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LUCIANA DUARTE MARTINS DA MATTA

SULFATASE DE FÍGADO DO MOLUSCO *Aplysia cervina* SOLÚVEL E IMOBILIZADA EM SUPORTES SÓLIDOS

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Tese apresentada ao Curso de Doutorado em Ciências Biológicas da Universidade Federal de Pernambuco, para obtenção do título de Doutor em Ciências Biológicas, área de concentração em Biotecnologia.

Orientador: Prof. Dr. Luiz Bezerra de Carvalho Junior
Co-orientador: Prof. Dr. Luiz Roberto Diz de Abreu

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RESUMO

Uma sulfatase (EC 3.1.6.1.), heparina específica, foi identificada no fígado do molusco *Aplysia cervina*, largamente encontrado na costa nordestina do Brasil. Esta enzima foi purificada por precipitações sucessivas com sulfato de amônio e acetona e por cromatografia de afinidade em Heparina-Sepharose CL-6B. Algumas das propriedades físico-químicas e cinéticas desta preparação purificada 89,7 vezes (rendimento de 5,37%) foram investigadas usando o p-nitrofenil sulfato (pNFS) como substrato. Seus valores ótimos de pH e temperatura foram 5,0 e 45^{o}C, respectivamente. Ela reteve mais de 90% de sua atividade quando incubada por 15 minutos a 45ºC enquanto que perdeu 60% a 55ºC. Seu K_m foi igual a 3,71 ± 0,41 mM. Sua atividade foi estimulada por $MgCl_2$, $CaCl_2$ e $FeCl_2$ e inibida por $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$ (citrato de sódio), $HgCl_2$, Na_2HPO_4 e NaH_2PO_4. Heparina de baixa massa molecular competiu com o pNFS pelo centro ativo da enzima mais do que a de massa molecular elevada. Esta enzima foi covalentemente imobilizada ao Dacron e a uma rede semi-interpenetrada de polisiloxano e álcool polivinílico (POS/PVA), ambos magnetizados, resultando em derivados com atividade específica e retenção de 3,17 unidades/mg proteína, 1,85 unidades/mg proteína, 36,5% e 21,23%, respectivamente. Estas preparações foram removidas facilmente da mistura de reação por um campo magnético e foram reutilizadas diversas vezes sem perda de suas atividades. Elas foram mais termoresistentes do que a enzima solúvel e apresentaram o mesmo pH ótimo, temperatura ótima e K_m aparente. O derivado sintetizado com o Dacron ferromagnético apresentou uma vida útil mais elevada do que aquele em POS/PVA. O $MgCl_2$, $CaCl_2$ e EDTA ativaram ambos os derivados de sulfatase insolúveis em água enquanto que Na_2HPO_4 e NaH_2PO_4 e a heparina inibiram. A ação pelos outros íons variou de acordo com o suporte.

Palavras chaves: Sulfatase, *Aplysia cervina*, enzima imobilizada, Dacron, polisiloxano, álcool polivinílico.

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ABSTRACT

A heparin specific sulfatase (EC 3.1.6.1.) was identified in the liver of the mollusc *Aplysia cervina*, widely found in the Northeastern Brazilian coast. This enzyme was purified by ammonium sulfate and acetone precipitations and by affinity chromatography using Heparin-Sepharose CL-6B. Some physical chemical and kinetics properties of this 89.7-fold purified preparation (5.37% yield) were investigated using p-nitrophenyl sulfate (pNPS) as substrate. The optima pH and temperature were found to be 5.0 and 45^{o}C, respectively. This enzyme retained more than 90% of its activity when incubated for 15 min at 45ºC while lost 60% at 55ºC. k_m equal to 3.71 ± 0.41 mM was found. Its activity was enhanced by $MgCl_2$, $CaCl_2$ and $FeCl_2$ and inhibited by $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$ (sodium citrate), $HgCl_2$, Na_2HPO_4 and NaH_2PO_4. The heparin low molecular weight competed with pNPS for the active site enzyme more than the high molecular one. This enzyme was covalently immobilized on ferromagnetic polysiloxane/polyvinyl alcohol composite and Dacron yielding derivatives with specific activity and retention of 1.85 units/mg protein, 3.17 units/mg of protein, 21.23% and 36.5 %, respectively. These preparations were easily removed from the reaction mixture by a magnetic field and were reused several times without loss in their activities. They were more thermal stable than the soluble enzyme and presented the same optima pH and temperature and apparent K_m. The derivative based on ferromagnetic Dacron presented higher shelf life than that on POS/PVA. $MgCl_2$, $CaCl_2$ e EDTA activated both immobilized sulfatase derivatives whereas Na_2HPO_4 and NaH_2PO_4 and heparin inhibited them. The action by the other ions varied according to the support.

Key words: Sulfatase, *Aplysia cervina*, enzyme immobilization, Dacron, polysiloxane, polyvinyl alcohol.

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1. Introdução

1.1. Imobilização de Enzimas

A imobilização de enzimas é, por definição, uma técnica que confina ou liga uma enzima numa determinada região do espaço, com retenção de suas atividades catalíticas (PEREIRA, 1999).

Existem diversas razões para se imobilizar uma enzima. Dentre estas podem ser citadas (TISHER & KASCHE, 1999):

- A separação da enzima do produto de reação;
- A reutilização da enzima.

Algumas vezes, dependendo das condições do processo de imobilização, a atividade da enzima imobilizada pode apresentar-se diminuída. Este fenômeno pode ser devido às perdas causadas após a reação de ligação da enzima ao suporte utilizado, como pode ser também devido à diminuição da acessibilidade das moléculas de enzima dentro de poros ou por diminuição da difusão do próprio substrato. Essas limitações levam a diminuição da eficiência enzimática. Contudo, a estabilidade do derivado imobilizado pode compensar os inconvenientes, resultando em benefício (TISHER & KASCHE, 1999).

1.2. Métodos de imobilização

Os métodos de imobilização podem ser classificados de acordo com o tipo de reação química envolvida na ligação. As propriedades da enzima imobilizada são governadas pelas propriedades da enzima e do suporte utilizado, ou seja, a interação entre os dois produz uma enzima imobilizada com propriedades química, bioquímica, mecânica e cinética específicas. Vários parâmetros devem ser levados em consideração para a imobilização de enzimas e dentre eles os mais importantes estão

listados no quadro 1 (TISHER & KASCHE, 1999).

Quadro 1: Parâmetros que Devem ser Levados em Consideração para Imobilização de Enzimas

Enzimas	**Propriedades Bioquímicas:** Massa molecular, grupos prostéticos, grupos funcionais, pureza. **Parâmetros Cinéticos:** Atividade específica, pH e temperaturas ótimas, parâmetros cinéticos para atividade e inibição, estabilidade contra pH e temperatura, solventes, contaminantes e impurezas.
Suportes	**Características Químicas e Estruturais:** Base química e composição, grupos funcionais, comportamento após turgescência, volume acessível da matriz, tamanho do poro e estabilidade química do suporte. **Propriedades Mecânicas:** Diâmetro da partícula, comportamento de compressão da partícula, resistência ao fluxo, velocidade de sedimentação e desgaste.
Enzima imobilizada	**Método de Imobilização:** Ligação da proteína**,** rendimento da enzima ativa e parâmetros cinéticos intrínsecos. **Efeito de transferência de massa:** Partição (concentrações diferentes de solutos dentro e fora das partículas catalíticas), difusão externa e interna (poros); **Estabilidade:** Estabilidade operacional e de armazenagem. **Desempenho:** Produtividade, gasto da enzima.

Os principais métodos utilizados para imobilização de enzimas são (figura 1): adsorção, ligação covalente, aprisionamento e encapsulação (BICKERSTAFF, 1997).

Ao se imobilizar uma enzima em determinado suporte é importante a escolha de um método de ligação que previna a perda de atividade enzimática, ou seja, que ligue a enzima ao suporte apropriado causando mínimos danos à sua estrutura. Neste processo é importante proteger o sítio ativo da enzima durante a ligação ao suporte, esta função de proteção pode ser dada por um substrato ou um inibidor competitivo da enzima (DORDIK & BUNGAY, 1998).

A atividade da enzima após imobilização depende, sobretudo da natureza do suporte utilizado. A seleção do suporte depende da natureza da própria enzima como

também do tamanho da partícula, área superficial, razão molar de grupos hidrofílicos e hidrofóbicos e composição química. O aumento na razão de grupos hidrofílicos geralmente resulta em uma alta atividade das enzimas imobilizadas. Alguns dos suportes mais utilizados para imobilização de enzimas são derivados de polissacarídeos como celulose, dextrana, agarose e gel de poliacrilamida (DORDIK & BUNGAY, 1998).

1.2.1. Adsorção

Pode ser considerado o método mais simples de imobilização. É baseado na adsorção da enzima sobre a superfície de suportes insolúveis em água (figura 1). Este método causa pouca ou nenhuma mudança conformacional no sítio ativo da enzima (FUENTES *et al*., 2001).

A maior vantagem da adsorção é que nenhum reagente é necessário e poucos passos são requeridos para o processo de imobilização em si. Este método é menos agressivo para a proteína que os métodos químicos, pois as principais forças que mantém a enzima imobilizada são as pontes de hidrogênio, ligações iônicas e de van der waals. Desta maneira, o método apresenta bastante similaridade com aqueles ocorridos naturalmente no ambiente celular (DORDIK & BUNGAY, 1998).

As desvantagens desse método estariam relacionadas à relativa facilidade de desorção da enzima do suporte durante seu uso devido às fracas interações entre eles. Mudanças na temperatura, pH, força iônica, ou mesmo a presença de substrato podem causar a desorção da enzima do suporte. Outra desvantagem seria a adsorção não específica de outras proteínas ou outras substâncias juntamente com a enzima. Isto pode alterar as propriedades da enzima imobilizada (WOODWARD, 1985). Diversas enzimas têm sido imobilizadas através deste método, entre elas:

proteases (KISE & HAYAKAWA, 1991), α-amilase, β-glicosidase, tripsina e fosfatase alcalina (SARDAR *et al.,* 1997), lipoxigenase (SANTONO *et al.*, 2002) e peroxidase (MELGAREJO-ROJAS *et al*., 2003).

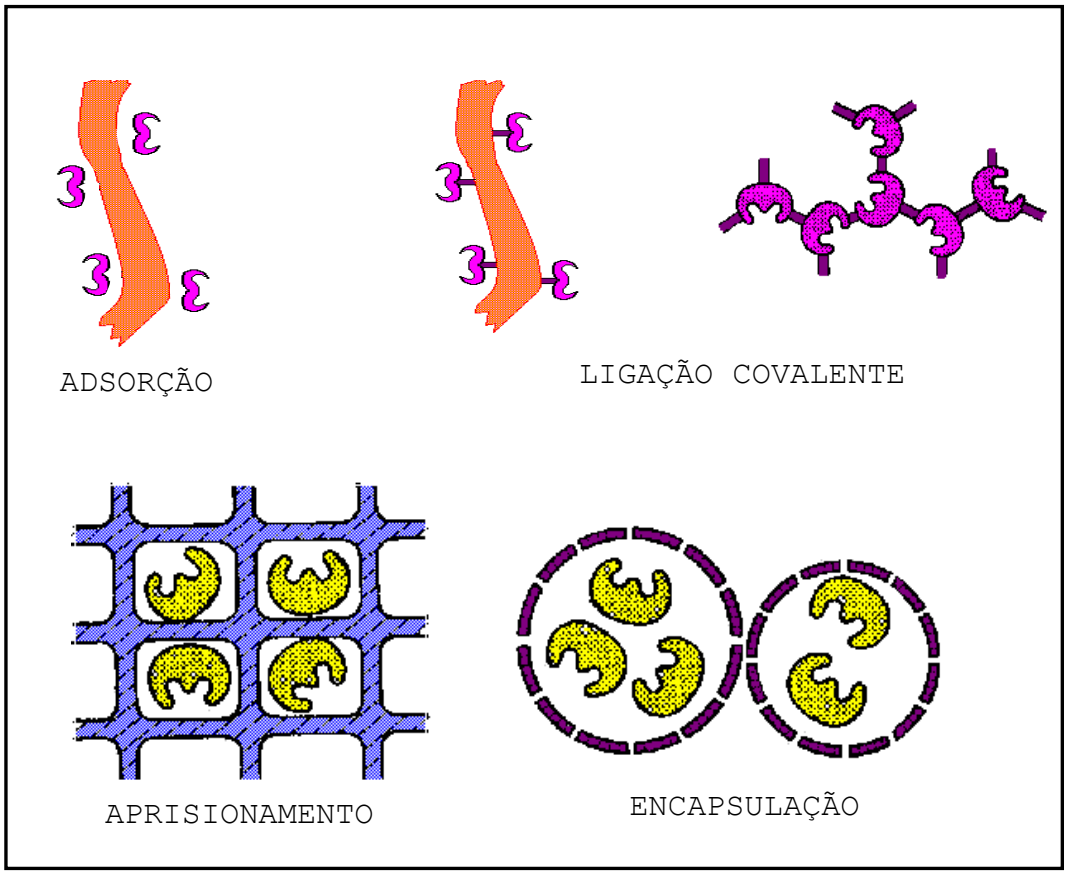

Figura 1: Métodos para imobilização de enzimas

1.2.2. Ligação Covalente

É a mais intensamente estudada das técnicas de imobilização e envolve a formação de ligações covalentes entre enzima e suporte (figura 1). Ao tentar selecionar o tipo de reação pela qual uma dada proteína pode ser imobilizada, a escolha deve ser limitada a duas características principais (DORDIK & BUNGAY, 1998).

1- A reação química de imobilização deve ser realizada sob condições que não causem perda da atividade enzimática;

2- O sítio ativo da enzima não pode ser afetado pelos reagentes utilizados no processo de imobilização.

Os grupos funcionais dos aminoácidos que podem ser utilizados nas ligações covalentes são: amino, hidroxila, tiol, carboxila, imidazol, sulfidrila, fenólico e indol.

A ligação covalente pode alterar a estrutura conformacional e o centro ativo da enzima, resultando em maior perda da atividade. Contudo, a força da ligação entre a enzima e o suporte é suficientemente forte para impedir a desorção da enzima tanto na presença do substrato, quanto em soluções de alta força iônica (DORDIK & BUNGAY, 1998).

Este método tem sido utilizado para imobilização de várias enzimas, tais como: sulfatases (BOPPANA *et al.*, 1989; LEONI *et al.,* 1998; GAO *et al.*, 2001; TOENNES & MAURER, 1999), β-glucuronidases (BOPPANA *et al.*, 1989; TOENNES & MAURER, 1999), peroxidase (AZEVEDO *et al.*, 2001) e lipases (CARNEIRO-DA-CUNHA *et al.,* 2002) dentre outras.

A ligação covalente pode também ser efetuada entre enzimas, constituindo uma técnica conhecida como ligação cruzada, na qual as enzimas estão interligadas através de reagentes bifuncionais entre os quais pode ser citado o glutaraldeído formando uma estrutura tridimensional grande e complexa (figura 1) (BICKERSTAFF, 1997; VILLENEUVE *et al.,* 2000). A desvantagem deste método está na baixa estabilidade mecânica e hidrodinâmica dos derivados obtidos (TISHER & KASCHE, 1999).

Amiloglicosidase e invertase (ULBRICHT & PAPRIA, 1996) e α-L-arabinofuranosidase (SPAGNA *et al.,* 1998) têm sido imobilizadas por este método.

1.2.3. Aprisionamento

Este método consiste na imobilização de enzimas dentro de matrizes poliméricas. A enzima é adicionada numa solução contendo monômeros, que, sob polimerização leva ao seu aprisionamento (figura 1). Os géis de poliacrilamida são matrizes comumente utilizadas para determinadas técnicas (VILLENEUVE *et al.*, 2000). Os géis formados a partir de alginatos de sódio ou carragenanas com cálcio ou cloreto de potássio são também utilizados (SANTOYO *et al.*, 1996; VILLENEUVE *et al.*, 2000).

Geralmente as enzimas que possuem pequenos substratos são mais utilizadas para a técnica de aprisionamento, pois substratos maiores são incapazes de atravessar os poros e chegar ao sítio ativo da enzima aprisionada (VILLENEUVE *et al.,* 2000).

Algumas enzimas têm sido imobilizadas por este método: glicoamilase, β-D-glicosidase e invertase (KENNEDY & KALOGERAKIS, 1984), β-glicosidase (BUSTO, 1998), β-galactosidase (KATO *et al.,*1998) e lipases (BETIGERI & NEAU, 2002; CHEN & HWANG, 2003).

1.2.4. Encapsulação

A encapsulação é, provavelmente, a técnica de imobilização menos desenvolvida. É similar ao aprisionamento, embora neste caso é a enzima e seu ambiente que são imobilizados (figura 1). A encapsulação cria células artificiais delimitadas por membranas. Moléculas grandes como as enzimas não são capazes de se difundir através da membrana enquanto que pequenas moléculas como substratos e produtos podem atravessá-la (VILLENEUVE *et al.,* 2000).

A vantagem deste método é que a enzima não interage quimicamente com o

polímero evitando assim sua desnaturação. A taxa de difusão (do substrato e do produto) através da membrana é o parâmetro limitante (VILLENEUVE *et al.,* 2000).

Este método permite a incorporação de diferentes enzimas ou enzimas e células no mesmo compartimento sem nenhuma modificação química (MANSFELD *et al.,* 1991).

Algumas enzimas e células já foram imobilizadas por este método, como: invertase (MANSFELD *et al.,*1991) e bactérias (LOPEZ *et al.,* 1997).

1.3. Suportes utilizados para imobilização de enzimas

Vários polímeros têm sido utilizados em nossos laboratórios para imobilização de proteínas, tais como: dacron (PINHEIRO *et al.,* 1999; MONTENEGRO *et al.,* 1999; CARNEIRO-LEÃO *et al.,* 1991; CARNEIRO-LEÃO *et al.,* 1994; BARBOSA *et al.,* 1995; CARVALHO JR *et al.,* 1987), discos de álcool polivinílico-glutaraldeído (ARAÚJO *et al.,* 1996), pérolas de polisiloxano-álcool polivinílico (COELHO *et al.,* 2003), polianilina (NADRUZ JR *et al.,* 1996) e poliacrilamida (CARVALHO JR *et al.,* 1987; CARVALHO JR *et al.,* 1988; BARBOSA *et al.,* 1995) dentre outros.

1.3.1. Dacron (polietilenotereftalato)

O Dacron é um poliéster, componente de fibras, de resinas e utilizado na manufatura de garrafas de refrigerante. É sintetizado a partir do etilenoglicol e ácido tereftálico (PAIVA, 1999). Caracteriza-se por apresentar a seguinte estrutura (Figura 2):

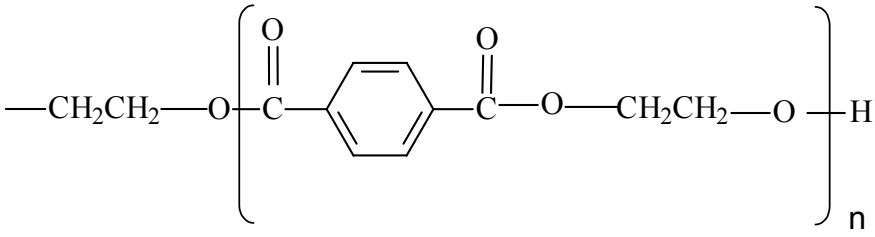

Figura 2: Estrutura do polietilenotereftalato, onde n pode assumir valores em torno de 15.000 (CARNEIRO-LEÃO *et al.,* 1991).

MELO (1984) propôs um mecanismo para imobilizar proteínas em Dacron que consistia em três passos: a primeira etapa consistia na hidrazinólise parcial dos filmes de Dacron com conseqüente transformação deste em pó; na etapa seguinte utilizava-se nitrito de sódio a fim de converter os grupos hidrazida a azida; o terceiro passo consistia na imobilização covalente da enzima ao suporte.

CARNEIRO-LEÃO (1991) propôs a imobilização de amiloglicosidase em Dacron em pó magnetizado por este apresentar maior facilidade operacional. Alguns suportes magnetizados têm sido descritos na literatura para imobilização de enzimas (KONERACKÁ *et al.,* 1999; KATO *et al.,* 1998).

1.3.2. Álcool polivinílico

O álcool polivinílico (PVA) é formado por uma longa cadeia de átomos de carbono unidos por ligações simples apresentando várias hidroxilas que se repetem ao longo de toda a cadeia (figura 3). Comparado com outros polímeros, o PVA é um material de baixo custo, que apresenta alta estabilidade, durabilidade e força mecânica (TING & SUNG, 2000).

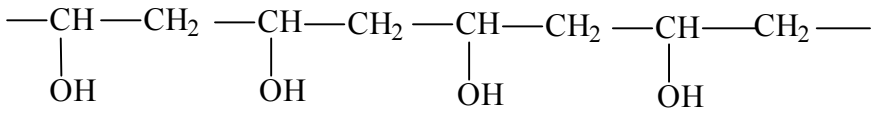

Figura 3: Estrutura do álcool polivinílico

A ativação do PVA com glutaraldeído é necessária como etapa anterior à imobilização enzimática. A síntese do PVA-glutaraldeído baseia-se em uma reação de acetalização em meio ácido envolvendo as hidroxilas do PVA e os grupos carbonilas do glutaraldeído (figura 4). O segundo grupamento carbonila do glutaraldeído pode reagir com uma outra molécula de PVA, como também pode formar ligações covalentes com grupamentos amina de moléculas como enzimas (ARAÚJO *et al.,* 1996).

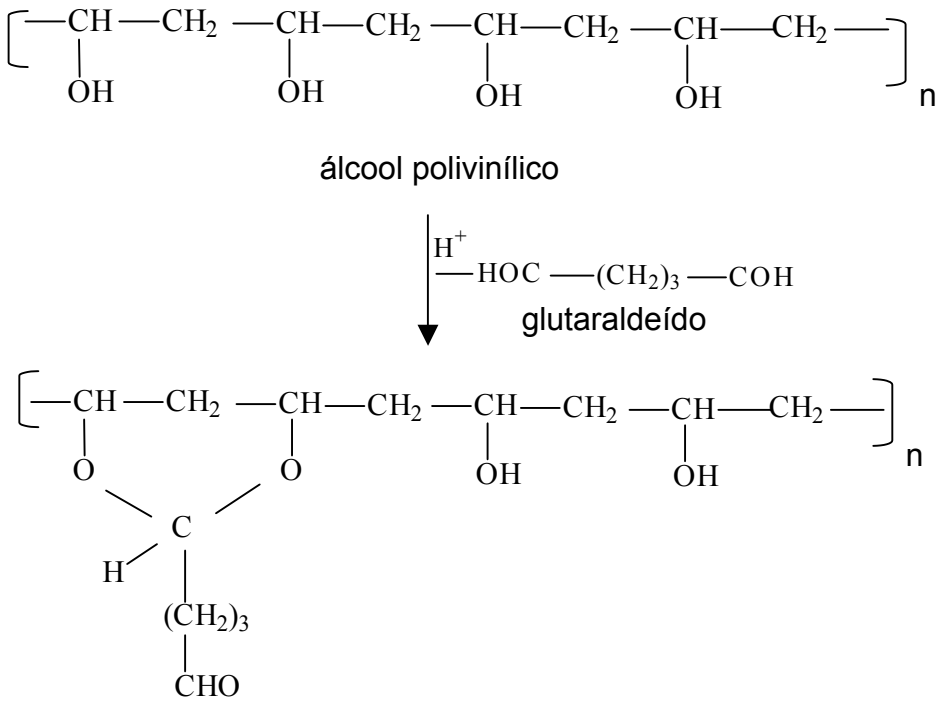

Figura 4: Reação do glutaraldeído com o álcool polivinílico via mecanismo acetal, sob catálise ácida.

1.3.3. Polisiloxano-álcool polivinílico-POS/PVA

O processo sol-gel para a formação de vidros inorgânicos tem sido conhecido há um século. Contudo o primeiro relato na literatura mostrando o uso de silicatos para a imobilização de proteínas foi proposto por DICKEY (1955), quando mostrou que várias enzimas podem ser aprisionadas em vidros derivados de ácido silícico com retenção de suas atividades biológicas. BRAUN e colaboradores (1990) demonstraram que uma série de enzimas podia ser aprisionada dentro de vidros derivados do tetraetilortosilicato (TEOS) com retenção de suas atividades enzimáticas.

A técnica sol-gel envolve a hidrólise parcial ou total de um precursor monomérico com a posterior condensação dos hidroxi derivados e posterior secagem, maturação e sinterização (CHEN & HWANG, 2003).

Silicatos modificados com material orgânico fornecem melhores propriedades, aumentando a hidrofobicidade, hidroficidade, cargas ou pontes de hidrogênio do material. Tais características podem ser usadas para aumentar a estabilidade de determinadas proteínas, particularmente as hidrofílicas ou hidrofóbicas (CHEN *et al.,* 2003).

O híbrido POS/PVA é obtido pelo processo sol-gel. Neste processo dois passos são necessários: A hidrólise e posterior condensação dos alquilsilicatos. O álcool polivinílico incorporado nos vidros resulta em um compósito contendo a propriedade de ligação covalente do álcool polivinílico e excelente estabilidade óptica, térmica e química da matriz (COELHO *et al.,* 2003).

1.4. Glicosaminoglicanos

Os glicosaminoglicanos são heteropolissacarídeos constituídos por unidades dissacarídicas repetitivas, unidas por ligação glicosídica, onde um dos resíduos é uma hexosamina (glicosamina ou galactosamina); e o outro, um ácido urônico (D-glicurônico ou L-idurônico) ou um açúcar neutro (galactose) (BRIMACOMBE & WEBER, 1964). Estes açúcares podem ainda apresentar grupamentos sulfato (exceto o ácido hialurônico), que juntamente com os grupamentos carboxílicos dos ácidos urônicos conferem a esses polissacarídeos alta densidade de cargas negativas.

Os principais tipos de glicosaminoglicanos são: ácido hialurônico (AH), condroitim 4-sulfato (C4S), condroitim 6-sulfato (C6S), dermatam sulfato (DS), heparam sulfato (HS), queratam sulfato (KS) e heparina (Hep) (Tabela I).

Existe grande heterogeneidade entre os glicosaminoglicanos, seja no grau de sulfatação, nas proporções das unidades dissacarídicas ou na massa molecular das cadeias. Na figura 5 estão esquematizadas as unidades estruturais mais frequentemente encontradas nestes compostos.

Nos tecidos animais, todos os glicosaminoglicanos, com exceção do ácido hialurônico, ocorrem covalentemente ligados às proteínas, formando os proteoglicanos (PG), os quais apresentam alto peso molecular (LOHMANDER *et al*., 1980; NILSSON *et al*., 1982; GOWDA *et al*., 1986).

Tabela I

Características estruturais dos glicosaminoglicanos

Gags (JEANLOZ, 1960)	P.M.[1](kDa)	Monossacarídeos[2]	Posição do Sulfato	Ligação Glicosídica
Ácido hialurônico (AH)	500 – 50.000	N-acetilglicosamina Ácido glicurônico	- -	β (1-4) β (1-3)
Condroitim 4-sulfato (C4S)	20 - 70	N-acetilgalactosamina Ácido glicurônico	4 -	β (1-4) β (1-3)
Condroitim 6-sulfato (C6S)	20 - 50	N- acetilgalactosamina Ácido glicurônico	6 -	β (1-4) β (1-3)
Dermatam sulfato (DS)	20 - 50	N-acetilgalactosamina Ácido glicurônico Ácido idurônico	4 - -	β (1-4) β (1-3) α (1-3)
Heparam sulfato (HS)	10 - 60	Glicosamina N-acetilglicosamina Ácido glicurônico Ácido idurônico	2 / 6 - / 6 - -	α (1-4) α (1-4) β (1-4) α (1-4)
Heparina (Hep)	5 - 50	Glicosamina Ácido glicurônico Ácido idurônico	2 / 6 - 2	α (1-4) β (1-4) α (1-4)
Queratam sulfato (KS)	10 - 30	N-acetilglicosamina Galactose	6 - / 6	β (1-3) β (1-4)

[1] o peso molecular médio varia neste intervalo de acordo com a origem dos glicosaminoglicanos
[2] todos os açúcares estão na configuração D, exceto o ácido idurônico que apresenta configuração L.
(-) representa ausência de éster de sulfato

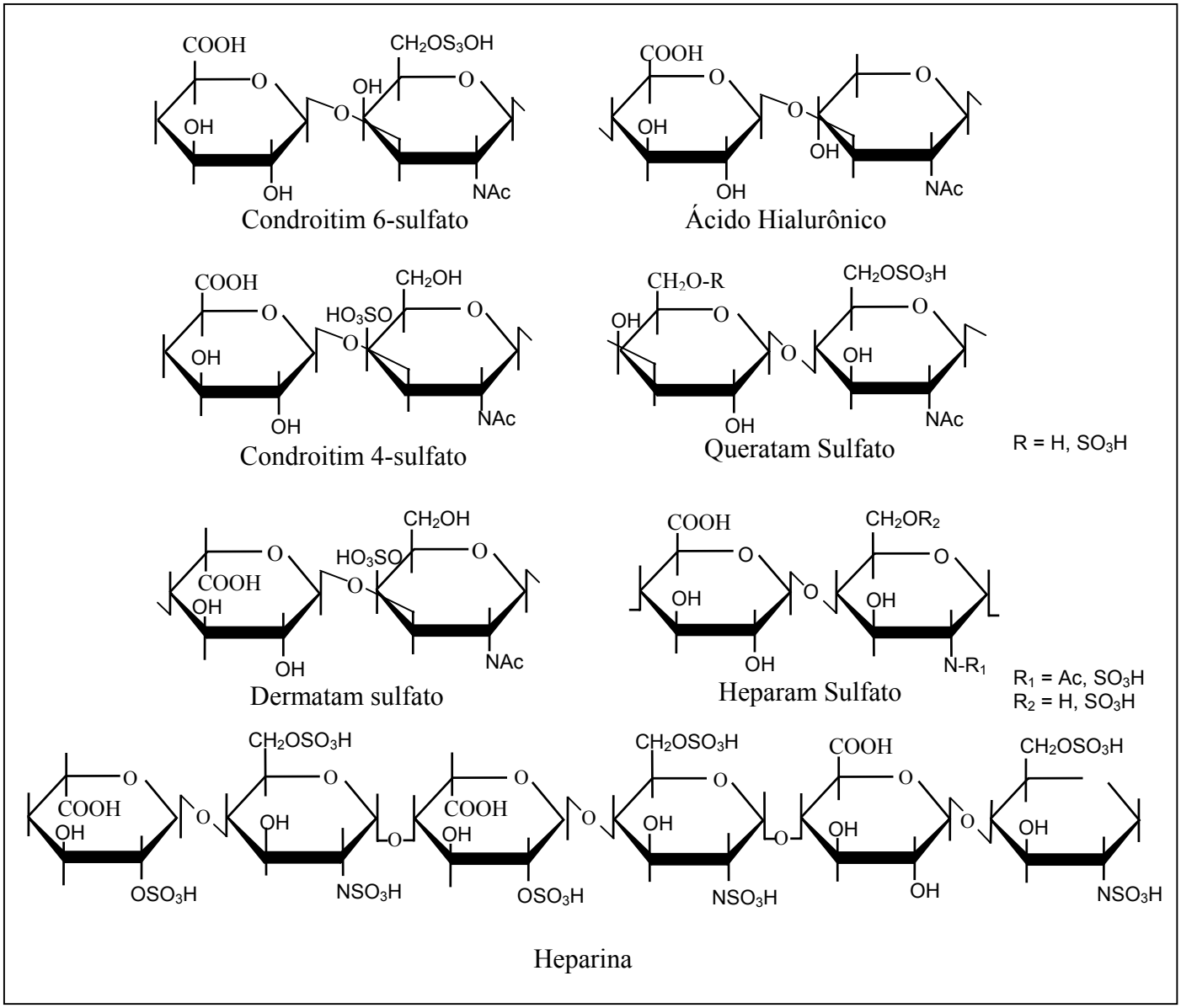

Figura 5: Unidades estruturais dos glicosaminoglicanos

1.5. Distribuição e função dos glicosaminoglicanos

O estudo da distribuição dos glicosaminoglicanos mostrou que, com exceção de bactérias, fungos e protozoários, os glicosaminoglicanos estão presentes em todos os filos do reino animal que apresentam organização tissular. Eles são encontrados em espongiários até mamíferos superiores, possibilitando formular novas hipóteses sobre o provável papel biológico evolutivo destes compostos (DIETRICH *et al*., 1976; DIETRICH *et al*., 1977; GEOCZE & NADER, 1982; MEDEIROS *et al*., 2000).

As funções atribuídas a esta classe de carboidratos sempre estiveram relacionadas ao órgão onde foram encontrados e a algumas de suas propriedades físico-químicas, tais como: viscosidade, peso molecular e caráter polianiônico. Cita-se, entre outras funções, resistência à infecção (WARREN & GRANHAM, 1950), controle de água e eletrólitos (DORFMAN, 1958), cicatrização (DORFMAN, 1958), divisão e crescimento celular (KRAEMER & TOBEY, 1972; OHNISHI *et al*., 1975; CHIARUGI & VANNUCHI, 1976; VANNUCCI & CHIARUGI, 1977; DIETRICH, 1984; BIANCO *et al*., 1990; PORCIONATTO *et al*., 1999), adesão celular (STALLCUP *et al*., 1990), reconhecimento intercelular (LÓPEZ-CASTILHAS *et al*., 1991), manutenção da transparência da córnea (KRESSE *et al*., 1993) e atividade anticoagulante (COLBURN & BUONASSI, 1982).

1.5.1. Glicosaminoglicanos em vertebrados

Os condroitins sulfatos são componentes característicos da matriz dos tecidos conjuntivos, representando cerca de 10% do peso seco de cartilagens. O condroitim 6-sulfato é o principal glicosaminoglicano de cartilagem articular adulta. Está também presente em grande quantidade em aortas (TOLEDO & DIETRICH, 1977). Com base nesses resultados, foi proposto que o condroitim 6-sulfato esteja relacionado com a manutenção da integridade dos tecidos sujeitos a forças mecânicas. Por outro lado, presume-se que o condroitim 4-sulfato, que é mais característico de cartilagem em crescimento esteja envolvido nos processos de crescimento e ossificação (MICHELACCI *et al.*, 1979; MICHELACCI *et al.*, 1981; MOURÃO *et al.*, 1973; MOURÃO *et al.*, 1976; MOURÃO *et al.*, 1979).

Os heparam sulfatos são componentes da membrana plasmática celular e estão presentes em tecidos de mamíferos e demais vertebrados, bem como de

invertebrados e células em cultura, o que sugere que estes compostos sejam componentes universais de células animais (CÁSSARO & DIETRICH, 1977; DIETRICH *et al.*, 1983; DIETRICH, 1984; NADER *et al.*, 1984, NADER *et al.*, 1988). Algumas funções foram sugeridas para os heparam sulfatos, tais como difusão de macromoléculas através de membranas basais, controle do acesso à superfície celular de moléculas regulatórias como fatores de crescimento, hormônios e neurotransmissores, influenciando o balanço catiônico local, especialmente de Ca++ (OBRINK *et al*., 1975), além de possível atuação na superfície celular como polímero de interação entre as células (DIETRICH *et al*., 1979; DIETRICH *et al*., 1980; DIETRICH, 1984).

O dermatam sulfato está amplamente distribuído em vários tecidos de mamíferos, ocorrendo predominantemente em tecidos conjuntivos densos, tais como pele e tendão (FRANSSON, 1985; MEYER & CHAFFEE, 1941; MEYER *et al.*, 1956; TOLEDO & DIETRICH, 1977). Sua função nesses tecidos está, possivelmente, relacionada à organização, velocidade de deposição e manutenção das fibrilas de colágeno (SCOTT & ORFORD, 1981; SCOTT, 1984; SCOTT & HAIGH, 1985).

Dados da literatura mostram que queratam sulfato do tipo I está presente no estroma da córnea, interagindo através de um padrão regular com as bandas “a” e “c” das fibras de colágeno, o que confere transparência óptica à córnea (SCOTT & HAIGH, 1985). Já o queratam sulfato do tipo II representa cerca de 25% do total de glicosaminoglicanos em cartilagem articular adulta e não é detectado em cartilagens de recém nascidos.

1.5.2. Glicosaminoglicanos em invertebrados

A maioria dos dados existentes na literatura sobre distribuição, isolamento e caracterização estrutural de glicosaminoglicanos e polissacarídeos sulfatados em invertebrados mostram apenas a existência desses compostos em tecidos histologicamente semelhantes ao conjuntivo (MATHEWS, 1975).

A presença de polissacarídeos sulfatados foi observada em poríferos (GROSS *et al*., 1956) e no crustáceo *Hemigrafus nudus* (MEENAKSHI & SCHEER, 1961; MATHEWS, 1975). Polissacarídeos ácidos contendo diversos tipos de açúcares como glicose, galactose, fucose, xilose, manose, arabinose, ramnose, ribose, hexosaminas (glicosamina e galactosamina) e ácido urônico foram identificados no cnidário *Physalia* (GROSS *et al*., 1958). Em poríferos acredita-se que estes polímeros estejam na matriz extracelular participando de processos de reconhecimento celular, agregação e adesão das células nas esponjas (MACLENNAN, 1974; ALBERTS *et al*., 1989).

No gastrópode *Charonia lampas* foi descrita a ocorrência do heteropolissacarídeo horatim sulfato, constituído por vários tipos de açúcares, como: glicose, galactose, manose, fucose, glicosamina e galactosamina, porém sem ácido urônico (INQUE, 1965).

Inicialmente, o ácido hialurônico foi considerado como o único glicosaminoglicano presente em invertebrados (HOGLUND, 1976). ESTES e FAUST (1964) descreveram que este era o único glicosaminoglicano existente no intestino de mariposas. Nas cartilagens do cefalópode *Loligo pealii* foi descrita a presença de compostos semelhantes ao condroitim 4-sulfato (MATHEWS, 1975). ASHURST e COSTIN sugeriram a presença de compostos semelhantes a ácido hialurônico, condroitim sulfato, dermatam sulfato e queratam sulfato em tecidos de alguns insetos

(ASHURST & COSTIN, 1971a; ASHURST & COSTIN, 1971b). Posteriormente, HOGLUND (1976) isolou além de ácido hialurônico, condroitim sulfato e compostos semelhantes a queratam sulfato e heparam sulfato do Arthropoda *Calliphora eritocephala*. Em moluscos, também foi observada a existência de compostos do tipo condroitim sulfato nos cefalópodes *Loligo sp.* (HABUCHI *et al*., 1977; HJERPE *et al.,*1983), *Loligo opalescens* e *Ommastrephes sloani pacíficus* (ANNO *et al*., 1964; SRINIVASAN *et al*., 1969). Nessa última espécie o condroitim sulfato mostrou em sua estrutura ramificações de glicose na posição C-6 da hexosamina (HABUCHI *et al*., 1977). Das espécies *Spissula solidissima* e *Cyprina islandica*, pertencentes à classe Lammelibranchia, foram isoladas substâncias com ação anticoagulante semelhante à heparina de mamíferos, que foram denominadas mactinas (BURSON *et al*., 1956). No crustáceo *Tachipleus tridentatus*, condroitim sulfato foi descrito como principal glicosaminoglicano sulfatado da cartilagem da guelra desse caranguejo e heparam sulfato na lagosta *Homarus americanus* (HOVINGH & LINKER, 1982).

Uma grande variedade de polissacarídeos sulfatados foi identificada em equinodermos. Inicialmente, polímeros de condroitim sulfato e fucose sulfatada foram extraídos do tecido conjuntivo de holotúrias (KATZMAN & JEANLOZ, 1969; KATZMAN & JEANLOZ, 1970). A existência de glicanos sulfatados foi descrita na túnica de várias espécies de ascídias (Chordata-Tunicata) e pepino do mar (Echinodermata-Holothuroidea) em concentrações semelhantes àquelas encontradas na cartilagem de vertebrados (ALBANO & MOURÃO, 1986; MOURÃO & BASTOS, 1987).

CÁSSARO & DIETRICH (1977) realizaram um estudo sistemático de distribuição desses compostos em 22 espécies de invertebrados pertencentes ao filo Porífera, Coelenterata, Annelida, Molusca, Arthropoda e Equinodermata. Este

trabalho demonstrou que todas as espécies continham quantidades variáveis de um ou mais tipos de glicosaminoglicanos sulfatados. Mais recentemente, MEDEIROS e colaboradores (2000) estendeu este estudo com mais 23 espécies de 13 filos de invertebrados e demonstrou que o heparam sulfato é encontrado em todas as espécies estudadas. A heparina foi encontrada em 10 espécies, sendo 5 de crustáceos.

No molusco *Pomacea sp.* todos os tecidos e órgãos analisados continham condroitim sulfato e heparam sulfato, bem como outros compostos de estrutura desconhecida (DIETRICH *et al*., 1983).

Em *Anomalocardia brasiliana* foi identificada uma heparina muito semelhante às purificadas de tecidos de mamíferos, mas com maior atividade anticoagulante, maior grau de afinidade pela antitrombina III e maior peso molecular (DIETRICH *et al.,*1985; PJELER *et al.,*1987). A heparina também foi isolada e identificada em mais duas espécies de bivalvia: *Tivela mactroides e Donnax striatus* (PJELER *et al*., 1987). A presença de heparina em moluscos, que não apresentam sistema de coagulação sanguínea relacionado com a antitrombina III demonstra que o papel biológico da heparina não tem relação com mecanismos de coagulação.

Estudos realizados recentemente com os moluscos *Aplysia californica e Helix aspersa* (HOVINGH & LINKER, 1998) demonstraram a presença de uma molécula denominada de “heparina” no intestino do molusco *Aplysia californica*. Este polissacarídeo foi assim denominado por migrar como heparina em eletroforese, por ser degradada por HNO_2, mas não ser bem degradada por heparinase, podendo indicar que este polissacarídeo trata-se de um heparam sulfato altamente sulfatado, semelhante a heparina. No molusco *Helix aspersa* foi encontrado maior conteúdo de condroitim sulfato no coração, manto e pulmão do animal.

A ampla distribuição destes polissacarídeos em invertebrados sugere possíveis papéis biológicos relacionados às características estruturais destes compostos, em particular suas propriedades aniônicas. A presença destes polissacarídeos em diversos órgãos dos invertebrados indica a possível presença de enzimas relacionadas ao processo de degradação destas moléculas.

1.6. Sulfatases

1.6.1. Definição

Sulfatases são enzimas que catalisam a hidrólise de ésteres de sulfato liberando sulfato inorgânico de uma ampla variedade de compostos sulfatados.

Algumas sulfatases são referidas como arilsulfatases (EC 3.1.6.1.) por liberarem sulfato inorgânico de um grande número de ésteres de arilsulfato. Esta característica serve de base para sua classificação (figura 6).

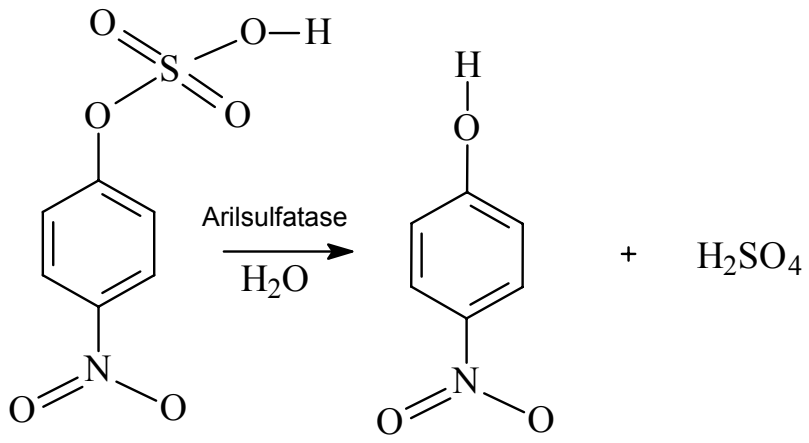

Figura 6: Reação geral catalisada por uma arilsulfatase sobre *p*-Nitrofenil sulfato

1.6.2. Funções e aplicações

Sulfatases de esteróides são distribuídas em tecidos de todos os mamíferos e sua função é hidrolisar vários sulfatos de esteróides, especialmente “estrona-sulfato”

que é o precursor dos estrógenos ativos. A sua deficiência resulta na síndrome da ictiose ligada ao X (MORTAUD *et al*., 1994).

Neuroesteróides exercem efeitos regulatórios sobre o sistema nervoso central através do receptor $GABA_A$. Tetrahidroprogesterona, hidroxitetrahidroprogesterona e androsterona são agonistas do $GABA_A$. Pregnenolona sulfato e deidroepiandrosterona sulfato são antagonistas ligando-se não competitivamente ao receptor $GABA_A$. Sulfatases de esteróides do cérebro convertem pregnenolona sulfato e deidroepiandrosterona sulfato a suas formas desulfatadas desempenhando assim papel chave na regulação do receptor $GABA_A$ (PARK *et al*., 2000).

Estrógenos desempenham papéis essenciais na fisiologia e patologia durante o desenvolvimento humano. As três enzimas mais importantes responsáveis pela biossíntese tecido-específica dos estrógenos são: citocromo P450 aromatase, estrona/deidroepiandrosterona sulfatases e hidroxiesteróide desidrogenase. Elas são primariamente responsáveis pela biossíntese dos hormônios femininos nos seios, ovários, placenta, endométrio e outros tecidos. A citocromo P450 aromatase catalisa a produção de estrógenos que são armazenados no corpo como ésteres de sulfato. Estrona sulfatase catalisa a hidrólise de sulfato da estrona e deidroepiandrosterona sulfatos liberando os esteróides não conjugados e a hidroxiesteróide desidrogenase é responsável pela redução da estrona a formas mais ativas dos hormônios femininos (estradiol). Modelos de vários tumores sugerem papéis críticos das três enzimas no mecanismo de biossíntese do estradiol em células tumorais de mama (HERNANDEZ-GUZMAN *et al*., 2001).

A conjugação de drogas e outros compostos endógenos com íon sulfato ou ácido glicurônico é um caminho metabólico comum em humanos e animais. Estes compostos são geralmente referidos como produtos metabólicos finais destinados

para excreção na urina ou bile. Eles desempenham papel chave para entender os destinos de determinadas drogas no corpo e necessitam de identificação e análise. Geralmente, são analisados de forma indireta, via hidrólise química ou enzimática, seguido por análise do aglicônio liberado.

Uma sulfatase (EC 3.1.6.1) e β-glicuronidase (EC 3.2.1.31) de *Helix pomatia* foram imobilizadas em pérolas de vidro e utilizadas com sucesso para hidrolisar conjugados glicurônicos e sulfatados do fenoldopam (BOPPANA *et al*., 1989). Mais recentemente, uma sulfatase e uma β-glicuronidase de glândulas digestivas do molusco *Helix pomatia* foram purificadas e imobilizadas no suporte Affi-Gel 10 para facilitar a clivagem e detecção de conjugados de morfina na urina (TOENNES & MAURER, 1999).

Em outros estudos, a sulfatase do molusco *Helix pomatia* tem sido utilizada como ferramenta adicional para auxiliar na detecção e quantificação de isoflavonas e seus metabólitos ativos na urina. As formas predominantes das isoflavonas e seus metabólitos na urina e plasma são os conjugados glicurônicos, sulfatados e sulfoglicurônicos. Vários métodos têm sido descritos na literatura para medir isoflavonas e seus metabólitos em fluidos corporais bem como nos alimentos e todos estes métodos requerem digestão ou hidrólise dos conjugados sulfatados e glicurônicos antes da análise, para que haja estudos completos farmacológicos a respeito destes compostos (CIMINO *et al*., 1999).

Um reator contendo sulfatase ácido biliar e 3-β-hidroxiesteróide desidrogenase imobilizadas foi proposto para determinação de ácidos biliares sulfatados na urina de pacientes com doenças do fígado. A amostra de urina é injetada em uma válvula e passa através da coluna contendo as enzimas imobilizadas. Os ácidos biliares sulfatados inicialmente são desulfatados sob ação da sulfatase ácido biliar e

convertidos a ácido biliar 3-β-hidroxiacil o qual reage com 3-β-hidroxiesteróide desidrogenase produzindo NADH que em seguida reage com um corante e é detectado a 550 nm (GAO *et al*., 2001).

Há crescente interesse industrial acerca dos óleos de sementes de crucíferas por serem mais biodegradáveis que os óleos minerais. A utilização desses óleos ao longo dos anos poderia levar a produção de alimentos contendo alto conteúdo de glicosinolatos (se apresentam em alta quantidade nas plantas crucíferas, são compostos tiossacarídicos) que não podem ser diretamente utilizados nos alimentos animais. Técnicas de desintoxicação estão sendo aplicadas nestes alimentos para torná-los acessíveis. Arilsulfatases têm sido amplamente utilizadas para produzir desulfo-glicosinolatos para análise quantitativa de glicosinolatos em extratos de plantas por HPLC. Uma arilsulfatase imobilizada sobre nylon foi capaz de remover todo o sulfato das moléculas produzindo assim desulfo-glicosinolatos, reduzindo muito o tempo de análise (LEONI *et al*., 1998).

1.7. Sulfatases no metabolismo de glicosaminoglicanos

1.7.1. Degradação de heparina e heparam sulfato em mamíferos e outros vertebrados

Em mamíferos e outros vertebrados o estudo da degradação de heparina e heparam sulfato, revelou a ação de várias sulfatases. Estas enzimas funcionam em alternância com as enzimas β-glicuronidase, α-L-iduronidase, α-N-acetilglicosaminidase e acetil-CoA-N-glicosaminiltransferase (KRESSE & GROSSL, 1987; HOPWOOD, 1989; DIETRICH, 1991; NEUFELD & MUENZEN, 1995). Os polímeros foram degradados a partir de seus terminais não redutores. A primeira enzima que atuava sobre estes compostos era uma sulfamidase que removia o

grupamento sulfato da posição C-2 da glicosamina (DIETRICH, 1970). Após esta etapa a enzima acetil-CoA-N-glicosaminiltransferase (KLEIN *et al*., 1978) promove a acetilação do grupo amino livre da hexosamina (Figura 7). Posteriormente, o grupamento sulfato da posição C-6 da N-acetilglicosamina é removido por ação da N-acetilglicosamina-6-sulfato sulfatase (KRESSE *et al*., 1980). Em seguida o resíduo de N-acetilglicosamina era retirado pela ação da α-N-acetilglicosaminidase e o polímero tornava-se então substrato para ação de uma outra sulfatase, a iduronato-2-sulfato sulfatase, que atuava removendo o sulfato do ácido idurônico (BACH *et al*., 1973). Após a hidrólise do ácido urônico pela α-L-iduronidase novo ciclo se iniciava até a completa degradação do polímero (NEUFELD, 1974).

Na maioria das sequências de heparam sulfato aparecem resíduos de ácido glicurônico, e também em alguns segmentos de heparina. A enzima responsável pela hidrólise deste açúcar é a β-glucuronidase (KRESSE & GROSSL, 1987; HOPWOOD, 1989; DIETRICH, 1991). Caso o ácido glicurônico esteja sulfatado na posição C-2, este sulfato será removido pela enzima glicuronato-2-sulfo-hidrolase (SHAKLEE, *et al*., 1985).

A deficiência de algumas enzimas envolvidas no catabolismo de glicosaminoglicanos, leva ao acúmulo de intermediários destes compostos nos tecidos e aumento da sua excreção urinária. Isto pode levar a sérias complicações tais como: retardo mental, problemas visuais, deformações físicas, podendo levar a óbito (MCKUSIC, 1969; NEUFELD & MUENZER, 1995; SUZUKI *et al*., 1997). Os estudos da análise do terminal não redutor de heparam sulfato em pacientes portadores da síndrome de Hunter, Sanfilippo B e Sanfilippo C monstraram a enzima deficiente em cada uma dessas mucopolissacaridoses (TOMA, 1992; TOMA, *et al*., 1996).

1.7.2. Degradação de heparina e heparam sulfato em moluscos

OLIVEIRA *et al*. (1994) relataram a presença de enzimas envolvidas na degradação de heparam sulfato durante o desenvolvimento embrionário do molusco *Pomacea sp*. As enzimas endoglicuronidase, β-glucuronidase e α-N-acetilglicosaminidase atuavam na região N-acetilada do heparam sulfato. Uma endoglicuronidase que agia sobre a região N-acetilada e N-sulfatada do heparam sulfato foi isolada e caracterizada, em ovas de 10 dias de desenvolvimento deste molusco.

ABREU (1994) demonstrou a presença de sulfatases e glucuronidase que agem sobre o polímero de heparam sulfato nos moluscos *Anomalocardia brasiliana* e *Mesodesma donacium*. As enzimas 2-O-sulfoglicuronato sulfatase, 6-O-sulfoglicosaminil sulfatase e uma glicuronidase reconheceram o heparam sulfato intacto e também dissacarídeos insaturados sulfatados produzidos pela ação de enzimas *Flavobacterium heparinum*.

Recentemente, foi relatada no molusco *Tagelus gibbus* a existência de enzimas que degradam heparam sulfato. Inicialmente o composto sofre ação de endo-*N*-glicosidases que o despolimeriza. Em seguida a iduronato-2-sulfatase e glicosaminil-6-sulfatase atuam removendo sulfato da posição C-2 do ácido idurônico e C-6 da glucosamina, respectivamente. As moléculas desulfatadas agora são substratos para a ação da iduronidase (glucuronidase) e *N*-sulfoglicosaminidase (MEDEIROS, *et al*., 1998) (Figura 8).

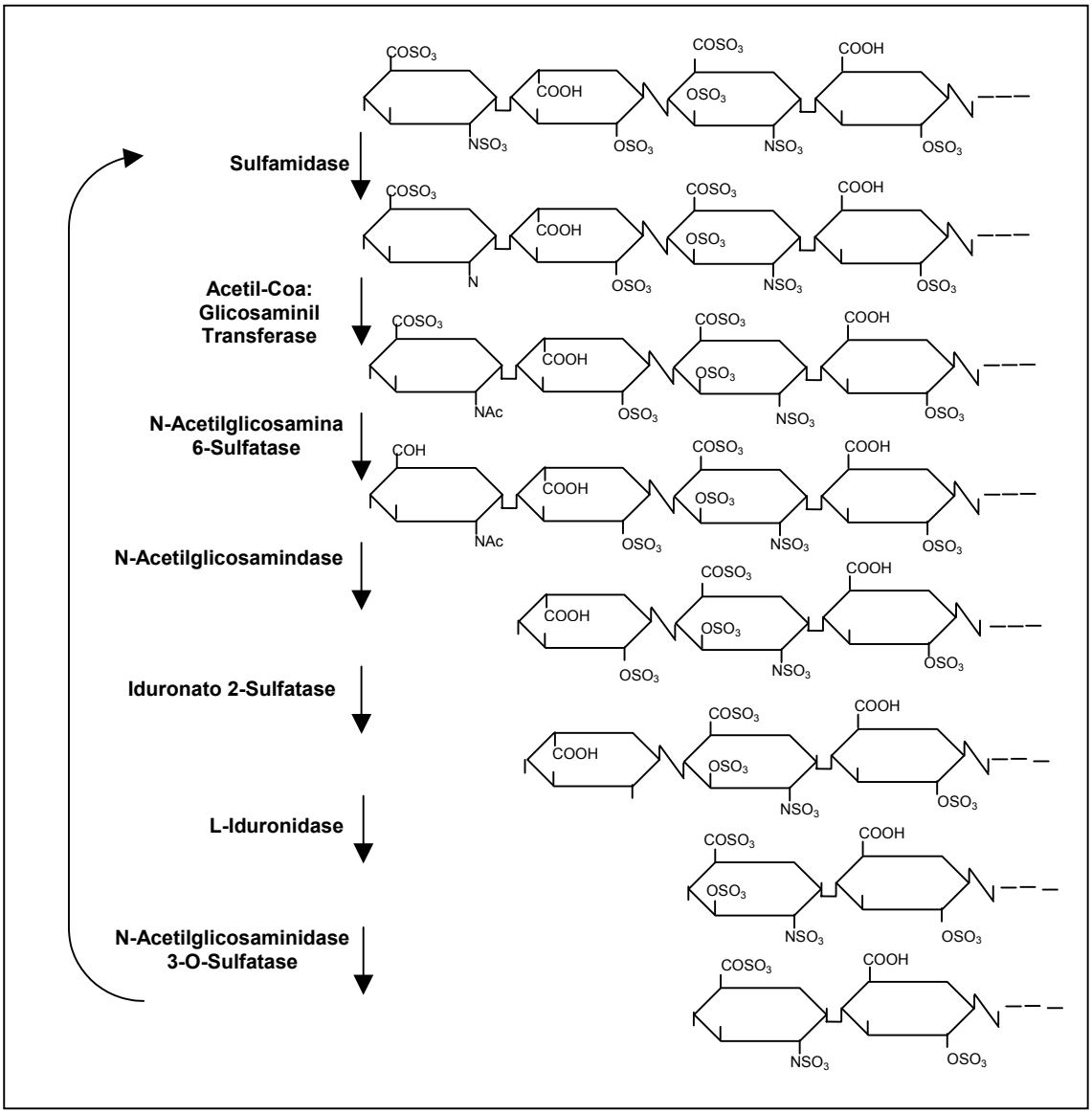

Figura 7: Degradação de heparina e heparam sulfato em mamíferos

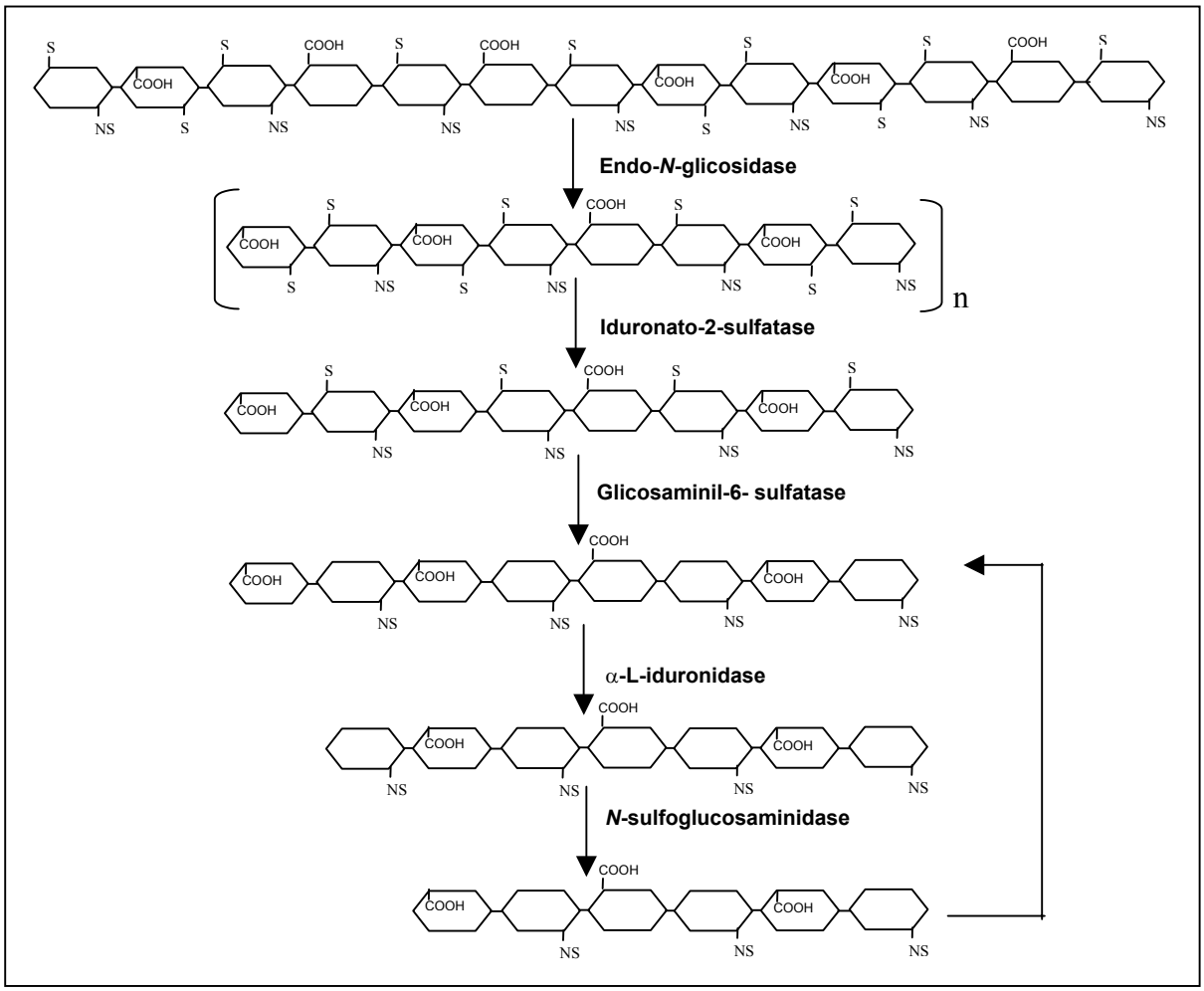

Figura 8: Degradação de heparina e heparam sulfato em moluscos

1.8. Considerações acerca do molusco *Aplysia cervina*

Filo: Mollusca

Classe: Gastrópoda

Sub-Classe: Opisthobranchia

Ordem: Aplysiacea

Gênero: *Aplysia*

Espécie: *Aplysia cervina*

As Aplysias, animais bastante comuns em nosso litoral, são moluscos relativamente grandes de um colorido verde-dourado, marmorado de branco no dorso

e nos lados (figura 9). Esse colorido varia uma pouco de indivíduo para indivíduo. Alguns chegam a 15 ou mesmo 17 cm de comprimento. Ao serem importunados soltam uma tinta roxa que certamente desempenha um papel defensivo, permitindo a camuflagem do animal. A tinta é produzida por glândulas situadas na parte posterior e interna da nadadeira esquerda do animal e, por isto, muitas vezes são conhecidas como “tintureiras” (ROSA, 1973).

Figura 9: *Aplysia cervina*

São animais comumente encontrados próximo às rochas ou do mar, pois são lá deixadas durante as marés altas e, impossibilitadas de voltar à água, aí permanecem, imóveis e encolhidas na areia.

As Aplysias são excelentes nadadoras e o fazem de forma graciosa com movimentos ondulatórios executados por duas largas expansões laterais do corpo. Contraindo e descontraindo o corpo, ela alonga a cabeça provida de expansões labiais e dois pares de tentáculos grandes, o que as fazem parecer-se com as lebres e daí vem seu nome vulgar “lebres do mar”. Sua concha é reduzida e localizada no dorso. A redução da concha nestes animais faz sentido, visto tratar-se de um molusco

nadador (ROSA, 1973).

As Aplysias constituem ótimo material para dissecação e estudo do aparelho digestivo dos moluscos. Apresentam celoma contendo um líquido claro e abundante. Seu aparelho digestivo é amplo e toma boa parte da cavidade do corpo. A boca é localizada na parte anterior e inferior da cabeça, comunicando-se com uma faringe contendo minúsculos dentes, a rádula, que auxilia o animal a cortar fragmentos de algas e de outros alimentos. Em seguida, tem-se um estreito e curto esôfago que se comunica com o papo e este com a moela, que é constituída por uma parede espessa e musculosa apresentando peças resistentes parecidas com cristais de quartzo, servindo como aparelho triturador para reduzir o tamanho dos fragmentos de algas ingeridos. Um estômago vem em continuidade e é comum encontrar pequenos pedaços de algas triturados neste local. Pouco adiante se encontra um intestino que ao final enrola-se com o fígado do animal que é uma grande massa cinzenta-esverdeada, enrolada em espiral. Na parte posterior do fígado encontra-se a glândula hermafrodita ou ovotestis. A descrição do aparelho reprodutor das Aplysias mostra que elas são hermafroditas (ROSA, 1973).

2. Justificativa

Os glicosaminoglicanos são polissacarídeos amplamente distribuídos em invertebrados e vertebrados. Suas variadas funções estão intimamente relacionadas à sua estrutura, mais precisamente à presença de grupos sulfatados especificamente distribuídos em determinadas posições. Na literatura há relatos da importância de um pentassacarídeo, existente na heparina, contendo glicosamina 3-*O*-sulfatadas essenciais para a ligação específica com a antitrombina III, interação esta necessária para a sua função como anticoagulante. Grupos 3-*O*-sulfatados no heparam sulfato também são importantes para a interação com um fator de crescimento dos fibroblastos (FGF-7). Em contraste a interação de FGF-1 com a heparina é mediada através de contato com grupos 6-*O* sulfatados (CAPILA & LINHARDT, 2002).

Há, atualmente, grande interesse relacionado à extração e purificação de enzimas que atuam sobre glicosaminoglicanos devido à necessidade de caracterizar quimicamente estes compostos originados de diferentes fontes e, que apresentam funções diversas ou ainda, produzir oligossacarídeos modificados quimicamente com a intenção de estudar as interações de determinadas regiões destas moléculas com proteínas específicas.

Na literatura não existem relatos da imobilização de sulfatases voltadas para a modificação química de polissacarídeos sulfatados. Por isso, neste trabalho imobilizou-se a sulfatase extraída do molusco marinho *Aplysia cervina*, com vistas ao estudo das características do derivado insolúvel obtido e à modificação química de polissacarídeos sulfatados e/ou moléculas relacionadas.

3. Objetivos

- ❖ Extrair, purificar e estabelecer algumas propriedades físico-químicas e cinéticas da sulfatase presente no fígado do molusco *Aplysia cervina*;
- ❖ Imobilizar a sulfatase purificada do molusco *A. cervina* em Polietilenotereftalato (Dacron-PET) e no híbrido Polisiloxano/Álcool polivinílico (POS/PVA) ferromagnéticos;
- ❖ Estabelecer algumas propriedades físico-químicas e cinéticas dos derivados enzimáticos insolúveis sintetizados;
- ❖ Comparar as propriedades obtidas nos derivados insolúveis com as obtidas para a enzima solúvel;

4. Referências

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CAPÍTULO I

[#]SULFATASE FROM MOLLUSC *Aplysia cervina* LIVER: ISOLATION, PHYSICOCHEMICAL AND KINETICS PARAMETERS

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Abstract

Heparin specific sulfatase was identified in the liver of the mollusc *Aplysia cervina*, widely found in the Northeastern Brazilian coast. This enzyme was purified by ammonium sulfate and acetone precipitations and by affinity chromatography using Heparin-Sepharose CL-6B. Some physical chemical and kinetics properties of this 89.7-fold purified preparation (5.37% yield) were investigated using p-nitrophenyl sulfate (pNPS) as substrate. The optima pH and temperature were found to be 5.0 and 45^{o}C, respectively. It retained more than 90% of its activity when incubated for 15 min at 45ºC while lost 60% at 55ºC. K_m of 3.71 ± 0.41 mM was found. Its activity was enhanced by $MgCl_2$, $CaCl_2$ and $FeCl_2$ and inhibited by $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$, $HgCl_2$, Na_2HPO_4 and NaH_2PO_4. The heparin low molecular weight competed with pNPS for the active site enzyme more than the high molecular one.

Keywords: sulfatase, *Aplysia cervina*, mollusc, kinetics

Introduction

Glycosaminoglycans (GAGs) are polymers of repeating disaccharides units that are usually O and/or N-sulfated. The most common GAGs are chondroitin sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid, heparan sulfate and heparin (Cechowska-Pasko *et al.*, 2002). They perform a wide range of biological functions. Heparin has the highest negative charge density of any known biological macromolecule. This is the result of its high content of sulfo and carboxyl groups. Heparin biological activities have been discovered to be regulated by the interaction of heparin with heparin-binding proteins. The specificity of its interactions with a diverse range of biologically important proteins suggests that it displays its sulfo and carboxyl groups in defined patterns and orientations to promote specific protein interactions (Capila and Linhardt, 2002).

Different enzymatic pathways of glycosaminoglycans degradation have been so far described. In mammals and others vertebrates the heparan sulfate and heparin are initially degraded to fragments of low molecular weight by endoglycosidases. These fragments are then degraded from the non-reducing end by the sequential action of *N*-sulfatase, *N*-acetyl Co-A-α-glucosamine acetyltransferase, *N*-acetyl glucosamine 6-sulfatase, α-*N*-acetylglucosaminidase, α-*L*-iduronate sulfatase, α-*L*-iduronidase and/or β-glucuronidase. This cycle is repeated in this sequence until the total degradation of the fragment (Neufeld, 1974, Glaser and Conrad 1979, Yanigishita and Hascall, 1992). In mollusc *Tagelus gibbus* heparin and heparan sulfate are depolymerized by the action of endo-*N*-glycosidases. The fragments are then *O*-desulfated by the action of an iduronate sulfatase and possibly a glucosaminil 6-sulfatase. These fragments are degraded by a *N*-sulfoglucosaminidase and glycuronidases to glucosamine *N*-sulfate, iduronic acid and glucuronic acid (Medeiros *et al.*, 1998). The preparation of modified oligosaccharides utilizing sulfatases and other enzymes changes the interaction of the glycosaminoglycans with specific proteins and thus theirs properties.

This paper reports the purification of sulfatase (EC 3.1.6.1) from the liver of the mollusc *Aplysia cervina* (Dall and Simpson, 1991) and some physicochemical and kinetics characteristics.

Materials and Methods

p-Nitrophenyl sulfate (pNPS) and Heparin Sepharose CL-6B were purchased from

Sigma (St. Louis, MO, USA) and Amersham Biosciences (Uppsala, Sweden), respectively. High (Liquemine, MW 15000 according Catani et al., 2001) and low (Clexane, MW 4500) molecular weight heparin were acquired from Roche Químicos e Farmacêuticos S.A. (Rio de Janeiro/RJ) and Rhodia Farma LTDA (São Paulo/SP), respectively.

Enzyme purification

The molluscs (*A. cervina*) were collected in Maracaípe beach, Pernambuco State, Brazil, and kept frozen at -20^{0}C until their utilization. Their livers were dissected and homogenized in 1:2, w/v, 0.1 M sodium acetate buffer, pH 5.0 and centrifuged for 30 min at 27.000 *x g*. To the supernatant, ammonium sulfate was slowly added up to 50% saturation and centrifugation was carried out at 27.000 *x g* for 15 min. The precipitate was discarded and ammonium sulfate was added to the supernatant up to 80% saturation and again centrifuged at 27.000*x g* for 15 min. The precipitate (50-80%) was suspended in 0.1 M sodium acetate, pH 5.0 and dialyzed against the same buffer overnight with four buffer changes. To the dialyzed preparation (4^{0}C), 30% of ice-cold acetone was added under gentle agitation. The mixture was reposed at 4^{o}C for 24 h and centrifugation (27.000 *x g* for 15 min) was carried out, the precipitate was discarded and to the supernatant 50% of acetone was added. This mixture was again kept and centrifuged as above described. Further acetone addition procedure was similarly performed for 70% and 90% volumes. The precipitate of the 90% acetone volume was dried under vacuum and suspended in 0.1 M sodium acetate buffer, pH 5.0. Finally, this fraction was applied to an affinity column (1.7 x 8 cm) of Heparin-Sepharose CL-6B pre-equilibrated with 0.1 M sodium acetate buffer, pH 5.0, and eluted with the same buffer, at a flow-rate of 0.33 mL/min until no absorbance at 280 nm be detected. Then, the enzyme was eluted from column with 0.1 M sodium acetate buffer, pH 5.0, containing 0.2 and 0.4 M NaCl (the fractions obtained were denominated according to the salt content). The fractions were pooled and dialyzed against 0.1 M sodium acetate buffer, pH 5.0, and its activity established as described below. All purification procedure was carried out at 4°C.

Determination of sulfatase activity and protein content.

The enzymatic preparation (50 µl) was incubated with pNPS (5 µl of 60 mM

solution) and 0.1 M sodium acetate buffer pH 5.0 (45 µl) at 45^0C for 30 min. The reaction was stopped by addition of 1 mL of 0.25 M NaOH and the p-nitrophenol formed measured at 405 nm (Hitachi 3200 spectrophotometer). One enzymatic unit was defined as the amount of enzyme capable to hydrolyze 1 µmol of pNPS per min (ε = 18.3 x 10^3 $M^{-1}.cm^{-1}$). Protein content was established according to Sedmak and Grossberg (1977). The kinetic constants, K_m and V_{max}, were calculated from the initial velocities and Lineweaver-Burk reciprocal plots.

pH, temperature and activation/inhibition effects.

Optimum pH value was determined over the pH range 3.7-6.5 using the following buffers (1mM): sodium-acetate buffer (pH 3.7-5.5) and bis-tris buffer (pH 5.5-6.5).

Optimum temperature assay for enzyme activity was measured at pH 5.0 by the standard method at temperatures ranging from 25 to 75°C. Thermal stability was studied by incubating an enzyme preparation at pH 5.0 for 15 min at temperatures varying from 25 to 100°C. The enzyme solution was transferred to room temperature for about 15 min and them the sulfatase activity was established at 45°C using the method described above.

Metal ions influence on the sulfatases activity was studied by incubating the enzyme preparation previously with 1 mM of the respective salts ($MgCl_2$, $CaCl_2$, $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$ (sodium citrate), $HgCl_2$, Na_2HPO_4, NaH_2PO_4, $FeCl_3$, $FeCl_2$ and NaCl) at 45°C for 15 min and assaying sulfatase activity as above described. The effect of EDTA, chelating agent, was also investigated.

Finally, an enzymatic preparation (50 µl) was incubated with high and low molecular weight heparin (4.5 mg) and pNPS (5 µl of 60 mM solution) in buffer (final volume of 100 µl) for 30 min at 45^oC. The reaction was stopped by adding 1 mL of 0.25 M NaOH and the formed p-nitrophenol was spectrophotometrically determined at 405 nm.

Results and Discussion

The sulfatase from *A. cervina* liver was purified 89.7-fold (yield of 5.37%) from crude extract by the sequential use of ammonium sulfate and acetone precipitations followed by Heparin-Sepharose CL-6B column chromatography (Figure 1 and Table 1). The elution profile showed the elution of two peaks of protein. The peak eluted with 0.2 M of NaCl presented higher specific activity and thus was further used for physicochemical and kinetics characterization. The second peak (0.4 M) has already been purified (31.8-fold, yield of 0.23%) and kinetically characterized (Matta, 2001). Heparin-Sepharose chromatography has been successfully used for the separation of lysosomal hydrolases, hyaluronidase and *N*-sulfatase (Farooqui, 1980).

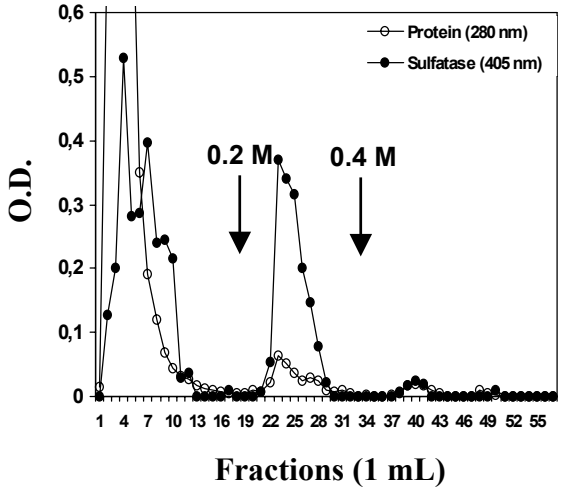

Figure 1 - Affinity chromatography on Heparin-Sepharose CL-6B. Chromatography was performed using a column (1.7 x 8 cm) pre-equilibrated with 0.1 M sodium acetate buffer, pH 5.0 and eluted with the same buffer, at a flow-rate of 0.33 mL/min. The enzyme was eluted from column with 0.1 M sodium acetate buffer, pH 5.0, containing 0.2 and 0.4 M NaCl.

Table 1 – Purification of sulfatase from *A. cervina* liver

Preparation	Total protein (mg)	Total activity (Unit)	Specific activity (Units/mg)	Purification (Fold)	Yield (%)
Crude extract	621.5	4599.1	7	1	100.00
Ammonium sulfate F-50-80 %	166.4	2662.4	16	2.2	57.89
Acetone F-0.9 v	15.2	380.0	25	3.4	8.26
0.2 M Heparin-Sepharose Cl-6B	0.374	246,8	660	89.7	5.37

One unit of enzyme was defined as the amount capable to hydrolyze one µmole of pNPS per min under the experimental conditions.

Previously, an arylsulfatase from *Helix pomatia* was purified 40-fold by tyrosine vanadate. The *N*-acetyl-*L*-tyrosine ethyl ester is rapidly esterified in the presence of vanadate to yield an arylvanadate. The strong inhibition of arylsulfatase by this complex could be used as an affinity matrix to purify arylsulfatase (Skorey, *et al.*, 1999).

In respect to the kinetic parameters, the K_m of this enzyme for pNPS was determined to be 3.71 ± 0.41 mM, which is higher than that (0.86 mM) estimated by Matta (2001) for the second peak, namely, Fraction 0.4 M. This result is consistent with the fact that this enzyme was eluted by a lower concentration of salt (0.2 M NaCl), indicating lower affinity for the heparin matrix when compared to the fraction eluted by 0.4 M NaCl.

pH profile is showed in Figure 2. An optimum pH of 5.0 was found for the *A. cervina* liver sulfatase. Others sulfatases presented the same optimum pH, such as the sulfatase from marine echinoderm *Holothuria polii* lysosomal (Canicatti, 1988), from mollusc *Anomalocardia brasiliana* (Sousa-Filho *et al.*, 1990), *Tagelus gibbus* (Medeiros *et al.*, 1998) and arilsulfatases A and B from mammals referred to as acid lysosomal hydrolases (Tobacman, 2002).

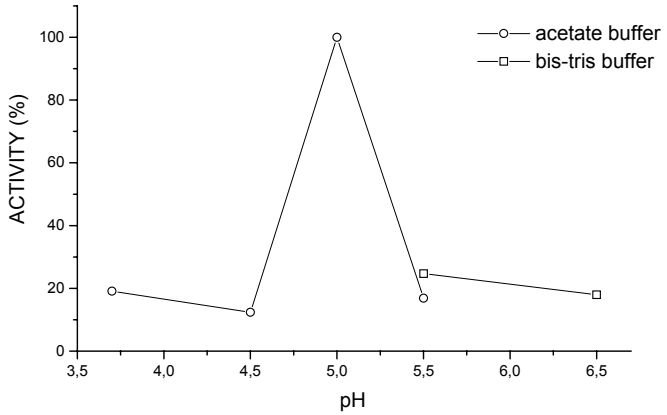

Figure 2 – Effect of pH on sulfatase from *A. cervina* liver. The activity was determined over the pH range 3.7-6.5 using the following buffers (1mM): Sodium-acetate buffer (pH 3.7-5.5) and Bis-Tris buffer (pH 5.5-6.5). Results shown are given as the relative activity in comparison to the observed maximum activity at the optimal pH for the hydrolysis of pNPS.

The optimum temperature for the sulfatase activity was 45^{o}C (Figure 3). In the thermal stability assays the enzyme retained more than 90% of its activity after 15 min of incubation at 45^{o}C whereas its activity decreased 60% at 55ºC (Figure 4). It is worthwhile to notice that the enzyme collected in the Fraction 0.4 M of the Heparin-Sepharose CL-6B column chromatography was completely inactivated after 30 min incubation at 50^{o}C (Matta, 2001).

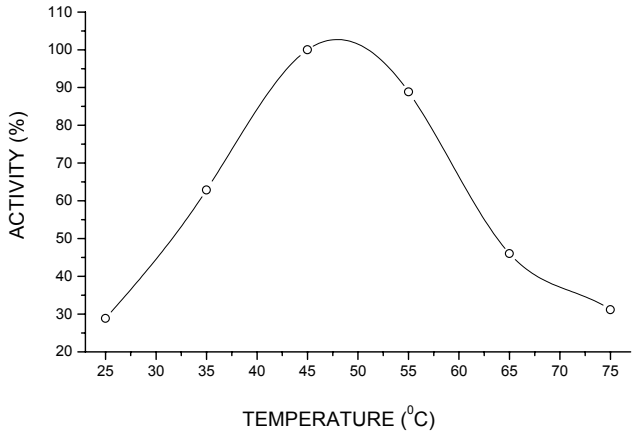

Figure 3 – Effect of the temperature on sulfatase activity. The enzyme activities were measured at pH 5.0 by the standard assays at various temperatures over the range 25-75^{o}C. Results shown are given as the relative activity in comparison to the observed maximum activity at the optimal temperature for the hydrolysis of pNPS.

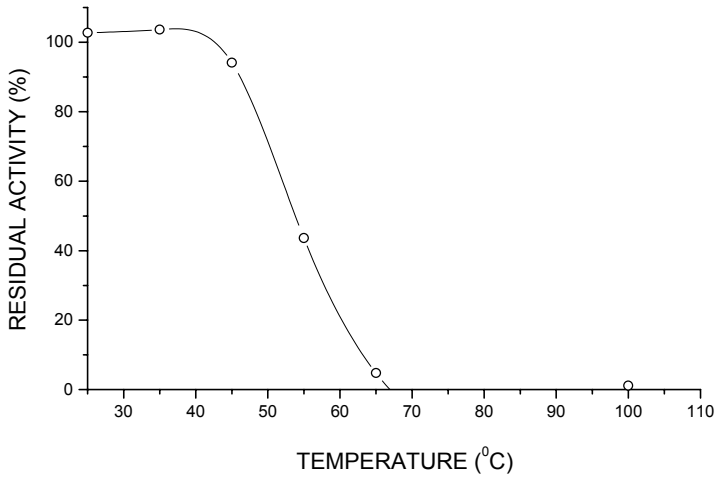

Figure 4 – Thermal stability of the sulfatase from *A. cervina* liver. Thermal stability was studied by incubating the enzyme at pH 5.0, for 15 min, at temperatures varying from 25 to 100°C. After resting for 15 min (room temperature equilibration), its activity was assayed at 45°C.

Effects of metal ions and EDTA are depicted in Figure 5. Among the salts tested, $MgCl_2$, $CaCl_2$ and $FeCl_2$ had stimulating effects on the activity. Arylsulfatase B structure has been determined and showed that residues conserved among the sulfatase family are involved with stabilization of a calcium ion and positioning of the sulfate ester in the active site (Tobacman, 2002). $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$, $HgCl_2$, Na_2HPO_4 and NaH_2PO_4 inhibited the *A. cervina* sulfatase. The addition of EDTA, $FeCl_3$ and NaCl had no significant effect in the enzyme activity. KCl, Na_2SO_4, $HgCl_2$ and NaH_2PO_4 inhibited a sulfatase from *Anomalocardia brasiliana* (Sousa-Filho *et al.*, 1990) while Na_2HPO_4 and NaH_2PO_4 inhibited 100% of sulfatase activity from *A. cervina* purified by the same chromatography system (Matta, 2001). Lysosomal arylsulfatases from mammals are inhibited strongly by phosphate ions as well (Allen and Roy, 1968).

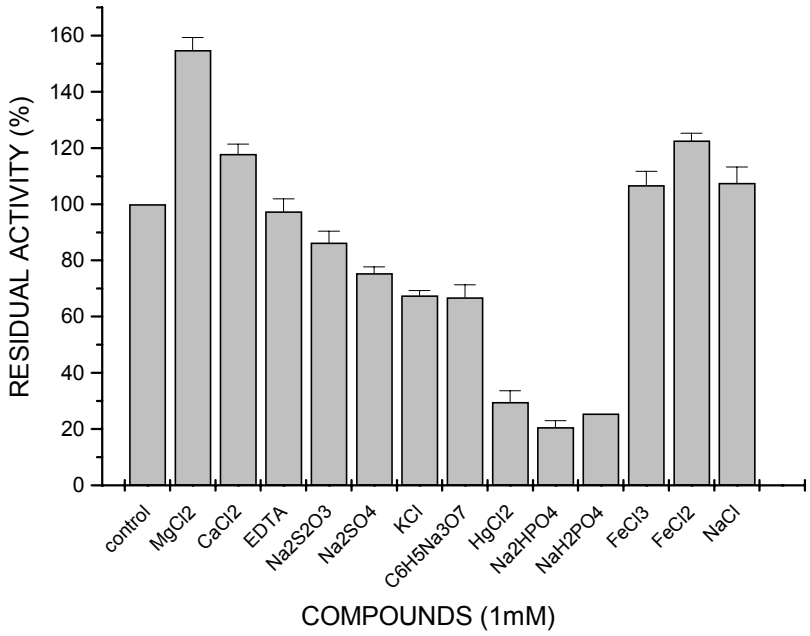

Figure 5 – Effects of metal ions and EDTA on sulfatase from *A. cervina* liver. These experiments were accomplished by previous incubation of the enzyme with 1 mM of the respective salts ($MgCl_2$, $CaCl_2$, $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$ (sodium citrate), $HgCl_2$, Na_2HPO_4, NaH_2PO_4, $FeCl_3$, $FeCl_2$ and NaCl) at 45°C for 15 min and assaying their activities as described in methods.

Results obtained by competition experiments are shown in Figure 6. Heparin of low molecular weight competed more with the pNPS for the enzyme active site. This result indicates that the enzyme binds with certain easiness to the heparin of low molecular weight; perhaps there would be some steric hindrance towards the binding with the heparin of high molecular weight. It is important to notice that the sulfatase employed in this work was purified by Heparin Sepharose CL-6B affinity chromatography. This last result indicates *A. cervina* sulfatase can be used in many applications to remove sulfate groups from glycosaminoglycans, sulfated bile acids, drugs, and to produce desulfo-glucosinolates from plant compounds for animal feed. Immobilization of these enzymes can be proposed in order to facilitate the optimization of the processes of desulfatation of molecules.

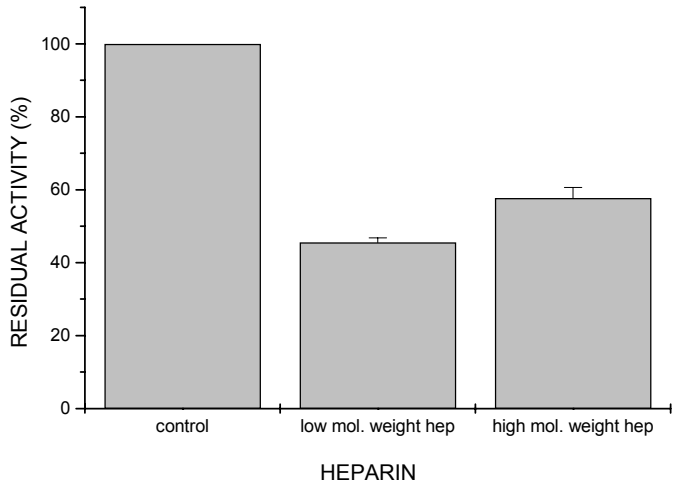

Figure 6 – Effect of low and high molecular weight heparin on the activity of the sulfatase from *A. cervina* liver. The enzyme (50 µl) was incubated with high and low molecular weight heparin (4.5 mg) and pNPS (5 µl of 60 mM solution) in buffer (final volume of 100 µl) for 30 min at 45°C.

Conclusions

A sulfatase from *A. cervina* was purified 89.7-fold (5.37% yield) by Heparin-Sepharose CL-6B. The optimal conditions for hydrolysis of the pNPS were at pH 5.0 and 45°C. $MgCl_2$, $CaCl_2$, and $FeCl_2$ activated the enzyme while Na_2HPO_4 and NaH_2PO_4 inhibited it strongly. K_m of 3.71 ± 0.41 mM was found. The heparin low molecular weight competed more with p-NPS for the active site enzyme than the high molecular weight one.

Acknowledgments

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CAPÍTULO II

[#]IMMOBILIZATION OF SULFATASE FROM *Aplysia cervina* LIVER ON FERROMAGNETIC POLYSILOXANE/POLYVINYL ALCOHOL

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Abstract

Sulfatase (EC 3.1.6.1) from the liver of *Aplysia cervina* was covalently immobilized on magnetic particles containing a semi-interpenetrated network of polysiloxane and polyvinyl alcohol (POS/PVA). This enzymatic preparation presented 1.85 units/mg protein and 21.23% of the soluble enzyme activity and was easily removed from the reaction mixture by a magnetic field. It was reused at least 7 times without loss in its activity and remained 50% active after 2 months stored at 4^{o}C in buffer. It was more thermal stable than the soluble enzyme and presented the same apparent K_m and optima pH and temperature. $MgCl_2$, $CaCl_2$, KCl, $FeCl_2$, $FeCl_3$, EDTA and NaCl activated the sulfatase-magnetic POS/PVA whereas Na_2HPO_4 and NaH_2PO_4 and heparin inhibited it.

Key words: Sulfatase, immobilization, ferromagnetic POS/PVA, glycosaminoglycans

Introduction

Enzymes have a wide variety of biotechnological, biomedical, and pharmaceutical applications. They are used as biosensors, in bioengineering, clinically as therapeutic agents, in modern diagnostics, and as catalysts for chemical or biochemical reactions. Sulfatases (EC 3.1.6.1) are hydrolytic enzymes that remove sulfate groups from all the different classes of sulfate ester.

Several techniques have been studied and utilized to optimize the various enzyme applications and therefore researchers have explored their immobilization on water insoluble supports (Betigeri and Neau, 2002). Sulfatases have been immobilized for different purposes such as tools for determination of sulfated bile acids in urine (Gao et al., 2001), for production of desulfo-glucosinolates (Leoni et al., 1998) and for desulfation of drug conjugates to increased and extend the detectability of the corresponding substances (Toenes and Mourer,1999; Boppana et al, 1989).

In our laboratory, glass beads composed of the semi-interpenetrated network of polysiloxane (POS) and polyvinyl alcohol (PVA) have been used as support to covalently immobilize antigen and used in enzyme-linked immunosorbent assays (ELISA) for diagnosis of plague and detection of human anti-*Toxocara canis* IgG (Barros et al., 2002; Coelho et al., 2003).

Glycosaminoglycans (GAG) are polymers of repeating disaccharides units that are usually O an/or N-sulfated. The most common GAG structures are chondroitin sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid, heparan sulfate and heparin (Hep) (Cechowska et al., 2002). They perform a wide range of biological functions. Heparin has the highest negative charge density of any know biological macromolecule. This is the result of its high content of negatively charged sulfo and carboxyl groups. Heparin biological activities have been discovered to be regulated by

the interaction of heparin with heparin-binding proteins. The specificity of its interactions with a diverse range of biologically important proteins suggest that is displays its sulfo and carboxyl groups in defined patterns and orientations to promote specific protein interactions (Capila and Linhardt, 2002). The preparation of modified oligosaccharides utilizing sulfatases and other enzymes would change the interaction of heparin with specific proteins and thus theirs properties.

This paper describes the immobilization of a sulfatase extracted from mollusc *Aplysia cervina* onto particles of ferromagnetic POS/PVA. Also, presents some of its physicochemical properties and kinetic parameters.

Materials and Methods

pNPS (p-Nitrophenyl sulfate; Sigma, St. Louis, MO), TEOS (tetraetylortosilicate; Merck), Heparin Sepharose CL-6B (Amesham Biosciences; Uppsala, Sweden), PVA (polyvinyl alcohol; MW 72000, Reagen). High (Liquemine, MW 15000 according Catani et al., 2001) and low (Clexane, MW 4500) molecular weight heparin were acquired from Roche Químicos e Farmacêuticos S.A. (Rio de Janeiro/RJ) and Rhodia Farma LTDA (São Paulo/SP), respectively.

Enzyme preparation.

The molluscs (*Aplysia cervina*) were collected in Maracaípe beach, Pernambuco State, Brazil, and kept frozen at -20^{0}C until their utilization. Their livers were dissected and homogenized in 1:2 (w/v), 0.1 M sodium acetate buffer, pH 5.0 (referred to as buffer A throughout) and centrifuged for 30 min at 27.000 x g. Ammonium sulfate was slowly added to the supernatant up to 50% saturation and centrifugation was carried out at 27.000 x g for 15 min. The precipitate was discharged and ammonium sulfate was added to the supernatant up to 80% saturation and again

centrifuged at 27.000 x g for 15 min. The precipitate (50-80%) was suspended in buffer A and dialyzed against the same buffer overnight with four buffer changes. To the dialyzed preparation held at 4^0C, 30% of ice-cold acetone was added under gentle agitation. After this step, the mixture was incubated for 24 h at 4^oC, and centrifugation (27.000 x g/15 min) was carried out. The precipitate was discarded and to the supernatant 50% of acetone was added. This mixture was again kept and centrifuged as described above. Further acetone addition procedure was similarly performed for 70% and 90% volumes. Finally, the precipitate of the 90% acetone volume was dried under vacuum and suspended in buffer A. This preparation was applied on a Heparin-Sepharose CL-6B column (1.7 x 8 cm) and unbound molecules were eluted utilizing buffer A at a flow-rate of 0.33 mL/min until no absorbance at 280 nm was detected. Then, the sulfatases bound were collected eluting the column with buffer A containing 0.2 M NaCl.

Solid support synthesis.

POS/PVA beads were synthesized according to Barros et al. (2002). Briefly, 6 mL of 2% w/v polyvinyl alcohol, 5 mL of ethanol (Merck) and 5 mL of tetraethylortosilicate were mixed in a beaker. After raising the temperature to 60ºC, under stirring, 100 μl of 1 M HCl were added and incubation lasted for 50 min. Then the solution was distributed in microplates (30 μl/well) and allowed to solidify for about 24 h. the beads were triturated until a powder was obtained.

Ferromagnetic POS/PVA powder synthesis.

The POS/PVA powder was stirred in deionized water (100 mL) and an aqueous solution (10 mL, v/v) containing $FeCl_3.6H_2O$ (1.1 M) and $FeCl_2.4H_2O$ (0.6 M) was

added dropwisely. Under vigorous stirring, the pH mixture was adjusted to 10 by addition of 28% v/v ammonium hydroxide solution and then incubated at 100ºC with stirring for 30 min. Then, the magnetic POS/PVA was washed with deionized water (twice) by using a magnetic filed (6000 Oe). Particles measuring lesser than 250 µm were selected by using an adequate sieve and dried until use.

Ferromagnetic POS/PVA powder activation with glutaraldehyde.

Ferromagnetic POS/PVA (10 mg, size <250 µm) was incubated with 2.0% w/v glutaraldehyde diluted in buffer A (1 mL) for 2 h at 25ºC, with mild stirring. Afterwards, the activated particles were washed with buffer A (1 mL, 10 times) using a magnetic field (6000 Oe) and kept in buffer A at 4ºC until use.

Immobilization of sulfatase on ferromagnetic POS/PVA powder.

The activated support was incubated with 1 mL of the sulfatase solution (30 µg) for 16h at 4^{o}C, under rotator agitation (20 rpm). Then the sulfatase-magnetic POS/PVA was collected under magnetic field (6000 Oe) and the supernatants used for protein determination according to Sedmak and Grossberg (1977). This sulfatase-magnetic POS/PVA was washed with buffer A (1 mL, 10 times) until no protein was detected in the supernatant. Afterwards, the sulfatase-magnetic POS/PVA was incubated with 1 M glycine solution (1 mL) for 16h at 4^{o}C, under rotator agitation (20 rpm) and washed 10 times with sodium acetate buffer.

Determination of sulfatase activity and protein content.

Sulfatase (50 µl) was incubated with pNPS (5 µl of 60 mM solution) and buffer A (45 µl) at 45^{0}C by 15 min. The reaction was stopped by addition of 1 mL of 0.25 M

NaOH and the p-nitrophenol formed measured at 405 nm (Hitachi 3200 spectrophotometer). The sulfatase-magnetic POS/PVA (10 mg) was assayed by adding it to 100 µl of the pNPS (3 mM) prepared in buffer A, at 45^0C for 15 min under stirring. After this time the immobilized enzyme was precipitated by a magnetic field (6000 Oe) for 10 s. The supernatant was added to 1 mL of 0.25 M NaOH and the p-Nitrophenol spectrophotometrically determined at 405 nm. Protein content was established according to Sedmak and Grossberg (1977). The amount of immobilized sulfatase was calculated by the difference between the offered protein and that presents in the washing supernatants. One enzymatic unit was defined as the amount of enzyme capable to hydrolyze 1 µmol of pNPS per min (ε = 18.3 x 10^3 $M^{-1}.cm^{-1}$). The kinetic constants, K_m and V_{max}, for the soluble and immobilized sulfatase were calculated from the initial velocities of Lineweaver-Burk reciprocal plots. The reaction was carried out at 45°C. An immobilized sulfatase sample had its activity assayed every time during eleven successive recoveries under magnetic field (reproducibility of reuse) and another one was assayed at times interval during two months of storage at 4°C in buffer (shelf life).

pH, temperature and activation/inhibition effects.

The optimum pH values for the soluble and sulfatase-magnetic POS/PVA activities were determined over the pH range 3.7-6.5 using the following buffers (1mM): Sodium-acetate buffer (pH 3.7-5.5) and bis-tris buffer (pH 5.5-6.5).

The optimum temperature values for the soluble and immobilized sulfatase activities were measured at pH 5.0 by the standard assays at various temperatures over the range 25-75°C. The thermal stability of enzymatic preparations were studied by incubating samples of the soluble and immobilized sulfatase, at pH 5.0, for 15 min

at temperatures varying from 25 to 100°C. After resting for 15 min (room temperature equilibration), their activities were assayed at 45°C using as above described.

The metal ions influence on the soluble and immobilized sulfatase was studied by their previous incubation with 1 mM of the respective salts ($MgCl_2$, $CaCl_2$, $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$, $HgCl_2$, Na_2HPO_4, NaH_2PO_4, $FeCl_3$, $FeCl_2$ and NaCl) at 45°C for 15 min and assaying their activities as above described. The effect of EDTA, chelating agent, was also included.

The soluble (50 µl) and immobilized (10 mg) sulfatase was incubated with high and low molecular weight heparin (4.5 mg) and pNPS in buffer (final volume of 100 µl) for 15 min at 45° C. The reaction was stopped by adding 1 mL of 0.25 M NaOH and the formed p-nitrophenol was spectrophotometrically determined at 405 nm either to the reaction mixture (soluble enzyme) or the mixture supernatant (immobilized enzyme).

Results and Discussion

In this paper, an active insoluble water derivative of sulfatase was synthesized by immobilizing the enzyme from *A. cervina* liver onto magnetic particles of a semi-interpenetrated network of POS/PVA. This preparation presented a specific activity of 1.85 units/mg protein and retained 21.23% of the soluble enzyme activity. Moreover, it was reused seven times without loss of activity (97.96% ± 2.84%; mean ± standard deviation). The immobilized enzyme lost about half (48.20% ± 1.97; mean ± standard deviation) of this initial activity after two months stored at 4°C in buffer (Figure 1). Immobilized sulfatase from *Helix pomatia* on aminopropyl control pore glass retained most of its activity (> 90 %) when stored at 4°C for 6 months (Boppana et al., 1989).

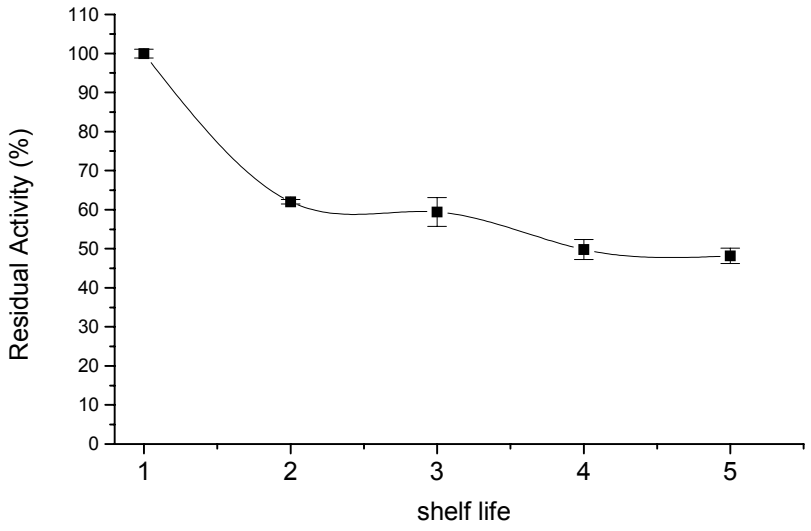

Figure 1 – Activity time course of the immobilized *A. cervina* liver sulfatase stored at 4°C and in buffer. An immobilized sulfatase sample had its activity assayed at times interval during two months of storage at 4°C in buffer (shelf life). Results shown are given as the relative activity in comparison to the observed initial activity. 1 (9/18/03), 2 (10/14/03), 3 (10/31/03), 4 (11/13/03), 5 (11/25/03).

The K_m values estimated for the soluble (3.7 ± 0.4 mM) and immobilized (3.7 ± 0.8 mM) sulfatase acting on pNPS did not show statistical difference. This results is different from that obtained for immobilized arylsulsulfatase on Concanavalin A where the constant increased by approximately 4-fold after immobilization (Farooqui and Srivastava, 1981). Usually immobilized enzymes present K_m values increased in relation to the soluble enzyme due to consequential conformational changes undergone by the enzyme molecule and or to the difficulty of diffusion of the substrate towards the active site of the enzyme.

The effects of pH on soluble and immobilized sulfatase are shows in Figure 2. These results showed that optimum enzyme activity for both the soluble and immobilized enzyme was equal to pH 5.0. The close similarity of pH profiles for both enzymes may show that no important conformational change occur upon immobilization of the enzyme, although the immobilized enzyme was less sensitive to

a slight change in the pH. From this result one can infer that this derivative may be more stable to pH changes. In spite of this pH stability experiment should be performed before this conclusion. This sulfatase present then similar comportment with arilsulfatases A and B from mammals has been referred to as acid hydrolases, due to this functionality at acid pH (Tobacman, 2002).

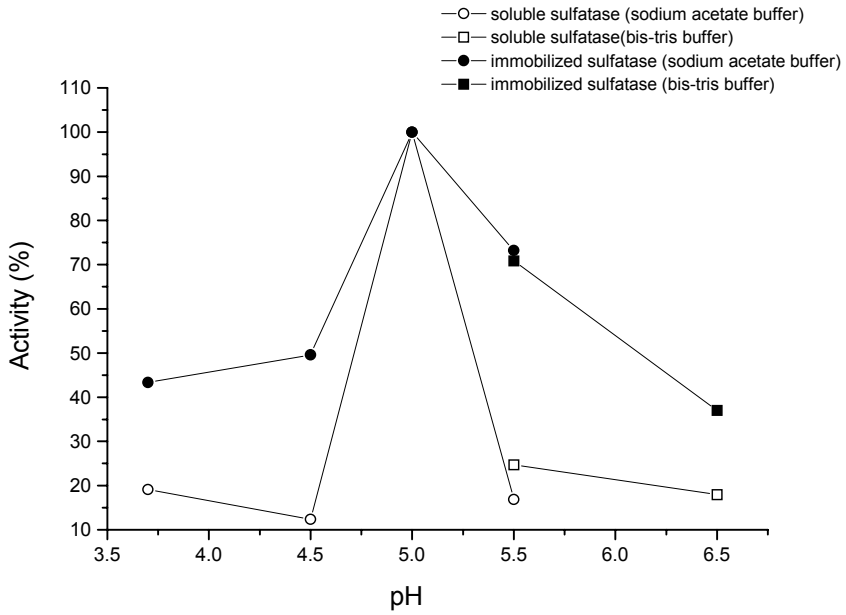

Figure 2 – Effects of pH on the soluble and immobilized sulfatase from *A. cervina* liver. The sulfatase activities were determined over the pH range 3.7-6.5 using the following buffers (1mM): Sodium-acetate buffer (pH 3.7-5.5) and Bis-Tris buffer (pH 5.5-6.5). Results shown are given as the relative activity in comparison to the observed maximum activity at the optimal pH for the hydrolysis of pNPS.

The effect of temperature over the range of 25-75ºC was investigated. Enzymatic activity increased with temperature reaching a clear maximum value at 45ºC and 55ºC for soluble and immobilized sulfatase respectively (Figure 3).

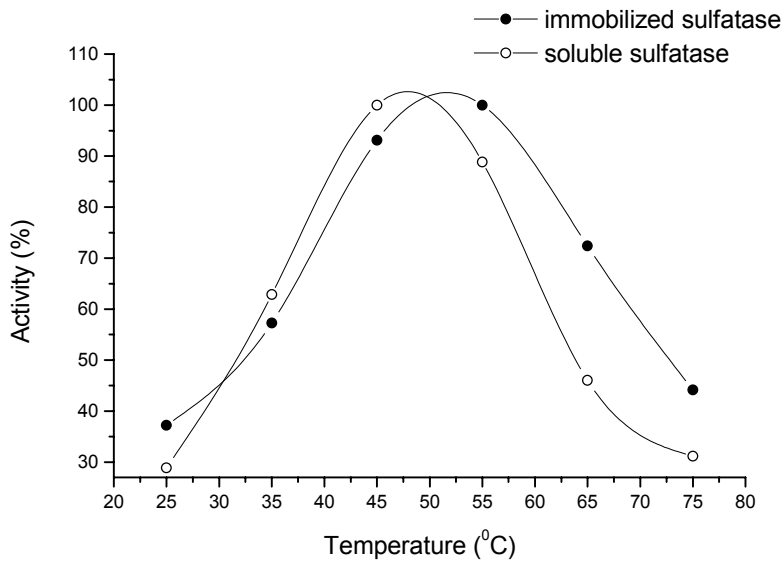

Figure 3 – Effects of the temperature on the soluble and immobilized sulfatase activities. The activities were measured at pH 5.0 by the standard assays at indicated temperatures. Results shown are given as the relative activity in comparison to the observed maximum activity at the optimal temperature for the hydrolysis of pNPS.

The immobilized enzyme showed a marked resistance to thermal inactivation (Figure 4), retained about 80% of its activity at 55ºC, while the soluble form at these temperature retained only 40%. The catalytic activity of enzymes increases with an elevation in temperature, as is usually the case for chemical catalysts. However, as enzymes have a protein nature and are generally unstable to heat, the enzymatic reaction cannot practically be carried out at a high temperature (Busto, 1998), but the formation of intermolecular covalent bonds between the enzyme molecules and between these and those of the support confer rigidity on the structure of the enzyme molecule, so that the enzyme is less affected by the denaturing effect of temperature (Spagna et al., 1998). Other enzymes showed similar results to these, which became stables after the immobilization process, such as: glucoamylase (Spagna et al., 1998), β-glucosidase (Busto, 1998) and peroxidase (Azevedo et al., 2001).

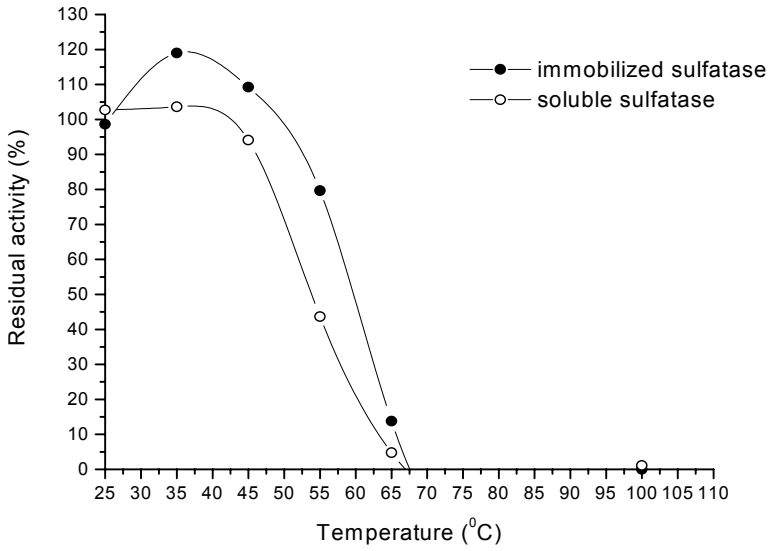

Figure 4 –Thermal stability of the soluble and immobilized sulfatase from *Aplysia cervina* liver. This phenomenon was studied by incubating samples of the soluble and immobilized sulfatase, at pH 5.0, for 15 min at temperatures varying from 25 to 100°C. After resting for 15 min (room temperature equilibration), their activities were assayed at 45°C.

Figure 5 shows the effects of metal ions and EDTA on the soluble and sulfatase-magnetic POS/PVA. Among the compounds tested, $MgCl_2$, $CaCl_2$ and $FeCl_2$ activating the soluble and immobilized sulfatase, althought higher increase was observed for immobilized enzyme. EDTA, KCl, $FeCl_3$ and NaCl were activators of the immobilized enzyme, while Na_2HPO_4 and NaH_2PO_4 had notable inhibition effects on the soluble and immobilized activities. These results are similar that one obtained for the lysosomal sulfatase from mammals which are strongly inhibited by phosphate ions (Allen and Roy, 1968). $HgCl_2$ inhibited the soluble enzyme and the results present similarity with the attained for immobilized sulfatase on Con-A where Hg^{2+} was strong inhibitor of this enzyme (Farooqui and Srivastava, 1981). The others compound presented no significant change on enzyme activities.

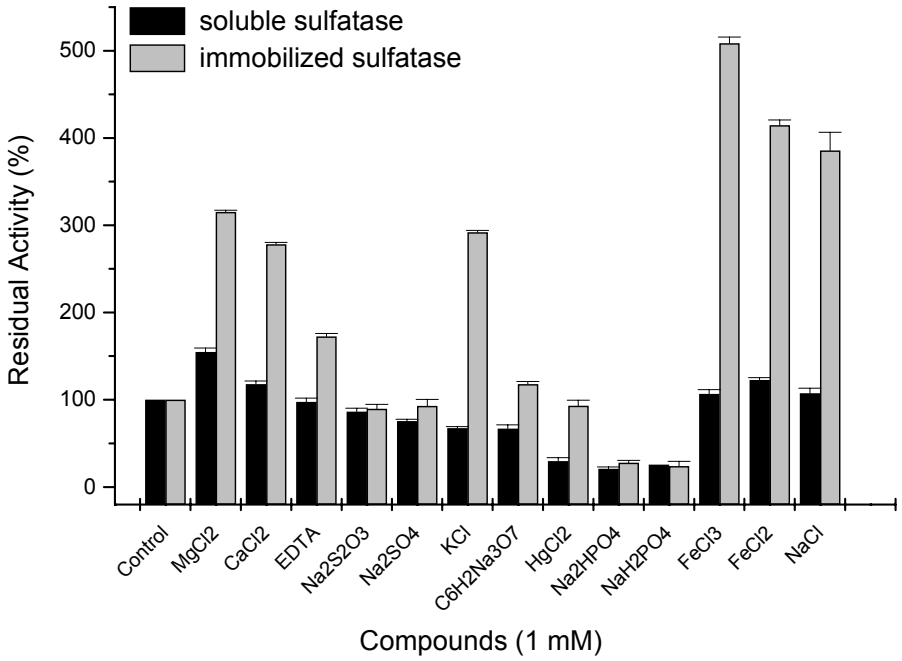

Figure 5 –Effects of metal ions and EDTA on the soluble and immobilized sulfatase from *A. cervina* liver. These experiments were accomplished by previous incubation of immobilized and soluble sulfatase with 1 mM of the respective salts ($MgCl_2$, $CaCl_2$, $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$ (sodium citrate), $HgCl_2$, Na_2HPO_4, NaH_2PO_4, $FeCl_3$, $FeCl_2$ and NaCl) at 45°C for 15 min and assaying their activities as described in methods.

The competition experiments are showed in figure 6. Heparin of low molecular weight competed more with the pNPS for the active site of soluble and immobilized enzyme. Although it can be observed that this competition is not very significant when compared to the competition using the heparin of high molecular weight. These results showed although the inhibition is larger for the soluble enzyme than for immobilized enzyme, it is interesting, because the soluble enzyme is probably with its catalytic site more available to the attachment of the heparins.

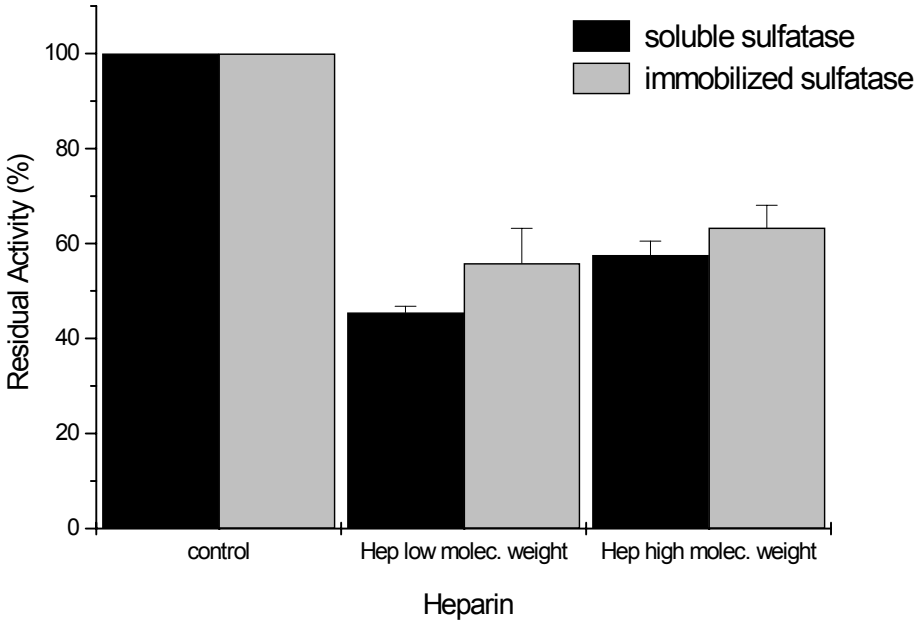

Figure 6 – Effect of low and high molecular weight heparin on the activity of the soluble and immobilized sulfatase from *A. cervina* liver. The soluble (50 µl) and immobilized (10 mg) sulfatase was incubated with high and low molecular weight heparin (4.5 mg) and pNPS in buffer (final volume of 100 µl) for 15 min at 45^{o}C.

Conclusions

The addition of partially purified sulfatase by Heparin-Sepharose CL-6B (0.2 M fraction) to ferromagnetic POS/PVA powder led to the formation of an insoluble but enzymatically active complex that could be separated by magnetic field (6000 Oe). The magnetic sulfatase-POS/PVA retained 21.23% of the soluble enzyme, exhibited higher thermal stability and presented the same apparent Km and optimum pH. The immobilized sulfatase could be reused at least seven times without loss in the hydrolysis of pNPS. $MgCl_2$, $CaCl_2$, KCl, $FeCl_2$, $FeCl_3$, EDTA and NaCl strongly activated magnetic sulfatase-POS/PVA derivative. The heparin low molecular weight competed more with pNPS for the active site immobilized enzyme. This last result indicate immobilized sulfatase can be used, among many applications, to remove sulfate groups from glycosaminoglycans, sulfated bile acids and drug and produce desulfo-glucosinolates from plant compounds for animal feed.

Acknowledgments

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APÍTULO III

[#] IMMOBILIZATION OF SULFATASE FROM *Aplysia cervina* LIVER ON FERROMAGNETIC DACRON

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Abstract

Sulfatase (EC 3.1.6.1) from the liver of *Aplysia cervina* was purified by ammonium sulfate and acetone precipitations and affinity chromatography on a Heparin-Sepharose CL-6B column. This enzyme was covalently immobilized on ferromagnetic Dacron yielding a derivative with 3.17 units/mg protein and 36.25% of the soluble enzyme activity. This preparation was easily removed from the reaction mixture by a magnetic field, was reused at least 11 times without loss in its activity and remained 80% active after 7 months stored at 4^{o}C in buffer. It was more thermal stable than the soluble enzyme and presented the same optimum pH and K_m. $CaCl_2$, $MgCl_2$, $FeCl_3$, EDTA and KCl activated the sulfatase-ferromagnetic Dacron whereas Na_2SO_4, $Na_2S_2O_3$, sodium citrate, Na_2HPO_4 and NaH_2PO_4 and heparin inhibited it. This immobilized sulfatase can be used to remove sulfate groups from glycosaminoglycans, sulfated bile acids and drugs and to produce desulfo-glucosinolatos from plant compounds for animal feed.

Key words: Sulfatase, immobilization, ferromagnetic Dacron, glycosaminoglycans

Introduction

The hydrolytic enzymes that are capable to release inorganic sulfate from all the different classes of "sulfate ester" are named as "sulfatases" (EC 3.1.6.1). They are widely distributed in animals and microorganisms and they remove sulfate from a wide variety of compounds. The most important sulfatases commercially available are contained in the intestinal juice of the snail *Helix pomatia*.

In our laboratory, a sulfatase has been isolated from the liver of a mollusc *Aplysia cervina* easily found in the Northeastern Brazil cost (Matta, 2001). The purification procedure ended with affinity chromatography step using Heparin-Sepharose CL-6B. Heparin has the highest negative charge density of any known biological macromolecule as a result of its high content of negatively charged sulfo and carboxyl groups. Consequently, the action of this heparin specific sulfatase would change the properties of this pharmacologically important polysaccharide. This contribution describes the immobilization of this enzyme on ferromagnetic Dacron, polyester chemically known as polyethyleneterephthalate and widely commercially employed, has been successfully used as support to immobilize amyloglucosidase (Carneiro Leão et al., 1991). Some physical-chemistry properties of the immobilized sulfatase are also reported. Immobilized sulfatase preparations have been previously proposed to remove sulfate groups from sulfated bile acids and drugs, important step to increase and extend the detection of these compounds (Gao, 2001; Toennes and Maurer, 1999). Also, it has been used to produce desulfo-glucosinolatos (Leoni, 1998). The glucosinolatos are plant compounds found in high amounts in the seeds of cruciferous, but cannot be directly used for animal feed.

Materials and Methods

p-Nitrophenyl sulfate (pNPS), Dacron (polyethyleneterephthalate) and Heparin Sepharose CL-6B were purchased from Sigma (St. Louis, MO, USA), Terphane, Inc. (Cabo, Brazil) and Amersham Biosciences (Uppsala, Sweden), respectively. High (Liquemine, MW 15000 according Catani et al., 2001) and low (Clexane, MW 4500) molecular weight heparin was acquired to Roche Químicos e Farmacêuticos S.A. (Rio de Janeiro/RJ) and Rhodia Farma LTDA (São Paulo/SP), respectively.

Enzyme preparation.

The molluscs (*A. cervina*) were collected in the beach of Maracaípe, PE, Brazil and kept frozen at -20^{0}C until their utilization. Their livers were dissected and homogenized in 1:2 (w/v), 0.1 M sodium acetate buffer, pH 5.0 and centrifuged for 30 min at 27.000 x *g*. To the supernatant, ammonium sulfate was slowly added up to 50% saturation and centrifugation carried out at 27.000 *x g* for 15 min. The precipitate was discarded and ammonium sulfate was added to the supernatant up to 80% saturation and again centrifuged at 27.000 x g for 15 min. The precipitate (50-80%) was suspended in 0.1 M sodium acetate buffer, pH 5.0 and dialyzed against the same buffer overnight with four buffer changes. To the dialyzed preparation held at 4^{0}C, 30% of ice-cold acetone was added under gentle agitation. After the mixture to rest at 4^{o}C for 24 h, centrifugation (27.000 *x g* for 15 min) was carried out, the precipitate was discarded and to the supernatant 50% of acetone was added. This mixture was again kept and centrifuged as above described. Further acetone addition procedure was similarly performed for 70% and 90% volumes. Finally, the precipitate of the 90% acetone volume was dried under vacuum and suspended in 0.1 M sodium acetate buffer, pH 5.0. This preparation was applied on the top of a Heparin-Sepharose CL-6B

column (1.7 x 8 cm) and eluted with 0.1 M sodium acetate buffer, pH 5.0, at a flow-rate of 0.33 mL/min until no absorbance at 280 nm was detected. Then, the sulfatases bound was collected eluting the column with 0.1 M sodium acetate buffer, pH 5.0 containing 0.2 M NaCl.

Ferromagnetic Dacron-hydrazide synthesis

Dacron was converted to ferromagnetic Dacron-hydrazide according to Carneiro Leão et al. (1991), except that: a) incubation time of Dacron-hydrazide with $FeCl_3.6H_2O/FeCl_2.4H_2O$ at 80ºC was extended from 10 min to 30 min and b) previous to the magnetization, a suspension of Dacron-hydrazide powder (2 g in 10 mL of water) was kept for 8 min under rest and the supernatant was decanted to remove fines (four times).

Ferromagnetic Dacron-hydrazide activation with glutaraldehyde

Ferromagnetic Dacron-hydrazide (10 mg) was incubated with 12.5% w/v glutaraldehyde (1 mL) for 2 h at 25ºC, with mild stirring, washed with sodium acetate buffer (1 mL, 10 times, until no glutaraldehyde was detected in the supernatant) using a magnetic field (6000 Oe) and kept in 0.1 M sodium acetate buffer, pH 5.0, at 4ºC until use.

Immobilization of sulfatase on ferromagnetic Dacron.

The activated support was incubated with 1 mL of the sulfatase solution for 16h at 4^{o}C, under rotator agitation (20 rpm). Then, the support was washed 10 times with 1.0 mL of 0.1 M sodium acetate buffer, pH 5.0. Afterwards, the sulfatase-magnetic Dacron was incubated with 1 mL of the 1 M glycine solution for 16h at 4^{o}C, under

rotator agitation (20 rpm) and washed 10 times with 0.1 M sodium acetate buffer, pH 5.0.

Determination of sulfates activity and protein content.

Sulfatase (50 µl) was incubated with pNPS (5 µl of 60 mM solution) and 0.1 M sodium acetate buffer pH 5.0 (45 µl) at 45^0C by 15 min. The reaction was stopped by addition of 1 mL of 0.25 M NaOH and the p-nitrophenol formed measured at 405 nm (Hitachi 3200 spectrophotometer). One enzymatic unit was defined as the amount of enzyme capable to hydrolyze 1 µmol of pNPS per min (ε = 18.3 x 10^3 $M^{-1}.cm^{-1}$). The sulfatase-magnetic Dacron (10 mg) was assayed by adding it to 100 µl of the pNPS (3 mM) prepared in 0.1 M sodium acetate buffer, pH 5.0, at 45^0C for 15 min under stirring. After this time the sulfatase-magnetic Dacron was precipitated by a magnetic field (6000 Oe) for 10 s. The supernatant was added to 1 mL of 0.25 M NaOH and the p-Nitrophenol spectrophotometrically determined at 405 nm. Protein content was established according to Sedmak and Grossberg (1977). The amount of immobilized sulfatase was calculated by the difference between the offered protein and that presents in the washing supernatants. The kinetic constants, K_m and V_{max}, for the soluble and immobilized sulfatase were calculated from the initial velocities of Lineweaver-Burk reciprocal plots. The reaction was carried out at 45°C. An immobilized sulfatase sample had its activity assayed every time during eleven successive recoveries under magnetic field (reproducibility of reuse) and another one was assayed at times interval during eight months of storage at 4^{o}C in buffer (shelf life).

pH, temperature and activation/inhibition effects.

The optimum pH values for the soluble and immobilized sulfatase activities were determined over the pH range 3.7-6.5 using the following buffers (1mM): Sodium-acetate buffer (pH 3.7-5.5) and Bis-Tris buffer (pH 5.5-6.5).

The optimum temperature values for the soluble and immobilized sulfatase activities were measured at pH 5.0 by the standard assays at various temperatures over the range 25-85°C. Thermal stability enzymatic preparations was studied by incubating samples of the soluble and immobilized sulfatase, at pH 5.0, for 15 min at temperatures varying from 25 to 100°C. After resting for 15 min (room temperature equilibration), their activities were assayed at 45°C using as above described.

The metal ions influence on the immobilized and soluble sulfatase was studied by their previous incubation with 1 mM of the respective salts ($MgCl_2$, $CaCl_2$, $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$ (sodium citrate), $HgCl_2$, Na_2HPO_4, NaH_2PO_4, $FeCl_3$, $FeCl_2$ and NaCl) at 45°C for 15 min and assaying their activities as above described. The effect of EDTA, chelating agent, was also included.

The soluble (50 µl) and immobilized (10 mg) sulfatase was incubated with high and low molecular weight heparin (4.5 mg) and pNPS in buffer (final volume of 100 µl) for 15 min at 45°C. The reaction was stopped by adding 1 mL of 0.25 M NaOH and the formed p-nitrophenol was spectrophotometrically determined at 405 nm either to the reaction mixture (soluble enzyme) or the mixture supernatant (immobilized enzyme).

Results and Discussion

Here, an active insoluble water derivative of *A. cervina* liver sulfatase was synthesized presenting 3.17 units/mg protein and 36.25% of the soluble enzyme activity. These results are consistent with those (33% ± 4% of soluble activity)

described for immobilized sulfatase from *Helix pomatia* on Affi-Gel support (Toennes and Maurer, 1999). The sulfatase-ferromagnetic Dacron derivative was easily removed from the reaction mixture by a magnetic field (6000 Oe). Furthermore, it was reused eleven times without loss of activity (97.42% ± 3.24%; mean ± standard deviation) and kept 80% of its initial activity after 07 months stored at 4^0C in buffer (Figure 1), and did not lose any activity during the final 05 months. This Figure also shows that the soluble enzyme presented 45% of the initial activity stored under the same conditions.

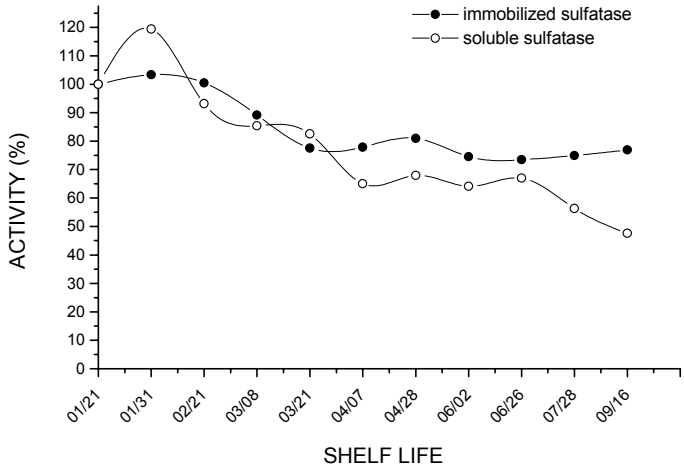

Figure 1 – Activity time course of the soluble and immobilized *A. cervina* liver sulfatase stored at 4°C and in buffer. An immobilized sulfatase sample and soluble enzyme had its activity assayed at times interval during seven months of storage at 4°C in buffer (shelf life). Results shown are given as the relative activity in comparison to the observed initial activity.

Regarding the kinetic parameters of the soluble and immobilized enzyme, the K_m values didn't come significantly different. K_m values for the immobilized and soluble enzyme acting on pNPS were 3.46 ± 0.36 mM and 3.71± 0.41 mM, respectively. These K_m values are not statistically different. Immobilized enzymes usually present higher K_m values compared to those estimated for the soluble form due to conformational changes undergone by the enzyme molecule, and to the difficulty of diffusion of the substrate towards the active site of the enzyme (Spagna et al., 1998).

Charged supports also influence kinetics parameters of immobilized enzyme acting on charged substrates either decreasing or increasing their apparent values (Goldstein, 1976). One can infer that such factors do not interfere with the sulfatase-ferromagnetic Dacron derivative acting on the used small and negatively charged substrate p-nitrophenyl sulfate.

Figure 2 shows the pH activity profiles for soluble and immobilized sulfatase. These results showed that optimum enzyme activity for both the soluble and immobilized enzyme was equal to pH 5.0. Notwithstanding, at more acid pH values the relative activity of the immobilized enzyme was found to be greater than that of the soluble one. Similar results were reported for immobilized sulfatase on aminopropyl controlled pore glass (pH 3.0-5.0; Boppana et al., 1989), on Affi-Gel (pH 5.2; Toennes and Maurer, 1999) and on Concanavalin-A (pH 5.2; Farooqui and Srivastava, 1981).

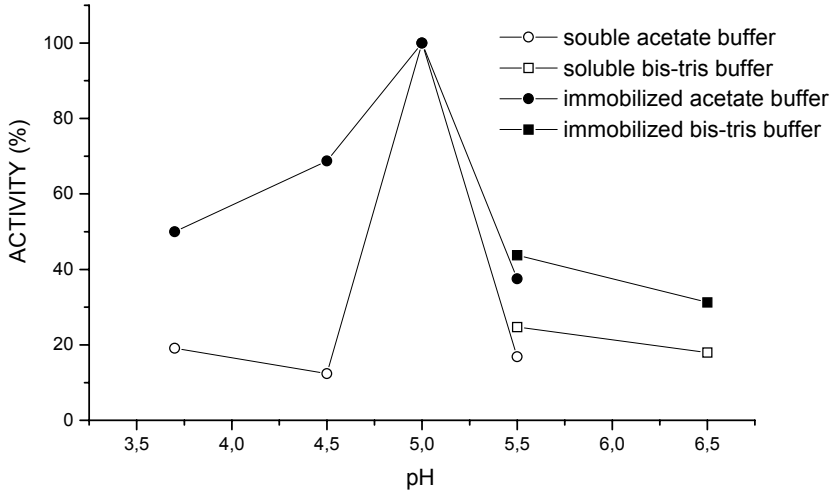

Figure 2 – Effect of pH on the soluble and immobilized sulfatase from *A. cervina* liver. The sulfatase activities were determined over the pH range 3.7-6.5 using the following buffers (1mM): Sodium-acetate buffer (pH 3.7-5.5) and Bis-Tris buffer (pH 5.5-6.5). Results shown are given as the relative activity in comparison to the observed maximum activity at the optimal pH for the hydrolysis of pNPS.

The optimum temperature for the immobilized and soluble sulfatase was 55^{o}C and 45^{o}C, respectively (Figure 3).

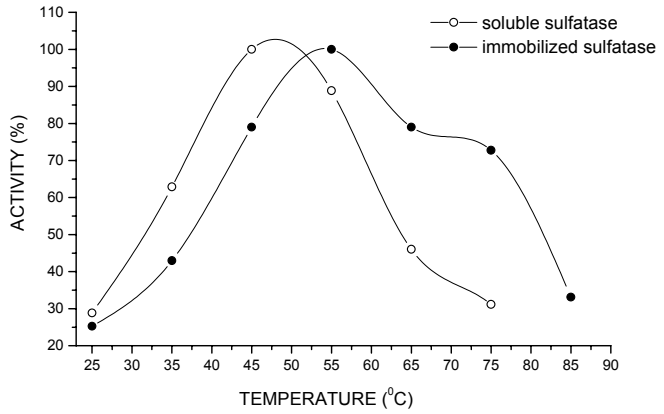

Figure 3 – Effect of the temperature on the soluble and immobilized sulfatase activities. The activities were established using pNPS as substrate at pH 5.0 at indicated temperatures and they are given as the relative to the maximum observed activity.

The immobilized was also more thermal stable than the soluble enzyme (Figure 4). Thus immobilized sulfatase retained 100% of its activity at 55ºC, while at this temperature the soluble sulfatase lost 60% of its activity. At 65ºC the soluble sulfatase almost lost all of its activity, whereas at this temperature the immobilized form retained 60% of its activity. The establishment of the intermolecular covalent bonds between the enzymes and the support confers rigidity on the structure of the enzyme molecule, so that the denaturing effects of the temperature on the enzyme are less effective (Spagna et al., 1998). These observations are in agreement with those reported by immobilized sulfatase on Concanavalin A (Farooqui and Srivastava, 1981).

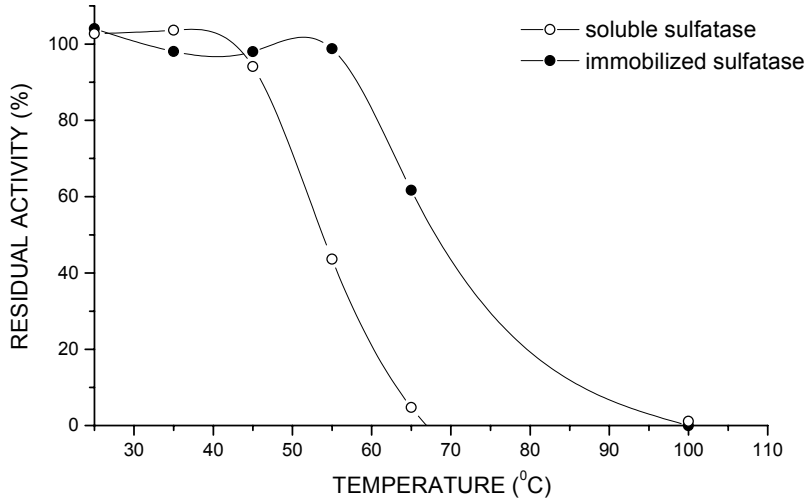

Figure 4 –Thermal stability of the soluble and immobilized sulfatase from *A. cervina* liver. This phenomenon was studied by incubating samples of the soluble and immobilized sulfatase, at pH 5.0, for 15 min at temperatures varying from 25 to 100°C. After resting for 15 min (room temperature equilibration), their activities were assayed at 45°C.

The effects of metal ions and EDTA on the soluble and immobilized enzyme are shown in the Figure 5. Among the tested salts, $CaCl_2$ and $MgCl_2$ activated both soluble and immobilized activities. EDTA and KCl activated the immobilized enzyme only. Na_2SO_4, $C_6H_5Na_3O_7$, Na_2HPO_4 and NaH_2PO_4 inhibited both soluble and immobilized enzymes. $HgCl_2$ inhibited in larger degree the soluble enzyme that the immobilized enzyme. $FeCl_2$ activated the soluble enzyme. The sodium phosphates inhibition has also been reported for the other immobilized sulfatases such as that on Concanavalin-A (Farooqui and Srivastava, 1981) and on aminopropyl control pore glass (Boppana et al., 1989).

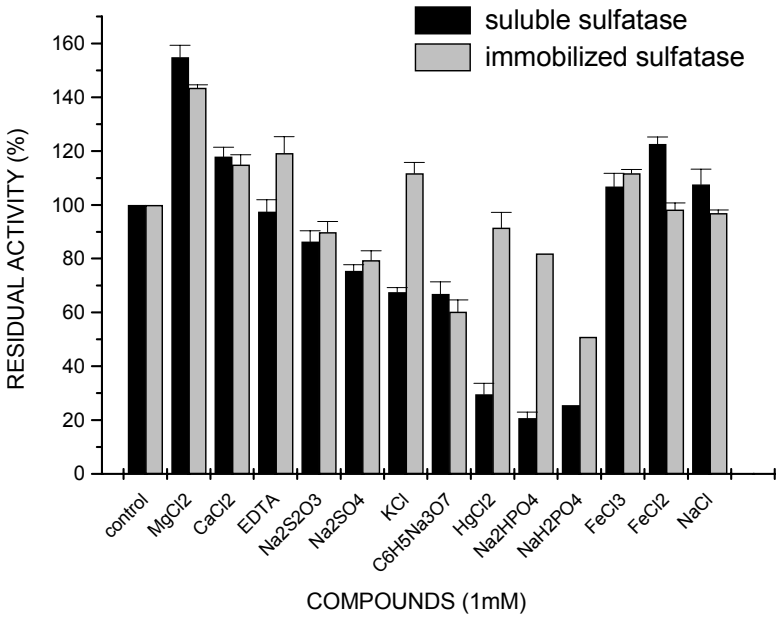

Figure 5 –Effect of metal ions and EDTA on the soluble and immobilized sulfatase from *A. cervina* liver. These experiments were accomplished by previous incubation of immobilized and soluble sulfatase with 1 mM of the respective salts ($MgCl_2$, $CaCl_2$, $Na_2S_2O_3$, Na_2SO_4, KCl, $C_6H_5Na_3O_7$ (sodium citrate), $HgCl_2$, Na_2HPO_4, NaH_2PO_4, $FeCl_3$, $FeCl_2$ and NaCl) at 45°C for 15 min and assaying their activities as described in methods.

Finally, Figure 6 displays the inhibition of the soluble and immobilized sulfatase by the heparin both of low and high molecular weight. The inhibition was stronger towards the soluble enzyme. Probably, the weaker inhibition effect of heparin on the immobilized enzyme is due to steric hindrance caused by the polymeric inhibitor and the decrease of its degree of freedom. It is important to notice that the sulfatase employed in this work was purified by Heparin Sepharose CL-6B affinity chromatography.

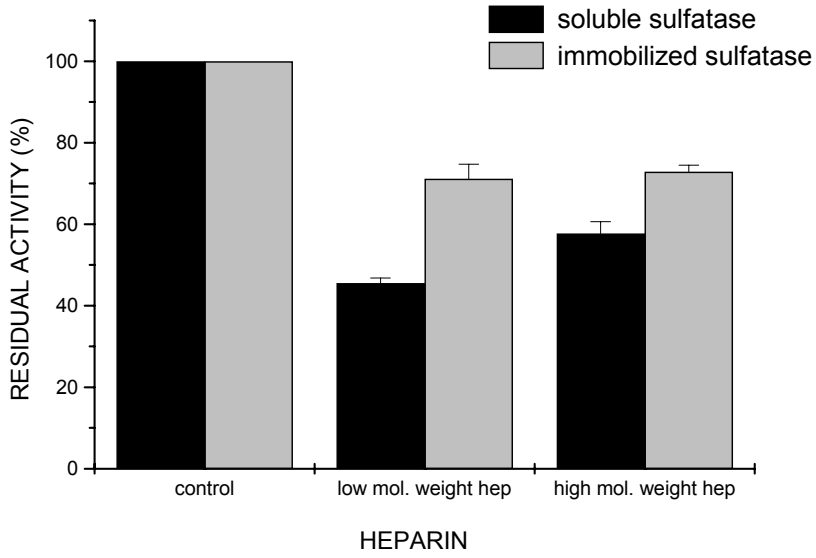

Figure 6 – Effect of low and high molecular weight heparin on the activity of the soluble and immobilized sulfatase from *A. cervina* liver. The soluble (50 µl) and immobilized (10 mg) sulfatase was incubated with high and low molecular weight heparin (4.5 mg) and pNPS in buffer (final volume of 100 µl) for 15 min at 45^{o}C.

Conclusions

From the results above reported one can conclude that *A. cervina* liver sulfatase can be covalently immobilized on ferromagnetic Dacron. The sulfatase-ferromagnetic Dacron is easily recovered from the reaction mixture by a magnetic field. This preparation retained 36.25% of the soluble enzyme, was more thermal stable and presented the same optimum pH and K_m. The immobilized sulfatase was reused at least 11 times without loss in its activity which remained 80% of that initially estimated after 7 months stored at 4^{o}C in buffer. $CaCl_2$, $MgCl_2$, $FeCl_3$, EDTA and KCl activated the sulfatase-ferromagnetic Dacron whereas $Na_2S_2O_3$, Na_2SO_4, sodium citrate, Na_2HPO_4 and NaH_2PO_4 and heparin inhibited it. This immobilized sulfatase can be used among many applications to remove sulfate groups from glycosaminoglycans, sulfated bile acids and drugs and to produce desulfo-glucosinolatos from plant

compounds for animal feed.

Acknowledgments

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CONCLUSÕES GERAIS

Os resultados dos trabalhos desenvolvidos nesta tese permitem concluir:

1. A existência de uma sulfatase (EC 3.1.6.1) no fígado do molusco *Aplysia cervina*, que purificada 89.7 vezes com rendimento de 5.37%, mediante precipitações sucessivas com sulfato de amônio e acetona, e cromatografia de afinidade em Sepharose-Heparina CL-6B, resultou numa preparação que apresentou as seguintes propriedades ao catalisar a hidrólise do p-nitrofenol sulfato:
    a) pH ótimo igual a 5.0;
    b) temperatura ótima igual a 45^{o}C;
    c) K_m igual a 3.71 $\pm$ 0.41 mM;
    d) ativação pelo $MgCl_2$, $CaCl_2$ e $FeCl_2$;
    e) inibição pelo $Na_2S_2O_3$, Na_2SO_4, KCl, citrato de sódio, $HgCl_2$, Na_2HPO_4 e NaH_2PO_4 e
    f) inibição pela heparina de baixo peso molecular maior do a promovida pela de elevado peso.

2. Esta sulfatase quando covalentemente imobilizada a partículas magnéticas contendo rede semi-interpenetrada de polisiloxano e álcool polivinílico, bem como Dacron, resultaram em derivados insolúveis detentores das seguintes propriedades:
    a) facilmente removíveis do meio de reação por um campo magnético;
    b) retenção de parte da atividade específica da enzima solúvel;
    c) reutilizações seguidas sem perda de atividade;
    d) retenção de atividade após meses de armazenamento a 4^{o}C em solução tampão;
    e) pH ótimo igual ao da enzima solúvel (5.0);
    f) Mais termoestável do que a enzima solúvel;
    g) K_m aparente igual ao da enzima solúvel (3.7 mM);
    h) ativação pelo $CaCl_2$, $MgCl_2$, $FeCl_3$, EDTA, KCl e
    i) inibição pelo Na_2HPO_4, NaH_2PO_4 e heparina.

PERSPECTIVAS

- Caracterizar físico-quimicamente os suportes utilizados para a imobilização da sulfatase (Dacron e POS/PVA);
- Modificar estruturalmente os glicosaminoglicanos (heparina e haparam sulfato) mediante o emprego da sulfatase imobilizada em Dacron e POS/PVA;
- Detectar por eletroforese em gel de agarose, ressonância magnética nuclear (RMN) ou infravermelho a desulfatação dos glicosaminoglicanos e
- Investigar as ações biológicas dos glicosaminoglicanos modificados.

ANEXOS

Comunicações em congressos a partir desta tese:

MATTA, L.D.M., AMARAL, I.P.G.ABREU, L.R.D. AND CARVALHO JR L.B. Immobilization of a sulfatase from mollusc Aplysia cervina onto Ferromagnetic Dacron In: XXXII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Caxambu MG, 2003.

MATTA, L.D.M., AMARAL, I.P.G., LIMA, T.R.M., ANTUNES, C.L., ABREU, L.R.D. AND CARVALHO JR L.B. Physical-Chemical and Kinetics Properties of Arylsulfatase from *Aplysia cervina* (Mollusc) Liver Immobilized on Ferromagnetic Dacron. XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Caxambu MG, 2004.

MATTA, L.D.M., LIMA, T.R.M., ANTUNES, C.L., ABREU, L.R.D. AND CARVALHO JR L.B. Purification, Physical-Chemical and Kinetics Properties of a Sulfatase from *Aplysia cervina* (Mollusc) Liver. XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Caxambu MG, 2004.

MATTA, L.D.M., AMARAL, I.P.G., LIMA, T.R.M., ANTUNES, C.L., ABREU, L.R.D. AND CARVALHO JR L.B. Immobilization of Arylsulfatase from *Aplysia cervina* (Mollusc) Liver on Ferromagnetic Polysiloxane/Polyvinyl Alcohol Composite. XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Caxambu MG, 2004.

MATTA, L.D.M., AMARAL, I.P.G., LIMA, T.R.M., ANTUNES, C.L., ABREU, L.R.D. AND CARVALHO JR L.B. Imobilização de uma Sulfatase Extraída do Molusco *Aplysia cervina* em Suportes Insolúveis em Água. Reunião da Sociedade Brasileira de Biotecnologia - Enzitec, Rio de Janeiro, abril de 2004.

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3. MacLean, D.C. and Schneider, R.E., Effects of gaseous hydrogen fluoride on the yield of field-grown wheat. *Environmental Pollution (Series A)*, 1981, **24** 39-44.

4. Mandl, R.H., Weinstein, L.H., Weiskopf, G.J. and Major, J.I., The separation and collection of gaseous and particulate fluorides. In *Proceedings of the 2nd International Clean Air Congress*, ed. H.M. Englund and W.T. Berry. Academic Press, New York, 1971, pp. 450-458.

5. Davison, A.W., Uptake, transport and accumulation of soil and airborne fluorides by vegetation. Paper presented at 6th International Fluoride Symposium, Logan, UT, 1-3 May 1982.

6. Chang, C.W., Effect of fluoride pollution on plants and cattle. PhD thesis, Banaras Hindu University, Varanasi, India, 1975.

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Example 2: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228, a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

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