8.0, for 2 hr at 4°C (1:25, w/w), then centrifugated at 5,000 g for 30 min. The supernatant was maintained at 4°C.

Determination of binodial curve

The binodial curves of the ATPS composed of PEG 4000/sodium sulphate and PEG 4000/potassium phosphate were constructed according to BAMBERGER *et al.* (1985) and ALBERTSSON (1986). Five grams of a concentrated stock solution (40% w/w) of potassium phosphate (or 20% w/w sodium sulphate) were put in a beaker with a magnetic stirrer and stirred constantly. A solution of 50% (w/w) PEG 4000 was then added drop by

drop to the flask. First a homogeneous mixture was obtained, but after a certain amount of PEG had been added, one additional drop caused turbidity and a two-phase system arose. The composition of the mixture was noted. Then deionized water was added drop by drop until the mixture lost its turbidity. The composition of the mixture was again noted. More PEG solution was then added and a two-phase system was again obtained. The composition of the mixture was noted and more water was added to obtain a one-phase system and so on. The procedure was followed in triplicate. In this way a series of compositions close to the binodial was obtained, and the concentration of phosphate or sulphate plotted versus PEG gave a binodial curve for the system. As a convention, the concentration of the compound whose distribution favours the bottom phase is plotted as the abscissa, and the concentration of the polymer which distributes into the top phase is plotted as the ordinate.

Determination of the tie-lines

The tie-line was constructed according to SNYDER *et al.* (1992). ATPS (45 g) were made by weighing stock polymer solutions and dry salts in a 50 ml equilibrium cell. The systems were mixed for 2 min and then separated at room temperature for 24 hr. Approximately 500 mg of phase were weighed in a glass tube using an analytical balance. Two volumes of water were added, and the solutions were shell-frozen in a mixture of dry ice and acetone. A lyophilizer was used to sublimate water at absolute pressure of 13.3 Pa for 24 h, after which the tubes were again weighed. The sample tubes were placed on the surface of a hot plate at approximately 450°C for 5 days. The PEG was oxidized and volatilized while the salt remained as a white ash. The tubes were repeatedly weighed until the mass was constant.

Synthesis of PEG-IDA-Cu²⁺

The PEG-IDA-Cu²⁺ were synthesized according to CHUNG *et al.* (1994).

Activation of PEG 4000 with thionyl chloride

(I) Solid PEG 4000 (30 g) was dried in vacuum at 55°C for 5 hr. Then 5.5 ml of thionyl chloride was added, and the melt was reheated at 65°C for 5 hr. Excess thionyl chloride was removed in vacuum, and the melt was dried (PEG-Cl).

Attachment of IDA to PEG-Cl

Twenty-five g of compound I (PEG-Cl) were dissolved in 100 ml of water and 15 g iminodiacetic acid (IDA) and 10 g potassium carbonate were added and mixed. The solution was refluxed for 48 hr. Ten g of sodium sulphate were added to the hot reaction mixture and allowed to cool and to separate into two phases. The PEG (top) phase was retained and diluted to 120 ml with water. Solid impurities were removed by filtration through a 0.45 μm membrane. The solution was ultrafiltered against 1% sodium bicarbonate and extensively against water over a period of 48 hr and finally lyophilized (II).

Attachment of Cu^{2+} to PEG-IDA

Five g of compound II and 3 g of copper sulphate were dissolved in 5 ml water and allowed to equilibrate for 24 hr. The solution was diluted to 20 ml, filtered through a 0.45 μm filter and then ultrafiltered with 1,000 ml of water to remove the copper excess. The contents (III) were lyophilized and used to prepare aqueous two-phase systems.

Aqueous two-phase system preparation

Aqueous two-phase systems were prepared according to FRANCO *et al.* (1996). They were prepared from stock solutions of PEG 4000 (50%, w/w), Na_2SO_4 (20%, w/w) and

potassium phosphate solution with $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (40%, w/w) with a molar ratio of 0.64. Solid PEG-IDA- Cu^{2+} was used to prepare the affinity ATPS. In the first step, the compounds were mixed to form 8.0 g of a system having a final concentration of 10% PEG-IDA- Cu^{2+} (compound III), 4% PEG-4000 and 8% sodium sulphate. In the second step, the bottom phase of the PEG-IDA- Cu^{2+} /sulphate system was discarded and replaced with a fresh phosphate phase, pH 6.0. The composition system (6.0 g total weight) was 14% PEG 4000-IDA- Cu^{2+} and 10% $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 6.0. The system was mixed and centrifuged. In the recycling of PEG-IDA- Cu^{2+} , the top phase of the PEG-IDA- Cu^{2+} /phosphate system was collected and washed ten times with water by ultrafiltration and concentrated at the initial concentration for reuse in the next cycle of purification.

Determination of enzyme activity and partition coefficient

Three hundred μl of enzyme extract were mixed with the PEG-IDA- Cu^{2+} /sulphate system, using a Vortex for 1 minute. Phase separation was achieved by centrifugation for 3 min. at 3,000 g, and the phases were carefully separated and the interface of each tube discharged. A known volume of each phase was transferred and the activity was determined. Peroxidase activity was assayed according to TJISSEN (1985). The assay mixture contained 3.0 ml of 100 mM potassium phosphate buffer, pH 7.0, 50 μl of guaiacol and 50 μl sample aliquots. The reaction was started by the addition of 40 μl of 8 mM hydrogen peroxide, and the absorbance at 436 nm was recorded within 0-5 min. intervals. Activity calculations were made as described by the above author.

The partitioning is described by the partition coefficient of the enzyme, K_E , which is defined as the ratio of enzyme activity found in the top phase to that found in the bottom phase at room temperature.

$$K_E = \frac{\text{Enzyme activity}_{\text{top phase}}}{\text{Enzyme activity}_{\text{bottom phase}}}$$

Protein assay

The main contaminant protein concentration was determined by the dye-binding technique of SEDMAK & GROSSBERG (1977). Fifty to 100 µl of the top phase were transferred from each prepared system to a cuvette containing 2.4 ml of water and 1.0 ml of Coomassie blue solution and mixed well, and the OD₅₉₅ was measured in a spectrophotometer, versus a blank which had 50 µl of a top phase of a system which has been equally prepared without any sample. The blanks were done to correct the interference of the phase components. The procedure was repeated for the bottom phase of each system. A BSA standard curve was used to calculate protein concentration. The partitioning of the main contaminant protein, K_p, was calculated as the ratio of protein in the top phase to that in the bottom phase at room temperature.

$$K_p = \frac{[\text{protein}]_{\text{top phase}}}{[\text{protein}]_{\text{bottom phase}}}$$

Electrophoresis

SDS electrophoresis (SDS-PAGE) was carried out in 12% homogeneous gel (LAEMMLI, 1970). The gels were stained with Bio-Rad silver. The molecular mass markers consisted of phosphorilase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa) available as a standard kit (Pharmacia Biotech).

Specific peroxidase activity (SA_{perox})

It is defined as the ratio of enzyme activity (U/ml) to total protein concentration (mg/ml) and is expressed in U/mg of protein (equation 1).

$$SA_{\text{perox}} = \frac{\text{Enzyme activity}}{\text{Protein concentration}} \quad (1)$$

Purification factor (PF)

The PF concept has been used in this work as a measurement of the purification operations (FROUND 1995; SILVA *et al.*, 1997) and is defined as the ratio of the specific peroxidase activity after a purification step to the initial specific peroxidase activity (from the crude enzyme extract or from a previous purification step) (equation 2).

$$PF = \frac{SA_{\text{perox}} \text{ in the collected phase}}{\text{Initial } SA_{\text{perox}}} \quad (2)$$

Recovery (R)

It is defined as the ratio of the enzyme activity of the aqueous phase after partitioning to the total enzyme activity added to the system (equation 3).

$$R (\%) = \frac{\text{enzyme activity of the phase}}{\text{total enzyme activity added to the system}} \quad (3)$$

Selectivity (S)

It is defined as the ratio of the partition coefficient of the enzyme, K_E , to the partition coefficient of the protein, K_P .

$$S = \frac{K_E}{K_P} \quad (4)$$

RESULTS AND DISCUSSION

Partitioning of peroxidase

In order to isolate peroxidase from the crude of the extract soybean enzyme, a metal affinity system was investigated. Initially the affinity polymer was prepared by covalent binding of IDA to the activated PEG 4000-Cl. Then the metal ion complex was prepared by dissolving copper sulphate salt in the PEG-IDA stock solution. The metal-loaded chelate PEG interacts with the accessible residues of histidine, cysteine and triptophane available on the protein surface (Figure 1) (BRENA *et al.*, 1994).

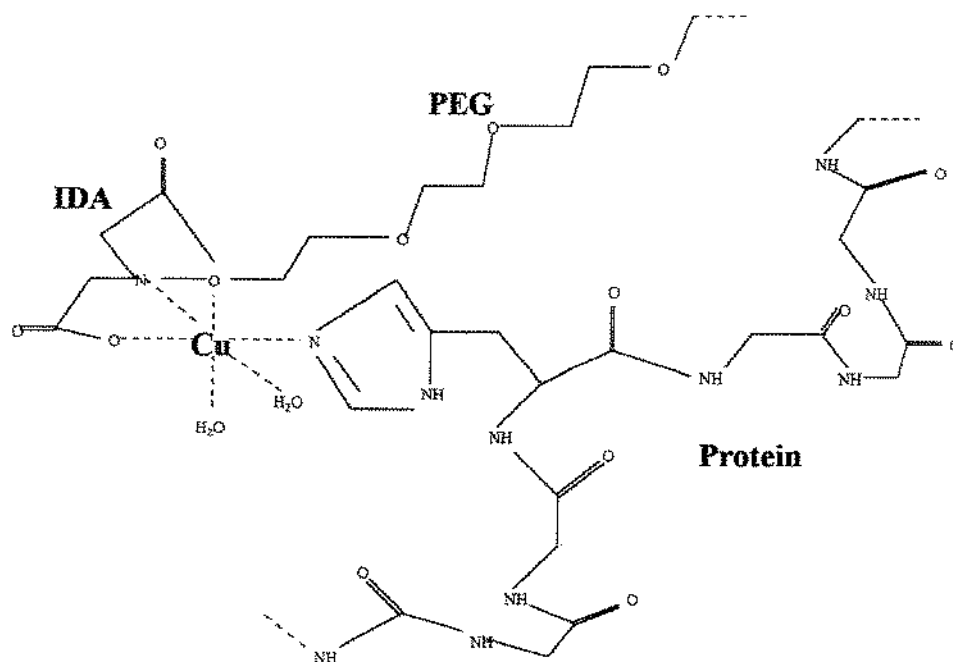


Figure 2. PEG-IDA-Cu²⁺-protein complex.

Preliminary experiments have shown that the soybean oil from the seeds inhibited the binding of peroxidase to PEG-IDA-Cu²⁺, therefore, the crude enzyme extract used was prepared from oil-free crushed seeds. The enzyme extract contained proteins of several different molecular masses in the range from 100 to 10 kDa, as it can be seen in Figure 3.

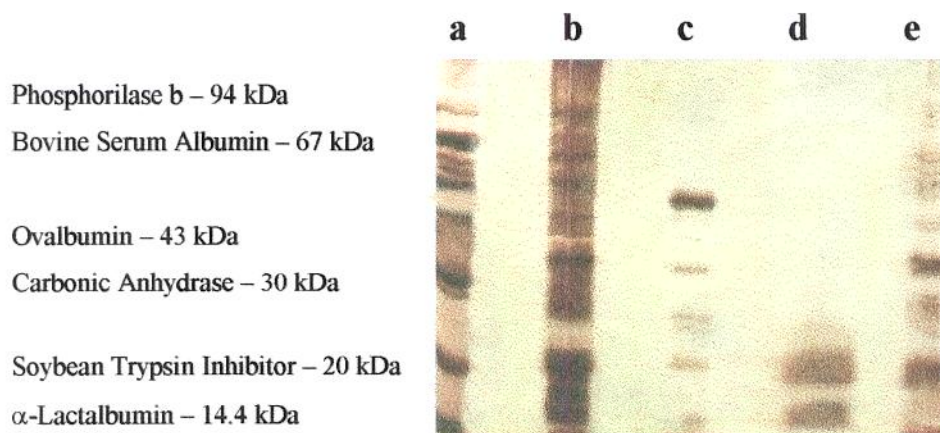


Figure 3. SDS-PAGE gel electrophoresis of soybean peroxidase: a) marker proteins; b) peroxidase (crude extract); c) soybean standard peroxidase; d) peroxidase extracted without ligand, top phase of ATPS; e) peroxidase extracted without ligand, bottom phase of ATPS.

When the enzyme extract was partitioned in the 14% PEG 4000 and 8% sodium sulphate system without a metal ligand, the partition coefficient of peroxidase was 1.0 and that of the main protein contaminants was 0.29, and 78% of the peroxidase activity was recovered in the bottom phase (Table 1). By replacing 10% of the plain PEG 4000 by the correspondent amount of PEG-IDA- Cu^{2+} , the peroxidase K_E value rose to 24 (a 24-fold increase), and the partition coefficient of the main contaminant proteins increased approximately sixfold ($K = 1.7$) and the peroxidase yield was above 100% in the top phase, indicating the preference of peroxidase for the IDA- Cu^{2+} . The amount of PEG-IDA- Cu^{2+} added to the system corresponded to about 71% of the total PEG content of the system.

In order to improve the purification of the peroxidase, the bottom sulphate-rich phase of the above ATPS was discarded and a new phosphate phase was prepared. Binodial curves and tie lines of PEG 4000/sodium sulphate and of PEG 4000/phosphate systems were then studied in order to calculate how much of each component should be added to the system.

The binodial curve of the PEG 4000 and sodium sulphate, shows that the top phase contained 31% PEG 4000 and 2.2% sodium sulphate and the bottom phase contained 1% PEG 4000 and 13% sodium sulphate (Figure 4).

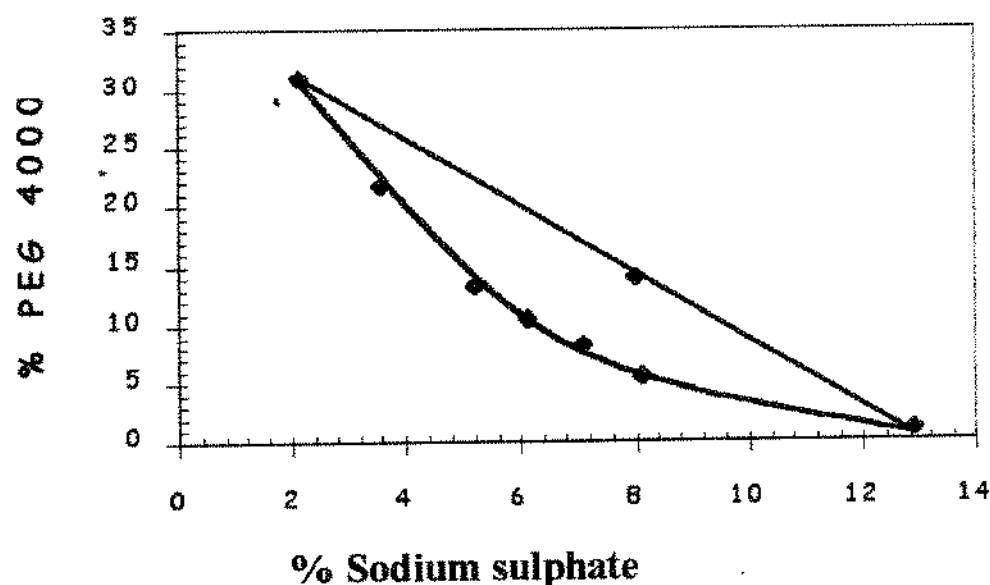


Figure 4. Binodial curve and tie line for the 14% PEG 4000 + 8% sodium sulphate + water system, at room temperature.

As a new PEG 4000/phosphate system, pH 6.0, had to be used in order to invert the partition coefficient of the peroxidase by disrupting the interaction between the copper complex and the enzyme, the top PEG-IDA-Cu²⁺ phase was mixed with a stock phosphate solution to give a final composition of 14% PEG-IDA-Cu²⁺ and 10% phosphate. According to the binodial curve and tie line (Figure 5), the new top PEG-rich phase contained 30% PEG 4000 and 3% phosphate and the bottom phosphate-rich phase contained 2% PEG 4000 and 15% phosphate. However, as the top PEG-rich phase of the PEG 4000/sulphate system was not washed or ultrafiltrated, the new PEG 4000-IDA-Cu²⁺/phosphate system still contained a low concentration of sulphate ion (< 2%). It was assumed that the binodial curve

of the PEG 4000-IDA- Cu^{2+} /sulphate system was similar to that of the PEG 4000/sulphate system.

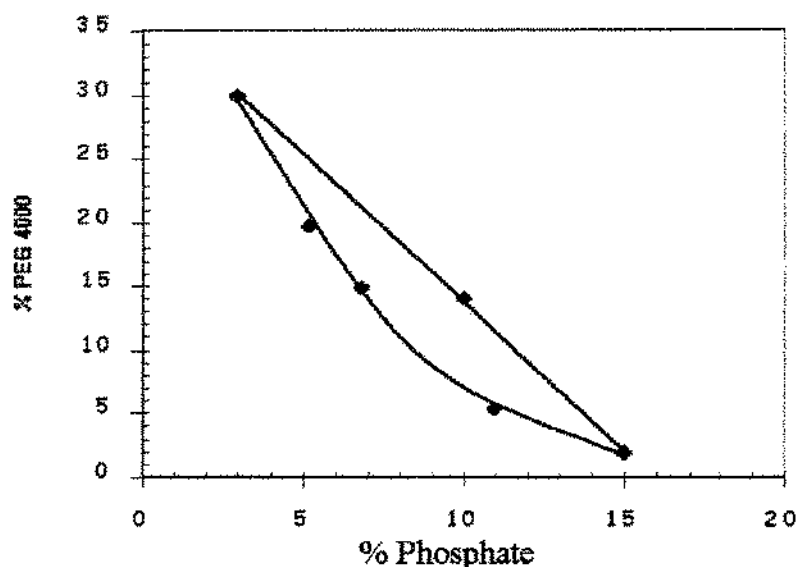


Figure 5. Binodial curve and tie line for the 14% PEG 4000 + 10% phosphate ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ —molar ratio 0.6) + water system, at room temperature.

Our studies showed that when the crude enzyme extract was partitioned in a 14% PEG 4000 and 10% phosphate, pH 6.0, system, the peroxidase was collected into a salt-rich phase and a K_E value of 0.05 was observed.

Therefore, the peroxidase, which had been previously extracted in the top-affinity phase of the PEG-IDA- Cu^{2+} /sulphate system, partitioned to the salt-phosphate rich phase in the second step. The dissociation of the enzyme from the affinity complex was performed by the addition of the salt phase (10% phosphate, pH 6.0) to the top phase such that the peroxidase was transferred to salt-rich phase.

Table 1. Liquid-liquid extraction process of soybean peroxidase.

Systems		K_E	K_P	R (%)	SA	PF	S
14% PEG 4000 8% Na ₂ SO ₄	-	1	0.29	78 (Bottom phase)	29	1.30	3.50
4% PEG 4000 8% Na ₂ SO ₄	10% PEG-IDA-Cu ²⁺	24	1.70	106 (Top phase)	35	1.50	14
4% PEG 4000 10% phosphate	10% PEG-IDA-Cu ²⁺	0.05	76	64 (Bottom phase)	3,333	145	7×10^{-4}
Recycle 4% PEG 4000 8% Na ₂ SO ₄	10% PEG-IDA-Cu ²⁺	22.80	1.40	85 (Top phase)	65	2.83	16

Peroxidase activity = 13 U/ml

Protein concentration = 0.58 mg/ml

Specific activity = 23 U/mg

It was observed that 60% of the total peroxidase added to the first system was collected in the bottom phosphate-rich phase in the second step partitioning. The purification factor achieved was 145 and the selectivity of the system was $7 \cdot 10^{-4}$, indicating that K_P was 1,520 times higher than K_E . The peroxidase specific activity found in the phosphate phase was 3,333 U/mg of protein. Figure 6 shows the SDS-PAGE of the affinity-purified peroxidase preparations obtained after the ATPS. The protein contaminant bands and the peroxidase band are shown at the top phase, and 106% of the peroxidase activity was recovered in the first step. A clear electrophoretic band of peroxidase in the material collected from this salt phase is observed. There are two other large bands corresponding to protein of low molecular masses which appear in the same material. This figure also shows that the commercial soybean peroxidase standard was more impure than the fraction obtained in the first partitioning step.

The recycling of the top phase PEG-IDA-Cu²⁺ was achieved by ultrafiltration. Table 1 shows that the partitioning of the peroxidase was not significantly affected by reusing this phase for the third time.

However, the comparison between the innumerable crude enzyme extract bands and those found in the material collected from the second purification step clearly shows the efficiency of the process developed.

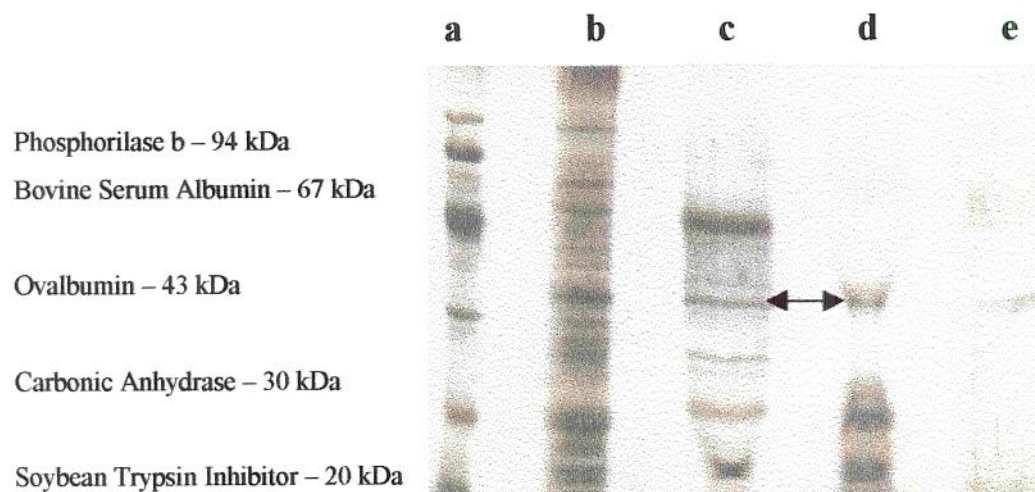


Figure 6. SDS-PAGE gel electrophoresis of soybean peroxidase: a) marker proteins; b) peroxidase (crude extract); c) soybean standard peroxidase; d) peroxidase extracted with ligand, top phase of ATPS; e) peroxidase extracted with ligand, bottom phase of ATPS.

Several researchers have used the metal chelate IDA-Cu^{2+} as the ligand attached to solid supports in affinity chromatography to recover and purify other enzymes: however, the use of this ligand in affinity partitioning is more easily scaled up than conventional affinity chromatography. An important factor in the protein-metal interaction is the accessibility of exposed histidine residues on the protein surface (PORATH *et al.*, 1975; BIRKENMEIER *et al.*, 1991; ARNOLD, 1991).

SESSA and ANDERSSON (1981) purified soybean peroxidase 628-fold by using a sequence of procedures consisting of ammonium sulfate fractionation, gel filtration

chromatography, ion exchange chromatography, affinity chromatography and hydrophobic chromatography (SESSA & ANDERSSON, 1981), obtaining a yield of 4%.

CHAGA (1994) modified horseradish peroxidase by covalent coupling of L-histidine to its carbohydrate moieties to recover it by affinity chromatography using metal as the ligand. The immobilized metal ion could be recovered and used in five consecutive cycles. The same approach was used in our preliminary experiments to extract horseradish peroxidase, soybean peroxidase and lactoperoxidase. Table 2 shows the partition coefficient of the peroxidases attached to L-histidines. This approach was initially used because the partition coefficients of lactoperoxidase and of horseradish peroxidase did not increase when PEG was replaced by PEG-IDA-Cu²⁺. It is suggested that the histidine, cysteine and tryptophane residues of these two enzymes were not available to interact with the affinity ligands of the PEG-rich phase.

Table 2. Partitioning of three peroxidases attached to L-histidines in metal affinity.

Systems (%)			Lactoperoxidase			Horseradish peroxidase		Soybean peroxidase		
PEG	Phosphate	PEG-IDA-Cu ²⁺	K _E	K _P	R (%)	K _E	R (%)	K _E	K _P	R (%)
13	9	-	0.16	0.06	86	5.42	70	2.03	0.03	90
12	9	1	0.17	0.05	73	150	54	3.74	0.04	100
8	9	5	0.18	0.93	95	180	55	37	2.99	75
3	9	10	3.31	1.10	95	-	-	-	-	-

According to several authors (ARNOLD, 1991; ZHAO *et al.*, 1991), it is necessary to have at least one accessible residue of histidine available to complex with the metal-chelate polymer.

Peroxidase from brown algae has been purified by partitioning in conventional ATPS (VILTER, 1990). A system made of 10% PEG 1550 and 15% carbonate, pH 11.8, was used and a partition coefficient of 97 with a yield of 93% was obtained. According to this author,

inactivation of peroxidase resulting from the treatment with potassium carbonate at high pH values was reverted when the enzyme was transferred into a vanadate containing Tris-buffer.

The extractive purification of soybean peroxidase in a temperature-induced ATPS system formed of Triton X-45, Triton X-100 and sodium acetate at pH 5.5 was developed by MIRANDA *et al.* (1998). A yield of 99% was obtained with a sixfold purification factor. Affinity chromatography was used to purify the top phase of the system above, increasing the purification factor 41-fold 28% yield of peroxidase.

CONCLUSIONS

A two-step extractive liquid-liquid process was developed with the objective of purifying the soybean peroxidase.

In the first step, the system was composed of 4% PEG 4000, 10% PEG-IDA-Cu²⁺ and 8% Na₂SO₄. In this system the value of the partition coefficient of peroxidase was increased 24-fold compared to the initial value in an ATPS without the metal ligand.

In the second step, a system formed by 4% PEG 4000, 10% PEG-IDA-Cu²⁺ and 10% phosphate was used to revert the value of the partition coefficient of the peroxidase ($K = 0.05$), thereby achieving the purification and recovery of 60% of the enzyme. The purification factor of the peroxidase in the phosphate phase was 145. Electrophoresis shows that only three main bands appear in the material collected in the several extraction processes. The purification procedure is also possible by using a non-expensive salt-rich phase composed of phosphate/sulphate. The experiments have also shown the possibility of reusing PEG-IDA-Cu²⁺ in two-phase systems at least for three times.

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CAPÍTULO 6

USO DE POLIETILENOGLICOL MODIFICADO PARA PURIFICAÇÃO DE LISOZIMA EM SISTEMA DE DUAS FASES AQUOSAS

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RESUMO

Partição por afinidade usando metal como ligante pode ser empregada na extração e purificação de proteínas, sendo que o metal mais comumente usado é o cobre na forma do complexo IDA- Cu^{2+} , por ser estável na presença de elevadas concentrações salinas, por interagir especificamente com biomoléculas ricas em histidina, cisteína e triptofano, pela fácil reciclagem e baixo custo. A enzima lisozima foi utilizada como modelo em SDFA de afinidade composto de PEG e fosfato de potássio, sendo o Cu^{2+} utilizado como ligante. As concentrações empregadas foram de PEG 4000 13% e fosfato de potássio 9% (m/m), pH 7,0 nos SDFA sem o ligante. Em sistemas de afinidade, as concentrações do PEG-IDA- Cu^{2+} foram de 1, 5 e 10% e as concentrações de PEG 4000 foram de 12, 8 e 3%, respectivamente, sendo que a concentração do fosfato permaneceu em 9%. O valor do coeficiente de partição, K , foi aumentado 9 vezes quando PEG-IDA- Cu^{2+} 5% foi usado nos SDFA de afinidade com rendimento de 45% da lisozima. Este é um trabalho pioneiro de extração de lisozima por partição em sistema de duas fases aquosas usando metal como ligante.

**USE OF MODIFIED POLYETHYLENE GLYCOL IN LYZOZYME
PURIFICATION IN AQUEOUS TWO-PHASE SYSTEMS**

ABSTRACT

Metal affinity partitioning in aqueous two-phase systems (ATPS) is a useful tool to extract the proteins which have accessible histidine, cysteine or tryptophan on their surfaces. Soluble chelating polymer prepared by covalent attachment of metal-chelating groups to PEG (PEG-IDA- Cu^{2+}) have been mostly employed for the extraction and separation of different proteins by ATPS.

This work investigated the extraction of the model protein lysozyme in PEG 4000/phosphate system, pH 7.0, in the presence and in the absence of PEG-IDA- Cu^{2+} . The composition of systems without ligands was 13% PEG 4000 and 9% phosphate. The concentrations of PEG-IDA- Cu^{2+} in the affinity systems were 1, 5 and 10% and PEG-4000

concentrations were 12, 8 and 3%, respectively, and the phosphate concentration remained 9%. The partition coefficient, K , of the lysozyme increased 9 fold when 5% PEG-IDA- Cu^{2+} was used and 45% of the enzyme was recovered in the top ligand-rich phase of the system. This is a pioneer work on liquid-liquid extraction of lysozyme in ATPS with PEG-IDA- Cu^{2+} .

Key words: aqueous two-phase system, lysozyme, downstream-processing, affinity.

ABREVIACÕES

SDFA – Sistema de Duas Fases Aquosas

IDA – Ácido Iminodiacético

PEG – Polietilenoglicol

K – Coeficiente de Partição

PEG-IDA- Cu^{2+} - Complexo polímero-ligante

INTRODUÇÃO

A extração e o isolamento de proteínas após fermentação em larga escala ou provenientes de uma suspensão de células são problemas críticos da biotecnologia moderna. Eles representam a maior parte do custo do processo de produção de uma molécula biológica e um ganho de competitividade dependerá não só das inovações da biologia molecular, imunologia e outras áreas de ciência básica, principalmente inovações e otimizações de processos pós-fermentativos (“downstream processing”). Partição em sistema de duas fases aquosas (SDFA) é um método usado para purificação de biomoléculas (Albertsson, 1971; Kula *et al*, 1982), tendo sido empregado por fornecer um meio adequado e não agressivo a materiais biológicos visto a maior parte do sistema ser constituída por 80% a 90% de água (Albertsson & Tjerneld, 1994).

Os primeiros sistemas PEG/sais a serem utilizados pela indústria preparadora de enzimas foram os contituídos de PEG/fosfato (Kula, 1979; Hustedt *et al*, 1983). Entretanto, com o objetivo de minimizar a poluição ambiental, diferentes maneiras de reciclar os sais foram desenvolvidas. Além de fosfato, outros sais podem ser empregados em SDFA tais

como sulfatos e citratos. Polímeros neutros ou carregados (ex. dextrana) formam duas fases com polietilenoglicol, sendo estes sistemas extensivamente utilizados em purificação e extração de biomateriais (Franco *et al.*, 1996).

Uma maneira de aumentar a seletividade de uma biomolécula é o uso de ligantes acoplados à uma das fases formadoras do sistema. Um método potencialmente útil para extração industrial de proteínas, incluindo as de interesse clínico e industrial (Wuenschell *et al.*, 1990) é a utilização de metais como ligantes em SDFA (Birkenmeier *et al.*, 1991; Suh & Arnold, 1990), por aumentar a seletividade da partição das mesmas. Este processo fundamenta-se na combinação da propriedade de reconhecimento de biomoléculas pelo ligante e a partição em SDFA das mesmas. O ligante é acoplado covalentemente à fase rica em polímero, e portanto sua partição ocorre em direção à fase superior de menor densidade (Figura 1). Se a biomolécula a ser isolada tiver afinidade pelo ligante, a formação do complexo ligante-biomolécula induzirá a alteração do coeficiente de partição da proteína para aquela fase enriquecida no ligante (Kopperschläger, 1994).

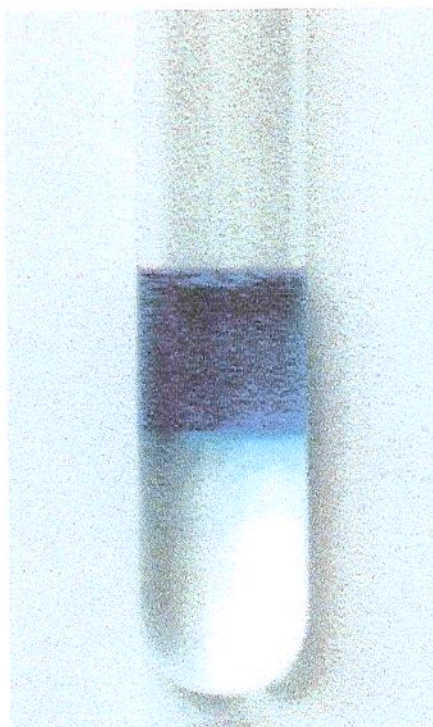


Figura 1. Fotografia de um sistema de duas fases aquosas constituído por 8% PEG-4000, 5% PEG-IDA-Cu²⁺ e 9% fosfato, pH 7,0. Fase superior é enriquecida em PEG-IDA-Cu²⁺ e fase inferior é enriquecida em fosfato.

As vantagens do uso de metais como ligantes são: 1) podem ser reciclados diversas vezes com insignificante perda do comportamento, 2) elevada concentração de metal imobilizado, conseqüentemente com elevada capacidade de “atração” da proteína, 3) relativa facilidade de desacoplamento da proteína e regeneração do ligante, e 4) baixo custo dos metais (Arnold, 1991).

A ativação do PEG se dá através do cloreto de tionila seguida pelo acoplamento do ácido iminodiacético (IDA), formando o complexo PEG-IDA, o qual age como quelante do íon cobre (Figura 2). O ácido iminodiacético é um quelante tridentado, ocupando 3 sítios de coordenação do metal. O uso de PEG-IDA-Cu²⁺ em SDFA aumenta seletivamente o coeficiente de partição de determinadas proteínas (K), direcionando a proteína alvo para a fase enriquecida em PEG, dependendo do número de resíduos de histidinas acessíveis presentes na superfície desta proteína (Wuenschell *et al*, 1990).

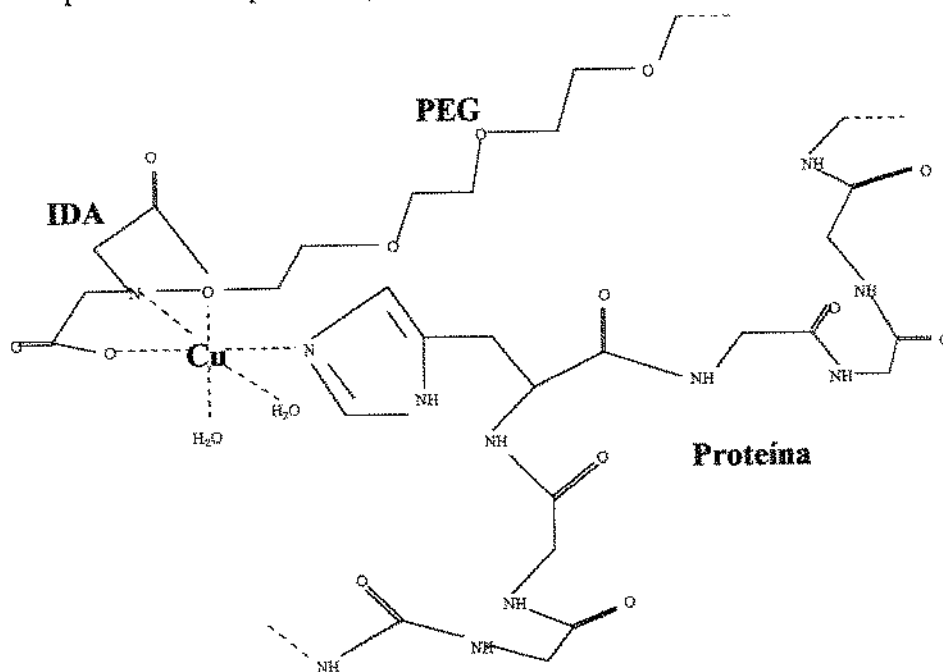


Figura 2. Sítio de ligação entre a proteína contendo histidina e o PEG-IDA-Cu²⁺.

Lisozima (muramidase E.C. 3.2.1.17) é uma enzima antimicrobiana produzida por bactérias, plantas, insetos, aves e mamíferos e hidroliza preferencialmente a ligação glicosídica β -1-4 entre o ácido N-acilmurâmico e N-acetilglicosamina, presente na parede celular de certos microrganismos, tal como o *Micrococcus lysodeikticus* (Macak, 1995).

A fonte comercial mais significativa da lisozima é a clara de ovo de galinha (Cunningham *et al.*, 1991). Aplicação potencial da lisozima à indústria de alimentos, tem sido descrita na literatura (Padgett *et al.*, 1998), por ser uma fonte segura para o consumo humano além de não causar problemas de poluição ambiental. A indústria de alimentos utilizaria a lisozima na prevenção do crescimento de microrganismos patogênicos por atuar como agente bacteriostático, especialmente em carnes e laticíneos (Guchte *et al.*, 1992; Fuglsang *et al.*, 1995), pois esta enzima destrói formas vegetativas de bactérias, especificamente *Clostridium tyrobutyricum* e parede celular de certas bactérias. Pesquisas mostram que esta enzima pode ter aplicação em veterinária, alimentação infantil e animal, além de possuir atividade contra infecção por HIV-1 (Lee-Huang *et al.*, 1999).

Com o objetivo de estudar a redução do número de etapas extrativas da lisozima, convencionalmente purificada por cromatografia (Igonina *et al.*, 1998; Awade & Efstathiou, 1999) e minimizar o custo final do processo de purificação, este trabalho investigou a partição em SDFA de afinidade, considerada uma técnica de elevada resolução, utilizando o sistema PEG-IDA-Cu²⁺/fosfato.

MATERIAL E MÉTODOS

MATERIAL

Os reagentes e sais utilizados nos experimentos foram adquiridos conforme descrito a seguir: PEG 4000 (Fluka, Suíça), cloreto de tionila (Aldrich, Steinheim, Alemanha), ácido iminodiacético (IDA), sulfato de cobre e lisozima de clara de ovo (Sigma, St. Louis, MO, USA), carbonato de potássio, sulfato de sódio e bicarbonato de sódio (Merck, Darmstadt, Alemanha), membrana de filtração 0,4 µm (Sartorium, Goettingen, Alemanha), membrana de ultrafiltração YMI Cut-off 1000 (Amicon, Beverly, MA, USA), kit proteínas padrões LMW (Pharmacia Biotech, Uppsala, Suécia).

MÉTODOS

Sistema de Duas Fases Aquosas

Os sistemas de duas fases aquosas foram preparados de acordo com Franco *et al.* (1996). Soluções estoques de fosfato $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ 40% (m/m) (relação molar = 0,64) pH 7,0 e de PEG 4.000 50% (m/m) foram preparados sob agitação magnética até completa solubilização. Os SDFA contendo o ligante foram preparados pela mistura de PEG 4000, PEG-IDA- Cu^{2+} e sal pH 7,0 em diferentes concentrações. Foram preparados 3 sistemas de afinidade, sendo a concentração final de PEG-IDA- Cu^{2+} de cada sistema de 1, 5 e 10%. Os sistemas foram preparados em duplicatas em tubos graduados de centrífuga de 10 ml contendo 2 g. Após a adição da lisozima (47.000 U) os tubos foram centrifugados a 3.000 g por 5 minutos. Aliquotas foram retiradas das duas fases para determinação da atividade enzimática. O coeficiente de partição (K) da enzima foi calculado como a relação entre a atividade enzimática determinada nas aliquotas retiradas de ambas as fases:

$$K = \frac{\text{Ativ. enzimática}_{\text{fase superior}}}{\text{Ativ. enzimática}_{\text{fase inferior}}}$$

A determinação da atividade da lisozima foi realizada de acordo com Decken (1977). Foram adicionados 0,006 g de *Micrococcus luteus* (Sigma, St. Louis, MO, USA) liofilizado à uma solução de 30 ml de tampão fosfato 0,067 mol/l contendo NaCl 0,0154 mol/l, pH 6,5. A reação foi iniciada pela adição da solução de lisozima e a absorbância anotada em intervalos de 30, 120 e 210 segundos. Uma unidade é definida como a quantidade de enzima capaz de provocar uma diferença de absorção de 0,001/minuto à 450 nm.

Ativação do PEG com Cloreto de Tionila (PEG-Cl)

solução 1 - 30 g de PEG 4000 foram aquecidos à 55°C sob vácuo por 5 horas e então 5,5 ml de cloreto de tionila foram adicionados e reaquecidos a 65°C por 5 horas. Em

seguida, o excesso de cloreto de tionila foi removido por destilação a vácuo em rota-evaporador e o produto (PEG-Cl) foi cristalizado pela adição de 200 ml de etanol e resfriado à -20°C e posterior filtração a 4°C.

Acoplamento do IDA ao PEG-Cl

A solução 1 (PEG-Cl) foi adicionada a 100 ml de água destilada, acrescentado 15 g de IDA e 10 g de carbonato de potássio. Esta solução foi mantida sob refluxo por 48 horas a 100°C. A seguir, foram adicionados 10 g de sulfato de sódio na mistura ainda quente e as fases separadas em funil de separação. A fase superior foi coletada (PEG-IDA – **solução 2**) e diluída a 120 ml de água destilada. Esta solução foi filtrada em membrana Millipore 0,45 µm para remoção de impurezas sólidas e a seguir ultrafiltrada em membrana YM1 (Cutt-off 1000) Amicon e lavada com solução de bicarbonato de sódio 1%, 0.5% e água durante 48 horas, para remoção do excesso de IDA e então liofilizado.

Acoplamento do metal Cu²⁺ ao PEG-IDA

O acoplamento do metal ao PEG-IDA foi realizado de acordo com Chung *et al* (1994). Cinco gramas de PEG-IDA foram dissolvidos em 5 ml de água destilada e 3 g de sulfato de cobre foram adicionados e mantidos em equilíbrio por 24 horas. Esta solução (**solução 3**) foi diluída a 20 ml, filtrada em membrana Millipore 0,45 µm e então lavada por ultrafiltração com 1.000 ml de água para retirada do excesso de cobre e então o material foi liofilizado.

Eletroforese

Eletroforese em gel de poliacrilamida (SDS-PAGE) 12% em condições desnaturantes foi realizada segundo LAEMMLI (1970). O gel foi revelado com uma solução de Coumassie Blue G250 (0,2% p/v) em H₂SO₄ 1M e misturada com KOH 10 M (9:1 v/v). Foram utilizados os seguintes marcadores de massa molecular: fosforilase b (94 kDa), soro bovina

albumina (67 kDa), ovoalbumina (43 kDa), anidrase carbônica (30 kDa), inibidor tripsina (20 kDa) e α -lactalbumina (14,4 kDa) disponíveis em kit (Amersham Pharmacia Biotech).

RESULTADOS E DISCUSSÃO

Os SDFA são utilizados para extração e purificação de proteínas de elevado valor biológico, devido à redução do custo operacional em relação a processos convencionais e possibilidade de integração aos mesmos, além da possibilidade de operação contínua e controle facilitado. Outras características desejáveis destes sistemas são a diferença de densidade entre as fases, o fato dos polímeros serem pouco tóxicos e serem biodegradáveis.

Inicialmente, para o acoplamento covalente de um ligante bioespecífico ao polímero PEG, é necessário que os grupos hidroxilas deste polímero sejam substituídos por grupos mais reativos, o que foi feito pela ativação do PEG com cloreto de tionila. Numa segunda etapa, o PEG-Cl reagiu covalentemente com o IDA e a seguir com sulfato de cobre, produzindo o polímero de afinidade PEG-IDA-Cu²⁺.

A Tabela 1 apresenta os resultados da partição da lisozima na presença e na ausência do ligante PEG-IDA-Cu²⁺ em SDFA.

Tabela 1. Coeficiente de partição da lisozima em SDFA composto de PEG 4000 e fosfato, pH 7,0.

Sistemas		K _{enzima}	Recuperação da lisozima (%)	K/K ₀
13% PEG 4000 9% fosfato	-	0,4	68 (fase inferior)	1,0*
12% PEG 4000 9% fosfato	1% PEG-IDA-Cu ²⁺	0,8	47 (fase superior)	2,0
8% PEG 4000 9% fosfato	5% PEG-IDA-Cu ²⁺	3,4	45 (fase superior)	8,5
3% PEG 4000 9% fosfato	10% PEG-IDA-Cu ²⁺	3,7	51 (fase superior)	9,3

* Ausência de ligante

O parâmetro K/K₀ caracteriza o aumento do coeficiente de partição da lisozima, onde K = coeficiente de partição em um sistema de afinidade e K₀ = coeficiente de partição em um sistema sem ligante.

Foi observado um aumento no coeficiente de partição da lisozima de 9,3, 8,5 e 2,0 vezes em sistemas compostos de 10%, 5%, 1% de PEG-ligante, respectivamente, em relação ao sistema que não possui o ligante PEG-IDA-Cu²⁺. Ao se comparar os sistemas contendo 5 e 10% de PEG-IDA-Cu²⁺, pode-se observar que a diferença entre os valores de K/K_0 é pouco significativa, sugerindo que a concentração de 5% de ligante naquele sistema já era suficiente para extrair aquela quantidade de enzima adicionada. Entretanto, ao aumentar a concentração de lisozima ao sistema, maior concentração do ligante seria necessária.

Quando a lisozima foi adicionada ao SDFA formado por PEG-4000 13% e fosfato 9%, sua partição se deu em direção à fase inferior. A presença de 1% de ligante no mesmo sistema teve pouca influência na partição da enzima. Na presença de concentrações mais elevadas de ligante, a partição da lisozima foi aumentada significativamente para a fase superior.

A Figura 3 representa um gel eletroforético em poliacrilamida em condições desnaturantes (SDS-PAGE) de amostras coletadas do SDFA na presença e na ausência de PEG-IDA-Cu²⁺.

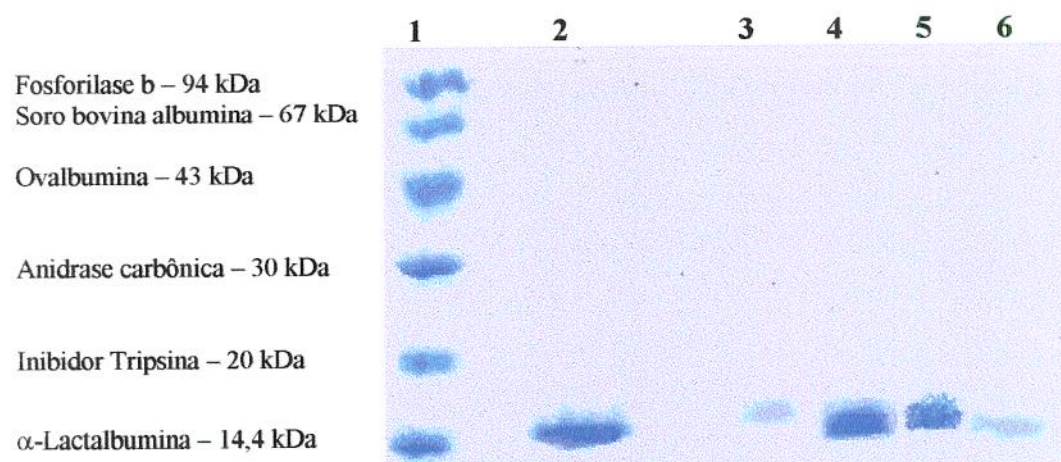


Figura 3. Eletroforese em gel de poliacrilamida (SDS-PAGE) em condições desnaturantes.

1) marcadores moleculares; 2) lisozima comercial; 3) fase superior coletada do sistema na ausência do PEG-IDA-Cu²⁺; 4) fase inferior coletada do sistema na ausência do PEG-IDA-Cu²⁺; 5) fase superior coletada do sistema na presença de PEG-IDA-Cu²⁺ e 6) fase inferior coletada do sistema na presença de PEG-IDA-Cu²⁺.

A coluna 3 do gel eletroforético representa a fase superior do sistema sem PEG-IDA- Cu^{2+} , enquanto que a coluna 4 representa a fase inferior do mesmo sistema, evidenciando que a lisozima particiona-se preferencialmente na fase enriquecida em fosfato na ausência do ligante no sistema. Entretanto, quando o sistema de afinidade é usado, a lisozima particiona-se para a fase enriquecida em PEG-IDA- Cu^{2+} , como pode ser visto na coluna 5 (fase superior) e coluna 6 (fase inferior).

Pode-se observar a inversão do K da lisozima, isto é, a enzima que é naturalmente coletada na fase inferior foi extraída na fase oposta na presença do ligante. Em um processo real de separação da lisozima dos demais contaminantes naturais presentes na clara de ovo, a lisozima seria seletivamente coletada na fase superior e os demais contaminantes seriam coletados na fase inferior, demonstrando a excelente capacidade de separação deste método.

A afinidade de uma enzima ao íon cobre é aumentada quando a proteína possui resíduos de histidina, cisteína e triptofano na sua superfície (Arnold, 1991). O princípio está baseado na interação destes aminoácidos presentes na superfície da proteína e metal ligado ao PEG (Chung & Arnold, 1991). A lisozima apresenta resíduos de histidina localizadas na sua superfície (Zhao *et al.*, 1991), e dependendo da fonte da enzima (espécie de ave) este número varia entre 0 à 4. Segundo estes autores, a lisozima de clara de ovo possui um resíduo de histidina, condição necessária para ocorrer a interação enzima-metal-ligante.

CONCLUSÕES

O uso pioneiro de SDFA por afinidade usando PEG-IDA- Cu^{2+} como ligante para extração de lisozima é descrito neste trabalho, embora, o uso de metalo-ligantes em SDFA já ser freqüentemente utilizado para purificação de outras biomoléculas de elevado valor comercial e tecnológico. É possível extrair a lisozima em SDFA por afinidade contendo PEG 4000 8%, PEG-IDA- Cu^{2+} 5% e fosfato 9%. O coeficiente de partição da lisozima teve um aumento de 9 vezes em sistema contendo 10% de PEG-IDA- Cu^{2+} , obtendo 51% de recuperação na fase superior. A otimização das condições de extração da lisozima de outras fontes que também possuam histidina em sua superfície, poderá conduzir a melhoria do rendimento e do fator de purificação.

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CAPÍTULO 7

MÉTODOS DE ATIVAÇÃO DO POLIETILENOGLICOL (PEG) PARA PURIFICAÇÃO DE ENZIMAS POR BIOAFINIDADE

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SBM Sociedade
Brasileira de
Microbiologia

ABEQ Associação Brasileira
de Engenharia Química

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MÉTODOS DE ATIVAÇÃO DO POLIETILENOGLICOL (PEG) PARA PURIFICAÇÃO DE ENZIMAS POR BIOAFINIDADE

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Resumo

O uso de ligantes bioespecíficos acoplados a polímeros solúveis (polietilenoglicóis - PEG) tem sido empregado para a recuperação de enzimas por partição em sistemas duas fases aquosas (SDFA). Estes polímeros são pouco reativos e necessitam ser ativados para posterior acoplamento do ligante, específico para a enzima a ser purificada. A aplicação de SDFA em "downstream processing" é simples, podendo ser realizada com equipamentos convencionais de processos de extração líquido-líquido. Com o intuito de diminuir o custo da obtenção de polímeros acoplados a ligantes, este trabalho investigou os principais mecanismos de ativação do PEG.

1. INTRODUÇÃO

A literatura relata as principais vantagens da utilização dos sistemas líquido-líquido de duas fases aquosas (SDFA) na extração e purificação de proteínas: possibilidade de integração ao processo, operação contínua, controle de processo facilitado e menor custo. A figura 1 representa o sistema de duas fases aquosas.

Estes sistemas são utilizados principalmente para o enriquecimento da proteína desejada ("alvo") em uma das fases ou para concentração dos resíduos celulares nas primeiras etapas do processo. Os contaminantes mais comuns (pigmentos, ácidos nucleicos, outras proteínas, polissacarídeos) podem ser removidos por partição na fase oposta à fase enriquecida na proteína alvo.

Para tornar o sistema mais econômico, a massa celular processada deve ser máxima possível, cuidando-se para evitar o deslocamento do equilíbrio entre as fases e a diminuição da eficiência da separação. Algumas vezes poderá ser necessária mais do que uma etapa de separação envolvendo SDFA: no caso da proteína alvo ser coletada na fase superior e a maior parte dos contaminantes na inferior. A seguir, numa segunda etapa, adiciona-se uma fase de composição diferente à fase enriquecida na proteína alvo, produzindo um SDFA completamente diferente, onde a proteína alvo poderá ser coletada, desta vez na fase inferior já com muito menos contaminantes. Outras etapas podem ser necessárias para melhor purificação, no entanto, em geral, não mais que três etapas. Ao fim do processo extrativo o polímero e a fase salina poderão ser reciclados (1, 2).

A seletividade dos SDFA pode ser significativamente aumentada com o uso de ligantes bioespecíficos acoplados ou não ao componente (fase) menos polar. A maioria dos contaminantes será coletada na fase mais polar enquanto que a proteína alvo será enriquecida na fase menos polar contendo o ligante. Os ligantes

utilizados podem ser específicos para uma determinada proteína (substrato, inibidores, anticorpos, proteína A) ou específicos para um grupo de proteínas (cofatores, corantes de triazina). Ao fim do processo os ligantes podem ser retirados por ultrafiltração, permeação em gel ou mesmo por partição nos sistemas de duas fases aquosas (2).

Com o intuito de diminuir o custo da obtenção de polímeros acoplados a ligantes bioespecíficos, este trabalho investigou os principais mecanismos de ativação do PEG.



Figura 1. Sistema de Duas Fases Aquosas.

2. PARTE EXPERIMENTAL

A obtenção dos polímeros acoplados a ligantes bioespecíficos envolve as seguintes etapas de reação:



Uma característica importante do grupo ativador é a facilidade de deslocamento pelo agente nucleofílico (ligante) na etapa subsequente.

2.1. Ativação com cloreto de tresila

Polietilenoglicol 4000 foi dissolvido em diclorometano contendo piridina e cloreto de tresila. A reação foi realizada sob agitação constante por 1.5 h. O diclorometano foi removido por pressão reduzida e o sólido obtido (TPEG) foi mantido em dessecador à vácuo à temperatura ambiente. O TPEG foi em seguida lavado com HCl-etanol (3 vezes) e após, mantido em dessecador à vácuo à temperatura ambiente (3). A ativação química do PEG com cloreto de tresila e subsequente acoplamento ao APGP (ligante) estão representados na Figura 2.

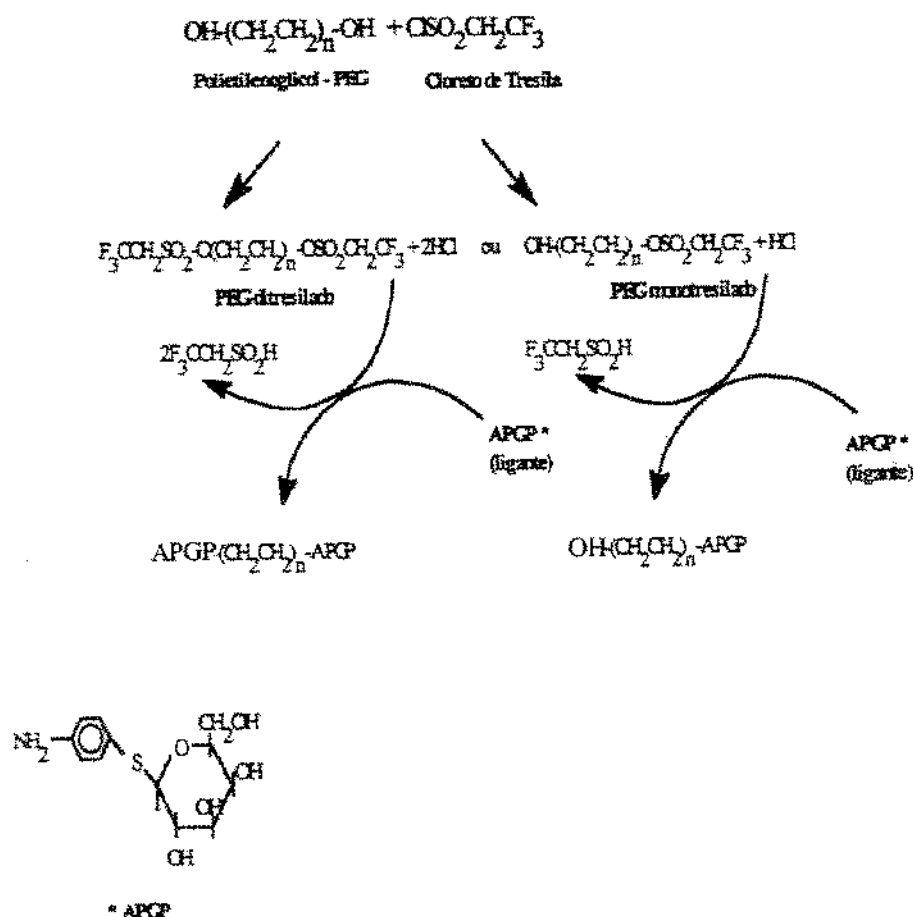


Figura 2. Ativação do PEG com cloreto de tresila e acoplamento subsequente do ligante APGP.

2.2. Ativação com cloreto de tionila

Polietilenoglicol 4000 foi aquecido à 65°C e adicionado cloreto de tionila e o meio reacional foi mantido à 65°C por 5 horas. Em seguida o excesso de cloreto de tionila foi destilado à vácuo e o produto foi cristalizado pela adição de etanol e resfriamento. Após filtração em funil de Büchner o produto foi seco em estufa à vácuo (4).

Polietilenoglicol 4000 e piridina foram obtidos da Labsynth, Diadema-SP, Brasil; cloreto de tresila da Sigma, St. Louis, MO, EUA; cloreto de tionila da Vetec, Duque de Caxias, RJ, Brasil; ácido clorídrico, etanol e diclorometano foram obtidos da Merck, Rio de Janeiro, RJ, Brasil.

Análise espectrofotométrica no I.V. foi realizada em equipamento Perkin Elmer 1600 Series F.T.I.R.

3. RESULTADOS E DISCUSSÃO

3.1 Avaliação do rendimento da ativação com cloreto de tresila

A análise de enxofre foi utilizada para determinar a ativação do PEG 4000. Para 100% de ativação, o teor de enxofre é 0,85% (m/m). Através de análise de enxofre do produto obtido, o teor de enxofre de 0,63% corresponde a 74% de ativação. A literatura (3, 5) cita rendimentos de 65-75% e 80%, respectivamente.

3.2 Avaliação do rendimento da ativação com cloreto de tionila

O rendimento obtido foi de 87% (m/m). Para caracterização do PEG ativado foi utilizado espectrofotometria de infra-vermelho. A figura 3 representa o espectro da amostra de PEG 4000 e PEG 4000 ativado, respectivamente. As hidroxilas são caracterizadas na região de 3400 cm^{-1} , devendo no entanto, estar ausente no PEG 4000 ativado. A observação do espectro da amostra de PEG 4000 ativado não demonstrou a ausência das hidroxilas, que pode ser resultante de quantidades mínimas de umidade durante a preparação da amostra. No momento, métodos para determinação de cloreto estão sendo avaliados. A literatura (4) cita rendimentos de 99%.

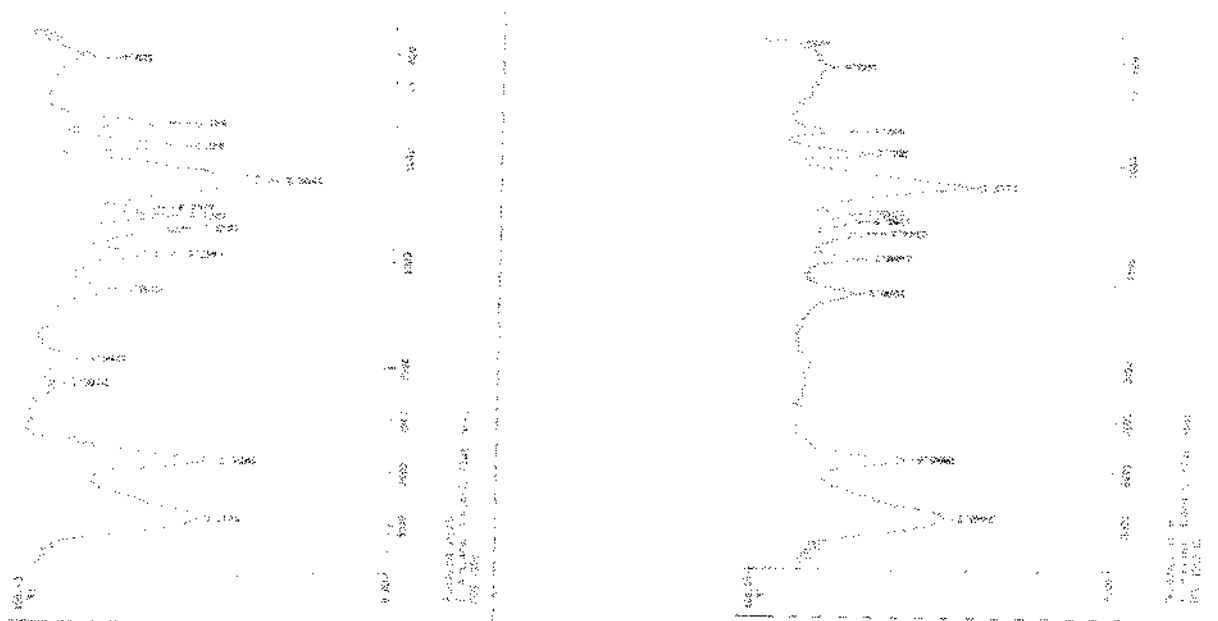


Figura 3. Espectro de infra-vermelho de PEG 4000 e PEG 4000 ativado.

4. CONCLUSÕES

Os rendimentos obtidos na ativação do polietilenoglicol 4000 com cloreto de tionila e cloreto de tresila foram bastante satisfatórios, obtendo-se valores muito próximos a literatura. Essas reações serão otimizadas visando melhorar os rendimentos. A avaliação do rendimento também é importante na etapa subsequente de acoplamento dos ligantes, o qual será avaliado posteriormente.

5. AGRADECIMENTOS

Agradecemos à FAPESP e ao CNPq.

6. REFERÊNCIAS BIBLIOGRÁFICAS

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ANEXO

1. Acoplamento de histidina à peroxidase

O acoplamento de histidina à peroxidase foi realizado segundo CHAGA (1994). Uma g de periodato de potássio foi adicionada à uma solução de 30 ml contendo peroxidase. Esta solução foi agitada por 1,5 hora na ausência de luz. O excesso de periodato de potássio foi removido em 50 mM fosfato sódio/0,15 M NaCl, pH 7, em uma coluna contendo Sephadex G-25. A modificação foi realizada adicionando-se 1 g de L-histidina nesta solução por 1h para equilibrar a amostra. Após a modificação esta solução foi lavada com tampão fosfato 50 mM pH 7 e então particionada nos SDFA. Segundo CHAGA (1994) o método da modificação da peroxidase é simples e foi aplicado à imobilização de peroxidase de nabo a suporte sólido. O mesmo procedimento para acoplamento da histidina à peroxidase descrito por este autor foi empregado para a peroxidase de soja, peroxidase de nabo e lactoperoxidase.