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**AGENTES QUELANTES E POLIAMINAS COMO
GRUPOS IONOGENICOS PARA A PURIFICAÇÃO DE
IgG HUMANA POR CROMATOGRAFIA**

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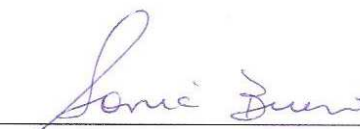
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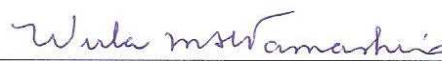
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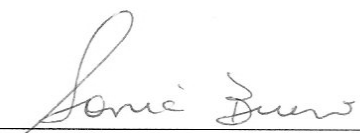


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PREFÁCIO

O presente trabalho inclui em seus capítulos artigos de minha co-autoria, em acórdância com a possibilidade aberta pelo artigo 2º da Informação CCPG/001/08, de 09/04/2008 (Anexo I).

Este trabalho consta de uma Introdução contendo a colocação do problema, os objetivos e a estratégia de trabalho utilizada. Na Revisão Bibliográfica se encontram detalhados aspectos relacionados à Imunoglobulina G humana (relevância, obtenção e purificação), bem como à técnica de IMAC e agentes quelantes, além do uso de aminas como ligantes em cromatografia. O capítulo de Resultados e Discussão está subdividido em três sub-capítulos distintos, cada qual contendo um artigo com resultados e discussão.

O artigo a ser submetido para publicação “Comparative evaluation of immobilized metal ion- and metal ion free- o-phosphoserine (OPS) adsorbents: Capture of IgG from human serum” apresenta resultados experimentais do uso do ligante OPS para a captura de IgG humana a partir do soro. Avaliou-se também capacidade de OPS em quelatar Cu(II), Ni(II), Co(II) e Al(III), bem como sua seletividade na adsorção de IgG humana, comparando os resultados com o ligante sem metal imobilizado.

Por sua vez, o artigo “Adsorption of human serum proteins onto TREN-agarose: Purification of human IgG by negative chromatography” (publicado em Journal of Chromatography B, v. 877, p. 17-23, 2009) aborda a utilização do agente quelante TREN (sem metal imobilizado) como ligante para purificação de IgG humana utilizando o princípio da cromatografia negativa.

O terceiro artigo, a ser submetido para publicação, tem como título “A comparison of IgG purification by negative chromatography in amine-based ligands: effect of L-lysine and poly-L-lysine”, apresentando o potencial de utilização destes ligantes (que também contém grupamentos aminas) visando a purificação de IgG humana por cromatografia negativa.

RESUMO

Dentre os hemoderivados disponíveis comercialmente, as imunoglobulinas do isotipo G (IgG) recebem destaque pelo seu uso em aplicações terapêuticas. Por esta razão são requeridas com elevado grau de pureza. Várias técnicas vêm sendo investigadas para a purificação de IgG a partir do soro ou plasma humano, desde a precipitação até métodos mais seletivos, como os cromatográficos. Neste trabalho, investigou-se o efeito de agentes quelantes de IMAC (cromatografia por íons metálicos imobilizados) como Tris-2(aminoetil)amina (TREN) e o-fosfoserina (OPS) como grupos ionogênicos (sem íon metálico imobilizado), além do aminoácido L-Lisina e seu polímero poli-L-Lisina (PLL) como ligantes visando a purificação de IgG a partir do soro humano. Para tanto, foram realizados experimentos de adsorção em diferentes sistemas tamponantes. A seletividade dos adsorventes foi verificada por eletroforese SDS-PAGE e análise nefelométrica. As melhores condições, para cada caso, foram utilizadas em experimentos de curvas de ruptura e isotermas de adsorção de albumina de soro humano (HSA) e IgG. No caso dos ligantes TREN e PLL, 73% e 86% da IgG alimentada foi recuperada nas frações não-retidas (cromatografia negativa) apresentando pureza superior a 90%. Quando o ligante OPS foi utilizado, por sua vez, a recuperação de IgG ocorreu nas frações retidas juntamente com IgM. Experimentos de curva de ruptura mostraram que um fator de purificação de 4.9 foi atingido, sendo a IgG recuperada com pureza de 88%. Este ligante se mostrou eficiente quando se deseja purificar IgG humana que possui pontos isoelétricos na faixa de 7,8 a 8,5. Para todos os ligantes, a recuperação de IgG a partir soro humano pode ser alcançado sob condições brandas de pH, baixa força iônica, e temperatura ambiente. De um ponto de vista de processo em larga escala, todos os ligantes apresentados neste trabalho apresentam potencial para serem usados como uma das etapas em um processo industrial de recuperação e purificação de IgG.

ABSTRACT

Among the commercially available hemoderivatives or blood products, the immunoglobulin G (IgG) is highlighted by its use in therapeutic applications, which need high purity IgG. Several techniques are being investigated aiming at the purification of IgG from human serum or plasma, usually starting with precipitation and then using more selective methods such as chromatography. In this study, we evaluated the effect of Tris-2 (aminoethyl) amine (TREN) and o-phosphoserine (OPS) – two chelating agents used in immobilized metal ion chromatography (IMAC) – as ionogenic groups (without immobilized metal ion), and the amino acid L-lysine and its polymer poly-L-lysine (PLL) as ligands aiming at the purification of IgG from human serum. For this purpose, adsorption experiments were performed using different buffering systems. The selectivity of the adsorbents was checked by SDS-PAGE and nephelometric analysis. The best conditions for each adsorbent were used in experiments of breakthrough curves and adsorption isotherms of human serum albumin (HSA) and IgG. In the case of TREN and PLL, 73% and 86% of IgG fed was recovered in the non-retained fractions (negative chromatography) with purity higher than 90%. When the ligand OPS was used, IgG was recovered in retained fractions. Experiments of breakthrough curve showed that a purification factor of 4.9 was reached, and IgG was recovered with a purity degree 88% (with IgM). This ligand is efficient when it is desired to purify human IgG with isoelectric points ranging from 7.8 to 8.5. For all ligands, the recovery of IgG from human serum was achieved under mild conditions of pH, low ionic strength and temperature. All ligands presented in this study have potential to be used in an industrial downstream processing of IgG from human serum.

CAPÍTULO 1: INTRODUÇÃO

Hemoderivados são produtos que têm o plasma ou soro humano como matéria-prima, sendo produzidos, mais comumente, pelo fracionamento industrial do plasma, cujos principais constituintes são a albumina, os fatores VIII e IX da coagulação, e as imunoglobulinas (Hemoderivados, 2006). Devido a sua gama de aplicações nos campos terapêuticos e diagnósticos, as imunoglobulinas assumiram o papel de destaque, dentre os hemoderivados, neste século.

De acordo com dados do Departamento de Assistência Farmacêutica e Insumos Estratégicos, vinculado à Secretaria de Ciência, Tecnologia e Insumos Estratégicos do Ministério da Saúde do Brasil, o país gastou, em 2008, cerca de 800 milhões de reais em importações de hemoderivados para abastecer hospitais (públicos e privados), sendo que 37% deste valor correspondem às imunoglobulinas (Hemoderivados, 2006; MARTINS, 2009).

Dentre todos os hemoderivados, a imunoglobulina G (IgG) é aquela que vem tendo a maior utilização em todo o mundo com um consumo anual médio per capita na ordem de 70 g por 1000 habitantes, devido, principalmente, a seu uso em indicações terapêuticas, que englobam o tratamento de imunodeficiências congênitas ou adquiridas, deficiências seletivas de anticorpos, doenças auto-imunes, diversas doenças infecciosas e no tratamento de alguns tipos de câncer (por exemplo, leucemia linfocítica crônica). Essas aplicações terapêuticas requerem, normalmente, grandes quantidades de IgG com grau de pureza superior a 90% (BURNOUNF, 1995; BURNOUNF e RADOSEVICH, 2001; Hemoderivados, 2006).

A IgG é normalmente obtida a partir do fracionamento do plasma humano pela precipitação com etanol de acordo com a metodologia proposta por Cohn e colaboradores (COHN et al., 1946). No entanto, o uso de métodos cromatográficos, complementarmente ou em substituição à precipitação, é visto pela indústria farmacêutica como uma operação indispensável antes da sua

utilização no campo terapêutico, pois, graças à elevada seletividade atingida pelas técnicas cromatográficas pode-se evitar que ocorram efeitos colaterais nos pacientes tratados com IgG (BURNOUNF, 1995; BURNOUNF e RADOSEVICH, 2001). Outro fator importante é o custo deste processo. Segundo LABROU e CLONIS (1994), o fator chave para o desenvolvimento dos processos biotecnológicos continua se encontrando nas etapas de recuperação e purificação (“dowstream processing”) que chegam a atingir de 50 a 80% do custo total de produção.

Como têm apresentado resultados promissores nos processos de purificação de anticorpos, técnicas cromatográficas de afinidade, principalmente com ligantes bioespecíficos como as proteínas A, G ou L (CHARCOSSET et al., 1995; BOYLE e REIS, 1987; NILSON et al., 1993) imobilizados foram propostas para a purificação de IgG devido à alta especificidade destes quando acoplados à fase estacionária, proporcionando IgG com grau de pureza superior a 90% (HUSE et al, 2002). No entanto, devido ao alto custo destes adsorventes, ligantes pseudobioespecíficos, apesar de menos seletivos, tem sido propostos e estudados devido ao seu custo menos elevado.

Ligantes pseudobioespecíficos como tiofílicos (BOSCHETTI, 2001), peptídeos, aminoácidos, ligantes biomiméticos (EL-KAK e VIJAYALAKSHMI, 1991; BUENO et al., 1995; YANG et al., 2003), e íons metálicos (PORATH e OLIN, 1983; VANÇAN et al., 2002) imobilizados em diversos suportes tem sido utilizados e estudados visando a purificação de IgG humana.

A técnica de cromatografia de afinidade por íons metálicos imobilizados (IMAC) aplicada à purificação de biomoléculas foi introduzida por PORATH et al. (1975). O princípio de IMAC está baseado na afinidade que íons metálicos imobilizados em uma matriz sólida apresentam por certos aminoácidos acessíveis situados na superfície de uma biomolécula em solução, por meio de ligações de coordenação (PORATH, 1988, WONG et al., 1991). No caso da biomolécula ser IgG, encontram-se relatos na literatura que a técnica de IMAC apresenta potencial para ser utilizada visando sua purificação devido, principalmente, a presença de resíduos de histidina acessíveis na superfície desta biomolécula (HALE e BEIDLER, 1994).

Diversos grupos de pesquisa vêm obtendo resultados promissores utilizando a técnica de IMAC, como alternativa aos métodos já existentes, para a purificação de IgG, atingindo-se teor de pureza superior a 90%. A maioria destes trabalhos foi realizada fazendo-se uso de agentes quelantes tradicionais, tais como os ácidos iminodiacético (IDA) e nitrilotriacético (NTA) (PORATH e OLIN, 1983; HALE e BEIDLER, 1994; MULLER et al., 1998; VANÇAN et al., 2002; RIBEIRO et al., 2008; BRESOLIN et al., 2010a). Agentes quelantes considerados não tradicionais, como é o caso da orto-fosfoserina (OPS) e do Tris(2-aminoetil)amina (TREN), também apresentam potencial para serem utilizados visando a purificação de IgG por IMAC (BODEN et al., 1995; RIBEIRO et al., 2008; ZACHARIOU et al., 1993; ZACHARIOU e HEARN, 2000).

OPS é um éster de serina e ácido fosfórico que, devido a presença de grupamentos fosfato, carboxila e amina em sua estrutura, apresenta a característica de quelatar íons metálicos, sendo utilizado em IMAC. Na ausência de íon metálico quelatado, estes grupamentos fazem com que OPS apresente carga líquida negativa em valores de pH superiores a 5,9 (SMIECHOWSKI, 2010), permitindo que o mesmo seja utilizado como um trocador catiônico fraco, sendo um potencial candidato para a adsorção de biomoléculas com carga positiva.

O TREN, uma amina ramificada, possui a peculiaridade de quelatar íons metálicos somente com átomos de nitrogênio presentes em sua estrutura (dois dos quais são primários, um é secundário e o quarto é terciário). Devido a presença destes quatro grupamentos amina, o TREN possui carga líquida positiva em valores de pH menores que 10, apresentando potencial para interação com biomoléculas fazendo também uso do princípio de troca iônica, porém, diferentemente do OPS, é considerado um trocador aniônico.

Com o objetivo de avaliar o efeito da presença de grupamentos amina em ligantes utilizados para a purificação de IgG humana, o grupo de pesquisa da Prof. Dra. Sônia Maria Alves Bueno realizou estudos sobre o uso de diaminas lineares com cadeia carbônica de 3, 6, 8, 10 e 12, comparando também possíveis efeitos hidrofóbicos devido a presença de braço espaçador de 1 ou 12 átomos (SOUZA, 2009; SOUZA et al., 2010; BRESOLIN et al., 2010b). Resultados indicaram que a presença do braço espaçador de 12 átomos fez com que os ligantes ω -aminohexil

e ω -aminodecil apresentassem os maiores rendimentos em termos de recuperação de IgG não-adsorvida com alta pureza a partir de amostras do soro humano (SOUZA, 2009).

O aminoácido L-Lisina e seu polímero poli-L-Lisina (PLL) são muito usados como ligantes em cromatografia de afinidade para promover a purificação de biomoléculas como plasminogênio, ativador do plasminogênio, apolipoproteína A e angiostatina (HOUEN, 2001). Estes ligantes também possuem grupamentos amina em suas estruturas, apresentando potencial para promover a adsorção de proteínas do soro humano e a consequente purificação de IgG por cromatografia negativa. A diferença entre aminas lineares estudadas anteriormente (aminohexil e aminodecil) e o aminoácido lisina é que, neste caso, após a imobilização, o grupo carboxila permanece livre para interação com biomoléculas. Já a PLL, por sua vez, apresenta braços hidrofóbicos que podem ser exploradas para purificação de proteínas usando também o princípio da interação hidrofóbica (HOUEN, 2001).

1.1. COLOCAÇÃO DO PROBLEMA

O desenvolvimento de processos alternativos de purificação de IgG por cromatografia é relevante pois constitui uma contribuição científica e tecnológica com potencialidade de aplicação industrial devido às vantagens previamente apresentadas. A literatura, por sua vez, apresenta escassos trabalhos que fazem uso de agentes quelantes atuando como grupos ionogênicos para a purificação de IgG, exceto resultados de experimentos controle, sem que haja uma abordagem mais detalhada das condições que proporcionam maior seletividade e pureza para a biomolécula a ser separada. Neste sentido, avaliou-se o desempenho de dois agentes quelantes com características de carga opostas na faixa de pH estudada: o TREN (poliamina ramificada, com carga líquida positiva) e o OPS (contendo grupamentos fosfato, carboxila e amina, com carga líquida negativa). O aminoácido L-lisina e seu polímero poli-L-lisina também foram utilizados como ligantes por conterem grupamentos amino e carboxila.

1.2. OBJETIVO

O objetivo deste trabalho foi contribuir para o desenvolvimento de novos processos de purificação de IgG humana alternativos aos tradicionais utilizando como ligantes os agentes quelantes OPS e TREN e o aminoácido L-lisina e seu polímero poli-L-lisina.

Quando os ligantes TREN, L-lisina e PLL imobilizados em géis de agarose foram utilizados como ligantes, investigou-se a técnica de cromatografia negativa, na qual a IgG foi recuperada nas frações não adsorvidas. Quando OPS foi utilizado, por sua vez, a IgG foi recuperada na fração adsorvida. Para todos os ligantes buscou-se purificar a IgG a partir do soro humano em uma única etapa, visando obter o produto com pureza superior a 90% e teor de IgA inferior a 37 $\mu\text{g mL}^{-1}$, conforme recomendações da World Health Organization (WHO) e a Food and Drug Administration (FDA) para uso humano.

1.3. ESTRATÉGIA DE TRABALHO

A estratégia de trabalho para todos os ligantes consistiu em verificar o efeito do sistema tamponante, da força iônica e do pH na seletividade e no grau de pureza da IgG obtida, sendo também avaliados parâmetros de adsorção. Sempre que possível, os resultados obtidos foram comparados com dados de purificação de IgG apresentados na literatura.

Ligante OPS:

Foram realizados experimentos utilizando o ligante OPS como agente quelante de IMAC, ou seja, com íons metálicos imobilizados. Foram avaliados a densidade dos íons metálicos Cu(II), Co(II) e Ni(II), bem como a seletividade destes ligantes utilizando tampões fosfato de sódio, Tris-HCl e Hepes, em valores de pH entre 6,0 e 8,5.

Por sua vez, quando o ligante OPS foi utilizado sem íon metálico imobilizado (como grupo ionogênico), foram avaliados os seguintes aspectos:

Seletividade: utilizando os tampões fosfato de sódio, Tris e Hepes em valores de pH entre 6,0 e 8,5;

Capacidade dinâmica de adsorção por meio de experimentos de curva de ruptura em condições saturantes para o adsorvente;

Capacidade máxima de adsorção, constante de dissociação e efeitos de cooperatividade, por meio do ajuste dos parâmetros dos modelos de Langmuir e Langmuir-Freundlich a dados de isothermas de adsorção de IgG e HSA.

Ponto isoelétrico e homogeneidade da IgG purificada por meio de eletroforeses nativa e de focalização isoelétrica (IEF).

Ligante TREN:

O ligante TREN foi utilizado somente como grupo ionogênico. Foram avaliados os seguintes aspectos.

Seletividade para a purificação de IgG por cromatografia negativa utilizando os tampões Mops, Mes, Tris-HCl e Bis-Tris, cobrindo uma faixa de pH de 5,5 a 9,0;

Efeito da força iônica (diluição do soro) na condição que proporcionou melhor seletividade. Alimentou-se soro humano sem diluir e diluído 5, 10 e 20 vezes, averiguando a influência de parâmetros como pH e condutividade;

Capacidade dinâmica de adsorção por meio de experimentos de curva de ruptura, ou seja, garantindo a saturação da coluna;

Capacidade máxima de adsorção e constante de dissociação, por meio do ajuste dos parâmetros do modelo de Langmuir a dados de isothermas de adsorção de IgG, HSA e soro humano total;

Aminoácido Lisina:

Foi avaliada a seletividade do aminoácido lisina na purificação de IgG, utilizando-se os tampões fosfato de sódio, Tris-HCl e Hepes, cobrindo a faixa de pH entre 5,5 e 9,0.

Poli-L-Lisina (PLL):

Para o ligante PLL foram estudados os seguintes aspectos:

Seletividade na purificação de IgG por cromatografia negativa, fazendo uso dos tampões Mops, Mes, Tris-HCl, Bis-Tris, Hepes e fosfato de sódio em valores de pH entre 5,5 e 9,0;

Capacidade dinâmica de adsorção por meio de experimentos de curva de ruptura, saturando-se a coluna;

Capacidade máxima de adsorção, constante de dissociação e efeitos de cooperatividade, por meio do ajuste dos parâmetros dos modelos de Langmuir e Langmuir-Freundlich aos dados de isotermas de adsorção de IgG e HSA.

CAPÍTULO 2: REVISÃO BIBLIOGRÁFICA

Nesta revisão bibliográfica serão abordados aspectos relacionados às propriedades biológicas da IgG humana, bem como das técnicas cromatográficas utilizadas para a sua purificação.

2.1. IMUNOGLOBULINA G HUMANA

2.1.1. Importância e relevância

Imunoglobulinas, γ -globulinas, ou anticorpos são glicoproteínas, compostas por cadeias polipeptídicas (82 a 96%) e carboidratos (4 a 18%) (HOLT et al., 2003). Estruturalmente, estas moléculas possuem uma configuração básica em forma de “Y” (Figura 2-1), sendo constituídas de duas cadeias leves e duas cadeias pesadas, sempre em pares idênticos, ligadas entre si por meio de ligações covalentes de dissulfeto (pontes de sulfeto). As cadeias leves de massa molecular aproximada de 25 kDa podem ser do tipo κ (kappa) ou λ (lambda) e são comuns a todas as classes de imunoglobulinas enquanto que as cadeias pesadas de massa molecular variando entre 50 e 70 kDa possuem estruturas e composição distintas em cada classe ou subclasse (HOLT et al., 2003).

Nos seres humanos, estes anticorpos são sintetizados e excretados por células derivadas dos linfócitos B, os plasmócitos, para o plasma, tecidos e secreções. Quando um antígeno entra no organismo humano proveniente de organismos como bactérias ou fungos, por exemplo, um mecanismo de resposta imune é desencadeado fazendo com que sejam produzidos anticorpos que se ligam ao antígeno, neutralizando-o e, posteriormente, eliminando-o, realizando assim a defesa do organismo (imunidade humoral) (ABBAS, 2003).

Enzimas proteolíticas clivam a molécula de anticorpo em diferentes fragmentos característicos. A papaína produz dois fragmentos Fab idênticos, cada um contendo um sítio que se liga ao antígeno, e um fragmento Fc. A pepsina

produz um fragmento $F(ab')_2$ e o restante da molécula é clivada em fragmentos menores, do tipo pFc' (NEWCOMBE e NEWCOMBE, 2007).

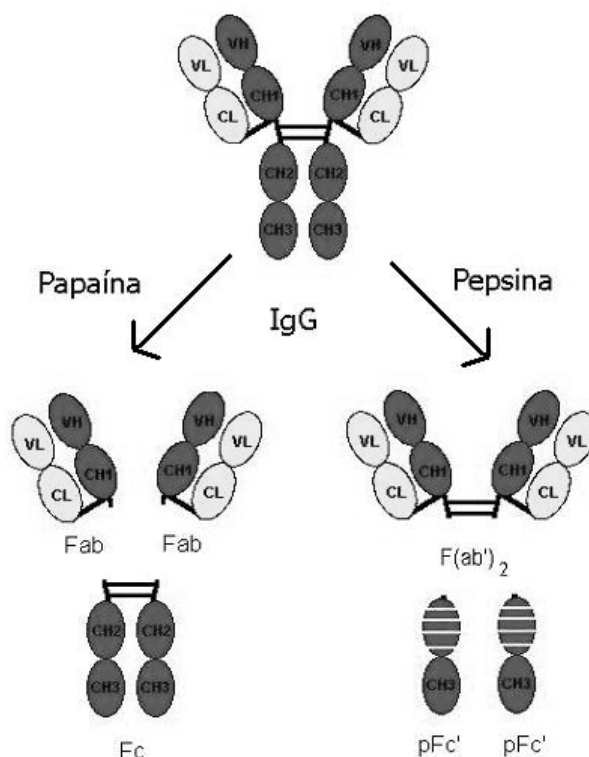


Figura 2-1. Estrutura da IgG e seus fragmentos proteolíticos produzidos pelas enzimas papaína e pepsina (adaptado de ALBERTS et al., 1997). Fab: fragmento que contém um sítio que se liga ao antígeno, localizado na região amino terminal (ab, “antigen-binding”); Fc: fragmento cristalizável que não possui atividade de anticorpo, localizado na região carboxila terminal (c, “crystallizable”); $F(ab')_2$: dois fragmentos Fab ligados covalentemente; pFc' : fragmentos menores no qual a molécula é clivada.

As cadeias leves são formadas por dois domínios, um variável e um constante. Por sua vez, as cadeias pesadas são características das regiões constantes de imunoglobulinas e diferem entre si a partir da estrutura das cadeias pesadas, originando as cinco classes de imunoglobulinas: IgG (γ), IgM (μ), IgA (α), IgD (δ) e IgE (ϵ) (VLUG e VAN REMORTEL, 1989; ALBERTS et al., 1997). A Tabela 2-1 apresenta as principais características destas classes de imunoglobulinas.

O fracionamento industrial do plasma humano, com o objetivo de produzir hemoderivados para fins terapêuticos, tem sido realizado em vários países do mundo a partir do material coletado de doadores pela separação do sangue, ou

diretamente do plasma pelo processo de plasmaferese. Visando suprir as necessidades de proteínas terapêuticas provenientes do plasma, 50 milhões de litros de plasma são coletados por ano no mundo para fracionamento, especialmente de albumina (cerca de 200 kg por milhão de habitantes por ano), de fatores de coagulação sanguínea (cerca de 2,3 UI por habitante por ano) e de IgG (cerca de 15 kg por milhão de habitantes por ano) (SOARES, 2001; Hemoderivados, 2006; SCHÄRER, 2010). É importante ressaltar que, neste século, a IgG passou a ser o produto chave dentre os hemoderivados provenientes do fracionamento do plasma humano, decorrente, por um lado, da sua crescente demanda em aplicações terapêuticas, e por outro, dos fatores anti-hemofílicos recombinantes terem alcançado cada vez mais mercado, substituindo o tradicional concentrado plasmático (COVAS, 2004; Hemoderivados, 2006).

As IgGs humanas são divididas nas subclasses IgG₁, IgG₂, IgG₃ e IgG₄ e são diferenciadas, principalmente, pela região flexível em termos de números de resíduos envolvidos e número de pontes dissulfeto inter-cadeias pesadas. As subclasses de IgG possuem valores de pI distintos: IgG₁ e IgG₃ possuem pI mais altos (7,8 a 9,0) que os da IgG₂ e IgG₄ (6,3 a 8,0). As concentrações relativas das subclasses no plasma humano variam de 68-71% para IgG₁, 19-31% para IgG₂, 4-8% para IgG₃ e 1-7% para IgG₄ (VLUG e VAN REMORTEL, 1989).

Dentre as principais aplicações da IgG, destaca-se o seu uso nos campos terapêutico (preparação de antissoro e anticorpos autocatalíticos), diagnóstico (imunotestes, testes ELISA) e pesquisa e desenvolvimento (uso como ligante em cromatografia de imunoafinidade para purificação de anticorpos e antígenos). Tais aplicações requerem sistemas eficientes para purificação e manutenção da atividade dos constituintes (TODOROVA-BALVAY et al., 2004). Várias técnicas têm sido empregadas para purificação de imunoglobulinas a partir do soro ou plasma humano, tais como precipitação e métodos cromatográficos de troca iônica, afinidade e exclusão molecular. O plasma humano é um fluido biológico constituído de várias proteínas em diferentes concentrações (Tabela 2-2), as quais exibem diferentes funções fisiológicas no organismo (PUTNAM, 1984).

Tabela 2-1. Principais características das cinco classes de imunoglobulinas presentes no plasma humano. Adaptado de VLUG e VAN REMORTEL (1989) e PRIN et al. (1995).

Classe	Massa molecular (kDa)	Forma de apresentação	pI ^a	% ^b	Observações
IgE	190	monômero	-	0,002	Pode estar associada a reações alérgicas e verminoses.
IgD	180	monômero	-	1,0	Sua função biológica ainda não é bem conhecida, mas possivelmente age como um dos principais receptores para antígenos na superfície dos linfócitos B maduros.
IgA	160	dímero	4,0-7,1	10-15	Desempenha um papel importante na defesa do corpo quando se verifica a invasão de microorganismos através da membrana mucosa. Predominante em secreções como saliva, lágrima, leite e muco intestinal.
IgM	900	pentâmero	4,0-9,1	10	Atua no início da resposta imune.
IgG	150	monômero	4,35-9,95	75-80	Predominante em todas as idades, sendo produzida nos últimos estágios da resposta imune. Sua concentração fisiológica média no adulto é 11,7 g L ⁻¹ .

^a Dados de PRIN et al. (1995).

^b Percentagem relativa às cinco classes de Imunoglobulinas presentes no plasma humano.

Tabela 2-2. Principais proteínas do plasma humano, suas massas moleculares e concentração plasmática. De acordo com dados apresentados por PUTNAM (1984).

Proteína	Massa molecular (kDa)	Concentração (mg mL⁻¹)
Albumina	66,3	35 – 55
α1-Lipoproteínas (HDL)	2000	2,9 – 7,7
β-Lipoproteínas (LDL)	170	1,9 – 7,4
α1 – Antitripsina	54,0	2 – 4
Transferrina	76,5	2 – 4
Fibrinogênio	341	2 – 4,5
Haptoglobulina	100-400	3,8 – 7,8
α2 – Macroglobulina	725	1,5 – 4,2
IgG	150	8 – 18
IgM	900	0,6 – 2,5
IgA	160	0,9 – 4,5

As prescrições terapêuticas de IgG humana são indicadas nos casos de imunodeficiências congênitas ou adquiridas, no tratamento de deficiências seletivas de anticorpos, doenças auto-imunes e alguns tipos de câncer (BERNARD et al., 1990; KEMPF et al., 2007). Estas doenças requerem, geralmente, grandes doses de IgG para o seu tratamento (vários gramas por paciente por ano). Como a demanda de imunoglobulinas é alta, os países que não as produzem em larga-escala necessitam importá-las, como era o caso do Brasil, cuja necessidade atual de Imunoglobulinas é de 8,5 toneladas (Hemoderivados, 2006). Felizmente este cenário está mudando para melhor com a criação de plantas industriais do Instituto Butantan e da Empresa Brasileira de Hemoderivados e Biotecnologia (Hemobrás). Criada pela Lei 10.972, de 02/12/2004, a Hemobrás é uma estatal vinculada ao Ministério da Saúde que visa o fortalecimento do complexo industrial da saúde no Brasil. Sua fábrica de hemoderivados estará localizada em Goiana/PE, cuja operação começará em 2014. De acordo com dados do Ministério da Saúde, o empreendimento estimado para a conclusão da obra e implementação da fábrica é de R\$ 540 milhões,

considerado o maior da América Latina no setor (Portal Hemobrás). Já no Instituto Butantan, há uma planta piloto em operação e outra de maior escala está sendo construída pelo Governo do Estado de São Paulo com investimentos de R\$ 60 milhões em uma área total de 10000 m² para processar 150 mil litros de plasma anualmente (JINZENJI, 2008).

É importante se destacar que para a utilização intravenosa de IgG humana, órgãos como a World Health Organization (WHO) e a Food and Drug Administration (FDA) recomendam que os métodos de recuperação e purificação devam garantir que a mesma deva cumprir os requisitos de apresentar pureza superior a 90%, com índice de agregados inferior a 4%, com teor de IgA inferior a 37 µg mL⁻¹ e, principalmente, garantindo a atividade biológica dos anticorpos seja preservada (TANAKA et al., 1998; TANAKA et al., 2000). Já para a IgG utilizada pra aplicações intramusculares, os requisitos são pureza superior a 90% e teor de agregados até 5%.

2.1.2. Métodos utilizados na purificação de IgG

Tradicionalmente as indústrias farmacêuticas purificam IgG a partir do plasma humano pela técnica de precipitação com etanol a frio, de acordo com o procedimento descrito por COHN et al. (1946). Este método baseia-se na mudança de solubilidade das proteínas por ajuste de pH, força iônica, temperatura e concentração de etanol. O plasma humano é descongelado até uma temperatura de 4°C, na qual algumas proteínas têm baixa solubilidade (fibrinogênio e fator VIII). Este precipitado formado (crioprecipitado) é removido e o sobrenadante (criossobrenadante) é fracionado por sucessivas precipitações com etanol. Os produtos resultantes do processo consistem num concentrado de IgG (na fração II ou nas frações II e III) e albumina (na fração V) (COHN et al., 1946, MARTIN, 2006).

Outros processos para purificação de IgG humana foram descritos na literatura, que representam variáveis do método de Cohn, por exemplo, o método de KISTLER e NITSCHMANN (1962). Este método foi desenvolvido para obtenção

de um melhor rendimento e menor tempo de manipulação do produto e é utilizado apenas pela empresa ZLB-Behring e empresas autorizadas (MARTIN, 2006).

Atualmente, métodos mais seletivos (como é o caso dos métodos cromatográficos) são utilizados pelas indústrias farmacêuticas após a precipitação ou em substituição à esta no processo de recuperação e purificação (“dowstream processing”) das proteínas do plasma. As etapas cromatográficas têm sido vistas como operações indispensáveis antes da utilização destas no campo terapêutico, uma vez que proteínas altamente purificadas limitam os riscos de efeitos colaterais no pacientes, aumentando a eficácia clínica (BURNOUF, 1995; BURNOUF e RADOSEVICH, 2001).

Dentre os métodos cromatográficos, destaca-se a cromatografia de afinidade. O primeiro relato de sua utilização é de 1910, entretanto, o grande impulso para o seu uso se deu a partir de 1967, por conta do desenvolvimento do método de imobilização de ligantes em gel de agarose por meio da ativação com brometo de cianogênio (PORATH et al, 1967). A cromatografia de afinidade é uma técnica de adsorção seletiva que está baseada na habilidade de moléculas em solução se ligarem específica e reversivelmente a moléculas complementares, os ligantes, normalmente imobilizados em uma matriz cromatográfica.

Os ligantes mais utilizados para a purificação de anticorpos de diversas fontes são as proteínas A, G e L, consideradas ligantes bioespecíficos (TODOROVA-BALVAY et al., 2004). A proteína A, secretada pela maioria das linhagens da bactéria *Staphylococcus aureus*, apresenta alta afinidade pela parte Fc das imunoglobulinas humanas IgG₁, IgG₂, e IgG₄ e afinidade média pela IgA e IgM humana (BURTON, 1985; BURTON et al., 1986; HUSE et al., 2002; VERDOLIVA et al., 2002), não apresentando, entretanto, nenhuma afinidade pela subclasse IgG₃ humana (LOGHEM, 1982). Esta interação entre proteína A e IgG parece estar caracterizada pela predominância de interação hidrofóbica, juntamente com algumas ligações de hidrogênio e pontes salinas (LJUNBERG et al., 1993; HAHN et al., 2003). Vários autores relatam o uso de proteína A imobilizada em diversos suportes para o isolamento e purificação de IgG humana (FÜGLISTALLER, 1989; MÜLLER-SCHULTE et al., 1991; CHARCOSSET et al., 1995; RODRIGO et al., 1997; DANCETTE et al., 1999; GARIPCAN e DENIZLI,

2002; ISHIHARA et al., 2006; BOI et al., 2008; MA e RAMAKRISHNA, 2008; ALKAN et al., 2009; WOZNIAK-KNOPP et al., 2010).

A proteína G produzida por linhagens de estreptococos do grupo G também é muito utilizada para a purificação de anticorpos por se ligar tanto a fragmentos Fc como Fab da IgG, apresentando, assim, afinidade por todas as subclasses de IgG humana, mas nenhuma afinidade pelas classes IgA, IgM, IgE e IgD (BJORCK e KRONVALL, 1984; ERNTELL et al., 1988). Por apresentar afinidade (alta ou média) por anticorpos de camundongo, rato, hamster, coelho, porco, ovelha, carneiro e galinha, a proteína G imobilizada nos mais variados suportes vem sendo usada como ligante visando a adsorção e purificação destes anticorpos (Sigma-Aldrich Technical Support; BJORCK e KRONVALL, 1984; OLSSON et al., 1987; BOYLE e REIS, 1987; KOCHAN et al., 1996; GOVORUKHINA et al., 2003; STEC et al., 2004; FLORESE et al., 2006; AOIKE, 2007; CHHATRE et al., 2007; PENHA et al., 2010).

A proteína L foi isolada da bactéria *Peptostreptococcus Magnus* em 1985 e possui afinidade apenas pelas cadeias leves do tipo κ das imunoglobulinas (MYHRE e ERNTELL, 1985). Ao se considerar que, no soro humano, aproximadamente 70% das imunoglobulinas apresentam cadeias leves deste tipo, é possível afirmar que o uso de proteína L imobilizada em matrizes cromatográficas permite que até 70% do total de imunoglobulinas do soro ou do plasma humano sejam adsorvidas em uma única etapa (AKERSTRÖM et al., 1994). Essa proteína é empregada como ligante na purificação de IgG e seus fragmentos Fc e Fab, sem interferir no sítio de ligação antígeno-anticorpo (BJORK, 1988; KASTERN et al 1990; KASTERN et al 1992; KIHLEBERG et al 1992; CHÂTEAU et al., 1993; BECKINGHAM et al., 1999; GRAILLE et al 2001; HAIGH et al 2009). A proteína L foi utilizada, também, para estudos *in vitro* visando o tratamento extracorpóreo de doenças auto-imunes por meio da remoção de IgG de cadeia leve kappa (NILSON et al., 1993; DUARTE et al., 2005).

Embora ainda muito utilizada, a cromatografia de afinidade com proteínas A, G ou L imobilizadas apresenta problemas de perda da capacidade da coluna, devido ao desprendimento do ligante após ciclos repetidos de utilização do processo cromatográfico aliada ao alto custo que as torna pouco utilizadas em

larga escala (ANSPACH et al., 1996; BLANK et al., 2001). Para contornar estes problemas, a utilização de ligantes que não possuem interação biológica, porém que apresentam a capacidade de se ligar às proteínas por meio de interações hidrofóbicas, eletrostáticas ou de coordenação (ligantes pseudobioespecíficos) vem sendo abordada na literatura e já é praticada em larga-escala (VIJAYALAKSHMI, 1989). Dentre estes ligantes, destacam-se os ligantes tiofílicos (OSCARSSON e PORATH, 1989, FINGER et al., 1996, KREUTZ et al., 1998, BOSCHETTI, 2001); peptídeos, aminoácidos e ligantes biomiméticos (EL-KAK e VIJAYALAKSHMI, 1991; BUENO et al., 1995; HAUPT et al., 1995; VERDOLIVA et al., 2002; HAIGH et al., 2009; YANG et al., 2009; BARROSO et al. 2010) e íons metálicos imobilizados (PORATH e OLIN, 1983; BODEN et al., 1995; HARI et al., 2000; VANÇAN et al., 2002; SERPA et al., 2005; DAS et al., 2005).

O princípio da técnica de cromatografia de afinidade por íons metálicos imobilizados (IMAC) está baseado na afinidade que íons metálicos quelatados em uma matriz sólida apresentam por certos aminoácidos acessíveis (tais como histidina, cisteína e triptofano) situados na superfície de uma biomolécula em solução (PORATH et al., 1975). Ligações reversíveis do tipo coordenação são formadas por meio da doação de elétrons dos aminoácidos aos íons metálicos imobilizados (PORATH, 1988, WONG et al., 1991). Por esta razão, a técnica de IMAC vem sendo utilizada na purificação de várias proteínas, inclusive anticorpos de diversas fontes.

Devido à presença de uma região rica em histidina presente na região CH3 (domínio constante 3 da cadeia pesada) de várias classes de IgG (humana, murina, cobaia e coelho), a técnica de IMAC vem sendo utilizada para a purificação de anticorpos (HALE e BEIDLER, 1994). Em virtude destes dados, pode-se afirmar que a retenção dos anticorpos em IMAC se daria, pela parte Fc da cadeia pesada. Corroborando os resultados de Hale e Beidler, TODOROVA-BALVAY et al. (2004) utilizando cálculos computacionais de superfície acessível dos resíduos de aminoácidos presentes no domínio Fc da IgG humana (realizada por meio do software Xplor) mostraram que a retenção da IgG ocorre, principalmente, pelos resíduos histidinas localizados nas posições 433 e 435 na parte Fc da IgG humana. É proposto também a presença de histidinas na estrutura

primária dos fragmentos $F(ab')_2$, porém não próximas umas das outras, mas que poderia contribuir para a retenção desses fragmentos por IMAC.

Resultados promissores obtidos por diversos pesquisadores fazem com que a técnica de IMAC seja uma boa alternativa aos métodos convencionas para a purificação de IgG. A literatura registra vários artigos que versam sobre a purificação de IgG de variadas fontes utilizando-se, principalmente, os agente quelantes ácido iminodiacético (IDA), ácido aspártico carboximetilado (CM-Asp), tris-2(aminoetil)amina (TREN), tris carboximetil etilenodiamina (TED) e ácido nitrilo triacético (NTA) e os íons metálicos Cu(II), Ni(II), Co(II), Zn(II) e Fe(II), podendo atingir, em muitos casos, teor de pureza superior a 90% (PORATH e OLIN, 1983; HALE e BEIDLER, 1994; BODEN et al., 1995; ANSPACH et al., 1996; HARI et al., 2000; YOSHIDA et al., 2001; TISHCHENKO et al., 2002).

Resultados do grupo de pesquisa da Profa. Dra. Sônia Maria Alves Bueno também vêm apresentado contribuição no que diz respeito à purificação de IgG por IMAC. VANÇAN et al. (2002) utilizaram géis de agarose com os íons Cu(II), Ni(II), Co(II) e Zn(II) imobilizados via agente quelante ácido iminodiacético (IDA) como adsorventes de IgG humana, mostrando que a técnica de IMAC com os quatro íons metálicos se mostrou promissora para a separação de albumina de soro humano (HSA) e IgG a partir experimentos realizados com a alimentação de plasma humano. RIBEIRO et al. (2008) avaliaram a purificação de IgG a partir do plasma humano, por sua vez, em membranas de afinidade de álcool poli(etileno)vinílico (PEVA) com Ni(II) imobilizado via agentes quelantes IDA e tris-2(aminoetil)amina (TREN). Visando a ampliação de escala, foram realizados experimentos em mini-módulos com as fibras-ocas, atingindo-se pureza de 99% para a IgG eluída.

A purificação de anticorpos monoclonais em membranas de fibra-oca de PEVA foi abordada nos trabalhos de SERPA et al. (2005) e BRESOLIN et al. (2010a), que utilizaram, respectivamente, os agentes quelantes IDA e ácido aspártico carboxi-metilado (CM-Asp). Tendo como alimentação o sobrenadante de cultura celular contendo o anticorpo monoclonal murino IgG₁ anti-TNP, foram alcançados fatores de purificação de 18,7 e 85,9, usando IDA e CM-Asp, respectivamente. Em ambos os casos, de acordo com resultados de ELISA, os

anticorpos puderam ser eluídos sem perda de atividade biológica, uma vez que foi utilizada uma condição de pH fisiológico devido ao uso de Tris como agente competidor na dessorção.

No entanto, RIBEIRO (2006) e BRESOLIN (2006) abriram novas perspectivas ao grupo ao apresentar resultados de experimentos controle utilizando agentes quelantes de IMAC (sem a presença do íon metálico) na purificação de IgG humana e de anticorpos monoclonais, respectivamente. Neste caso, os agentes quelantes atuam como grupos ionogênicos, e, em alguns casos, há a recuperação do anticorpo nas frações não retidas, em um processo denominado cromatografia negativa. Nesta técnica a proteína alvo é recuperada na fração não adsorvida, enquanto todas as outras proteínas contidas na amostra permanecem adsorvidas na matriz, sendo recuperadas nas etapas de eluição e regeneração.

2.1.3. Cromatografia Negativa para purificação de IgG

O princípio da cromatografia negativa para a purificação de IgG a partir do plasma humano sem pré-tratamento foi primeiramente apresentada por PITIOT et al. (2001b) que demonstraram a potencialidade do emprego do ligante aminohexil-histidina imobilizado em gel de agarose, no qual a IgG foi recuperada nas frações não retidas. No entanto, os autores dosaram somente IgG e albumina nas frações cromatográficas, não fornecendo informações sobre a adsorção de outras proteínas que se encontram relativamente em alta concentração no plasma (não dosaram IgM, IgA, transferrina, por exemplo). Além disso, para obter IgG eletroforéticamente com alta pureza, a amostra de plasma necessitou ser diluída vinte vezes antes de ser alimentada na coluna. Para menores fatores de diluição, IgG foi obtida nas frações não retidas contaminada com outras proteínas, principalmente albumina e transferrina.

Segundo os mesmos autores, proteínas do plasma humano são adsorvidas em aminohexil-histidina-agarose a baixos valores de força iônica. A afinidade destas moléculas pelo ligante não se deve a uma função biológica, mas devido a interações hidrofóbicas e de carga. Os grupamentos NH_3^+ e o anel imidazol contribuem predominantemente nas interações eletrostáticas (carga-

carga e carga-dipolo) em cooperação com outras interações tais como as de natureza hidrofóbica, pontes de hidrogênio e van der Waals. A combinação adequada de interações de naturezas distintas pode resultar na obtenção de alta seletividade, reduzindo o número de etapas dos processos de recuperação e purificação e, em alguns casos, solucionando problemas de purificação (MAJORS et al., 2009; ZHAO et al., 2009). No entanto, por causa deste modo misto de operação (“mixed-mode”), o desenvolvimento do método pode ser mais complexo e ainda não é completamente bem entendido (GILAR et al., 2008).

Com base nos estudos de PITIOT e colaboradores (2001b), conclui-se que ligantes com carga positiva contendo grupamentos amina (primária, secundária ou terciária), são excelentes candidatos a serem empregados em processos de purificação de IgG por cromatografia negativa. No entanto, a literatura não registra nenhum processo de purificação de IgG por cromatografia negativa que empregue adsorventes de troca-aniônica ou adsorventes com poliaminas imobilizadas (exceto os trabalhos realizados pelo nosso grupo de pesquisa, com o ligante TREN imobilizado (capítulo 3.2 deste trabalho), e com as diaminas lineares ω -aminohexil e ω -aminodecil imobilizadas (SOUZA, 2009; SOUZA et al., 2010; BRESOLIN et al., 2010b).

2.2. IMAC: CROMATOGRAFIA DE AFINIDADE POR ÍONS METÁLICOS IMOBILIZADOS

A afinidade de biomoléculas por íons metálicos é conhecida desde que Everson e Parker demonstraram que íons metálicos presentes em metaloproteínas eram os principais responsáveis pela adsorção dessas em matrizes contendo quelantes imobilizados (EVERSON E PARKER, 1974). Esta afinidade foi, então, explorada na separação de metaloproteínas e a técnica se popularizou com o trabalho de PORATH et al. (1975), quando o termo Cromatografia de Afinidade por Íons Metálicos Imobilizados (Immobilized Metal-Ion Affinity Chromatography) foi introduzido. A separação e purificação de diferentes biomoléculas (peptídeos, proteínas e ácidos nucleicos), a separação de células a partir de extratos biológicos e estudos de estrutura-função de proteínas são

algumas das aplicações de IMAC em escalas analítica, preparativa e industrial de IMAC (BRESOLIN et al., 2009).

Grupos doadores de elétrons presentes em resíduos de aminoácidos expostos na superfície da biomolécula (tais como o anel imidazol de histidina) são os principais responsáveis pela interação entre a biomolécula e o íon metálico imobilizado, por meio da formação de ligações de coordenação. Entretanto, além das ligações de coordenação, forças como van der Waals, hidrofóbicas e eletrostáticas também podem contribuir no processo de adsorção da biomolécula (SHARMA e AGARWAL, 2002; UEDA et al., 2003; GUTIÉRREZ et al., 2007; BRESOLIN et al., 2009).

Quando a técnica de IMAC é utilizada como parte integrante de um processo de recuperação e purificação de uma determinada biomolécula, é importante se avaliar aspectos como a escolha do íon metálico, do agente quelante e das condições operacionais (vazão, concentração protéica da alimentação, sistema tamponante, força iônica, pH e temperatura) para que ocorra, de fato, a purificação biomolécula de modo a se atingir elevada recuperação e pureza, fazendo com que o processo se torne economicamente viável (PORATH, 1988; WONG et al., 1991; GUTIÉRREZ et al., 2007; BRESOLIN et al., 2009).

Muitos compostos são empregados como agentes quelantes em IMAC, sendo que o mais utilizado é o ácido iminodiacético (IDA), que é tridentado (possui um átomo de nitrogênio e dois átomos de oxigênio para a coordenação), isto é, quelata o íon metálico ocupando três sítios de coordenação. No caso de íons metálicos hexacoordenados, com três sítios ocupados, há disponibilidade para a biomolécula interagir com um dos outros três sítios remanescentes. Agentes quelantes tetradentados, como, por exemplo, o ácido aspártico carboximetilado (CM-Asp), o tris-2(aminoetil)amina (TREN) e o ácido nitrilotriacético (NTA) ocupam quatro sítios de coordenação, quando quelatam íons metálicos hexacoordenados. Agentes quelantes pentadentados, como o tris(carboximetil)etilenodiamina (TED), apresentam uma elevada estabilidade nas ligações de coordenação, porém, apresenta capacidade de adsorção bastante

reduzida (GABERCK-POREKAR e MENART, 2001; GUTIÉRREZ et al., 2007; BRESOLIN et al., 2009).

A maioria das pesquisas desenvolvidas se concentra sobre a utilização de agentes quelantes tridentados ou tetradentados, mas há também uma série de outros ligantes não-tradicionais que também foram sintetizados e usados com sucesso para a separação de proteínas, como, por exemplo, 8-hidroxi-quinolina (8-HQ), dipicolamina (DPA), 1,4,7-triazociclononano (TACN), N-methacrilolil-L-histidina metil éster (MAH) e orto-fosfoserina (OPS), entre outros (GUTIÉRREZ et al., 2007).

O TREN (Figura 2-2a) é um agente quelante tetradentado utilizado em IMAC, que apresenta a peculiaridade de quelatar íons metálicos somente com átomos de nitrogênio presentes em sua estrutura (dois dos quais são primários, um é secundário e o quarto é terciário). Tem sido utilizado quelatado a íons metálicos como Cu(II), Ni(II), Co(II) e Zn(II) para a purificação de diversas proteínas (BODEN et al., 1995; SHARMA e AGARWAL, 2001a; SHARMA e AGARWAL, 2001b; BRESOLIN, 2006, RIBEIRO et al., 2008; PEREIRA, 2009; GÓES et al., 2010)

Dentre os agentes quelantes não-tradicionais, destaca-se o OPS (Figura 2-2b), que é um éster de serina e ácido fosfórico, e, devido a presença dos grupamentos fosfato, carboxila e amina em sua estrutura, apresenta a característica de quelatar íons metálicos. Na ausência de metal imobilizado, entretanto, OPS comporta-se como um trocador de íons e esta habilidade pode também ser explorada para a adsorção de biomoléculas.

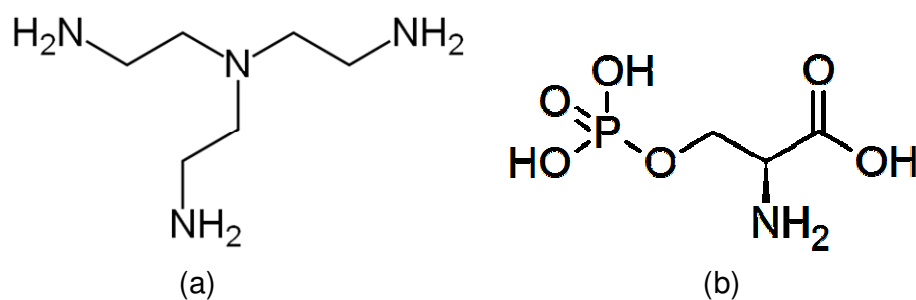


Figura 2-2. Estruturas químicas dos agentes quelantes de IMAC: (a) TREN, (b) OPS

2.3. AMINAS COMO LIGANTES EM CROMATOGRAFIA LÍQUIDA

Com o objetivo de se purificar biomoléculas que apresentam afinidade por grupamentos amino (como, por exemplo, amino oxidases, lactoferrina, plasminogênio e proteínas contendo grupamentos tioésteres), a literatura apresenta vários trabalhos que utilizam adsorventes com aminas imobilizadas para esta finalidade, destacando-se as diaminas 1,5 pentanodiamina (aminopentil), 1,6 hexanodiamina (aminohexil) e 1,10 decanodiamina (aminododecil) (HOUEN et al., 1996; HOUEN e SVENDSEN, 1998; HOUEN, 2001; THOMAS et al., 2001).

Não apenas as diaminas em si, mas também poliaminas com maiores quantidades de grupamentos amino são utilizadas como ligantes em cromatografia líquida. Dentre elas, a literatura enfoca o uso da poli-L-lisina e da espermina. A primeira é empregada como ligante para a remoção de endotoxinas e bilirrubinas encontradas em soluções protéicas como o plasma humano (ANSPACH e PETSCH, 2000; SHI et al., 2005) enquanto que a segunda é utilizada para purificação de receptor de rianodina (SHOSHANBARMATZ e ZARKA, 1992). Poliaminas também podem atuar como braços espaçadores entre a matriz cromatográfica e o ligante propriamente dito, como, por exemplo, ocorre nos adsorventes histidina-aminohexil-Sepharose e N ω -homocisteína-aminohexil-agarose, utilizados, respectivamente, para purificação de IgG (PITIOT et al., 2001) e de anticorpos contra as proteínas N-homocisteinadas (PERTA et al., 2004).

Por possuírem dois ou mais grupamentos amino em sua estrutura, as poliaminas estão sujeitas a interações de natureza eletrostática com as biomoléculas em solução. Quando os ligantes apresentam grupamentos amina primária e secundária, eles são considerados trocadores de íons fracos, uma vez que estes grupos normalmente estão protonados nos valores de pH utilizados na adsorção de biomoléculas (pH < 8,0).

Segundo a literatura, a quantidade de carbonos, bem como o agente de ativação utilizado para a derivatização da matriz cromatográfica apresentam

efeitos significativos na purificação, em adição às condições cromatográficas (sistema tamponante, pH, temperatura e força iônica), devendo ser minuciosamente avaliados para que se atinja elevados rendimento, pureza e fator de purificação. Os métodos de ativação mais empregados em suportes como o gel agarose são a ativação por brometo de cianogênio (CNBr), epícloridrina e bisoxiranos, que reagem com os grupos hidroxila da matriz, tornando-a ativada (GABERC-POREKAR e MENART, 2001; GUTIÉRREZ, 2007).

Com o principal objetivo de evitar impedimentos estéricos, braços espaçadores são acoplados covalentemente à matriz, cujo principal papel é distanciar o ligante da matriz propriamente dita (GABERC-POREKAR e MENART, 2001; GUTIÉRREZ, 2007). A ocorrência de interações hidrofóbicas entre proteínas e matrizes com poliaminas imobilizadas, por sua vez, depende do tamanho do braço espaçador, e estas propriedades têm sido exploradas para purificação de proteínas por cromatografia de interação hidrofóbica, ou ainda, explorando a combinação adequada de interações de naturezas distintas (“mixed-mode”), como eletrostática e hidrofóbica (MAJORS et al., 2009; ZHAO et al., 2009). Contudo, braços espaçadores longos e hidrofóbicos podem também contribuir para ocorrência de interações não específicas entre a biomolécula e o espaçador (HOUEN, 2001).

CAPÍTULO 3: RESULTADOS E DISCUSSÃO

3.1. AGENTE QUELANTE OPS

Neste tópico estão apresentados os resultados experimentais do uso do ligante orto-fosfoserina (OPS) visando a captura de IgG a partir de soro humano. Foi avaliada a capacidade da OPS em quelatar íons metálicos como Cu(II), Ni(II), Co(II) e Al(III), bem como sua seletividade na adsorção de IgG humana, comparando os resultados com o ligante sem metal imobilizado (que se mostrou mais seletivo que na presença de metal imobilizado). O artigo está intitulado como “Comparative evaluation of immobilized metal ion- and metal ion free- o-phosphoserine (OPS) adsorbents: Capture of IgG from human serum” que será submetido para publicação.

Este trabalho teve como objetivo realizar uma avaliação do potencial deste agente quelante visando a captura e/ou purificação de IgG a partir do soro humano, comparando os resultados experimentais com agentes quelantes tradicionais como o IDA e o CM-Asp. Na ausência de metal imobilizado, entretanto, OPS comporta-se como um trocador de íons, sendo utilizado para realizar a adsorção de IgG.

Inicialmente, avaliou-se a densidade dos íons metálicos Cu(II), Ni(II) e Co(II) imobilizados em OPS-agarose, e os dados obtidos foram comparados com os dos agentes quelantes IDA e CM-Asp. Resultados mostraram que a densidade de íons metálicos em OPS seguiu a ordem Cu(II) > Co(II) > Ni(II), sendo significativamente inferior à IDA e CM-Asp para todos os íons metálicos estudados, exceto para CM-Asp-Co(II), que se mostrou inferior a OPS-Co(II). Comparações foram realizadas com dados de densidade de metais destes mesmos agentes quelantes já apresentados na literatura.

A fim de avaliar a seletividade dos adsorventes OPS-Me(II), realizaram-se experimentos cromatográficos alimentando-se 50 µL de soro humano diluído 20

vezes (1,0 mL de solução de soro com, aproximadamente, 3 mg de proteína total) em tampão fosfato de sódio 25 mmol L⁻¹ pH 6,5, pois este tampão proporcionou maiores recuperações de IgG nas frações retidas em um grupo de experimentos que contou também com os tampões Tris-HCl e Hepes, cobrindo uma faixa de pH de 6,0-8,5. Análises de eletroforese SDS-PAGE e nefelometria demonstraram que a IgG foi adsorvida com IgM e traços de HSA quando Co(II) e Ni(II) foram usados. Já quando o Cu(II) e Al(III) foram imobilizados, a IgG adsorvida foi recuperada com impurezas como HSA, Trf e IgM, indicando que estes adsorventes não são seletivos para a purificação de IgG, nas condições estudadas.

Em uma segunda etapa, avaliou-se o desempenho do gel OPS-agarose sem metal imobilizado, aproveitando as características do ligante OPS que lhe permitem atuar como um trocador de íons. Novamente, alimentou-se 50 µL de soro humano diluído 20 vezes (1,0 mL de solução) nos tampões fosfato de sódio, Tris-HCl e Hepes, cobrindo a faixa de pH de 6,0 a 8,5. Quando o tampão fosfato de sódio foi utilizado, observou-se que IgG foi recuperada nas frações retidas juntamente com IgM (em todos os valores de pH avaliados), além de ter ocorrido uma diminuição na quantidade de IgG adsorvida quando o pH foi aumentado de 6,0 até 7,5. HSA, Trf e IgA não foram detectadas nas frações retidas, sendo recuperadas nas frações não-retidas, fazendo com que a IgG recuperada alcançasse uma pureza máxima de 90% em pH 6,5. Esta redução na quantidade de proteína adsorvida com o aumento do pH pode ser explicada em termos da existência de interações eletrostáticas entre proteínas em solução (cada qual com seu pI ou faixa de pI) e o ligante OPS (pK_a = 5,9). No entanto, quando Tris e Hepes foram utilizados, além de IgG e IgM, traços de HSA e Trf também foram detectados nas frações retidas.

Experimentos de curva de ruptura (com a alimentação de 1,25 mL de soro diluído 20 vezes em fosfato de sódio 25 mmol L⁻¹ pH 6,5, totalizando 75,5 mg) mostraram que se conseguiu atingir um fator de purificação de 4,9, sendo a IgG recuperada com pureza de 88%, devido a presença de IgM (33% da IgM alimentada foi recuperada nas frações retidas). A capacidade dinâmica do OPS-agarose obtida foi de 5,8 mg de proteínas totais mL⁻¹ de gel.

A análise da isoterma de adsorção monocomponente (com IgG humana comercial), por meio do ajuste dos parâmetros dos modelos de Langmuir e Langmuir-Freundlich, mostrou que o gel OPS-agarose apresenta uma capacidade máxima de adsorção entre 24,2 e 28,5 mg mL⁻¹, valores muito parecidos com o gel de afinidade Proteína A-Sepharose (20 a 30 mg mL⁻¹), porém com uma constante de dissociação de 10⁻⁵ mol L⁻¹, caracterizando-o como um ligante de média afinidade. Esta análise também mostrou que ocorrem efeitos de cooperatividade positiva, provavelmente devido à natureza heterogênea da adsorção (presença de interações multiponto). A realização dos experimentos de eletroforese nativa e de focalização isoeletrica (IEF) mostrou que ocorre a predominância de interações eletrostáticas na adsorção de IgG humana em OPS-agarose, uma vez que foi detectada IgG apresentando uma faixa de pI de 7,8 a 9,3 nas frações retidas.

A análise conjunta de todos estes dados sugere que, quando se deseja IgG com valores mais elevados de pureza, medidas complementares são necessárias no processo de purificação. Pode-se afirmar que o uso do ligante OPS sem metal imobilizado serviu como uma etapa de captura de IgG a partir de amostras do soro humano utilizando tampões de baixa força iônica, temperatura ambiente e condições de pH próximas à neutralidade. O ligante OPS se mostrou eficiente quando se deseja purificar IgG humana que possui pontos isoeletricos na faixa de 7,8 a 8,5.

O artigo está apresentado na forma do manuscrito a ser enviado para a submissão. Para facilitar o entendimento do leitor, as figuras e tabelas encontram-se inseridas no manuscrito.

**COMPARATIVE EVALUATION OF IMMOBILIZED METAL ION- AND METAL
ION FREE- O-PHOSPHOSERINE (OPS) ADSORBENTS:
CAPTURE OF IgG FROM HUMAN SERUM**

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Abstract

The ortho-phosphoserine (OPS) - a nontraditional chelating agent used in IMAC - immobilized in agarose gel complexed with the borderline Lewis metal ions Ni(II), Co(II), and Cu(II) was evaluated for adsorption of immunoglobulin G (IgG) from human serum in the presence of low ionic strength buffers. The agarose-OPS-Ni(II) and agarose-OPS-Co(II) had higher selectivity for human IgG than the agarose-OPS complexed with the hard Lewis metal ion Al(III), but an identical protein binding capacity and IgG purity values that OPS metal ion free. The agarose-OPS not complexed with metal ions captured IgG with high pI values (7.8 to 9.3) from human serum in a single step. Through breakthrough curve analysis (feeding of 75.5 mg of total protein) an enrichment factor of 4.9 with an IgG purity of 88% was obtained. IgG adsorption equilibrium studies showed that these data followed the Langmuir-Freundlich model, with the fitted cooperativity parameter (n) found to be greater than one (1.74), indicating the presence of positive cooperativity, probably due to multipoint interactions. The maximum IgG binding capacity was 24.2 mg mL^{-1} , near the value of the bioaffinity ligand protein A. The agarose-OPS metal ion free adsorbent provides an attractive alternative for capturing of IgG from human serum.

Keywords: o-phosphoserine, human IgG, adsorption, IMAC, purification, human serum

1. Introduction

O-phosphoserine (OPS) is an ester of serine and phosphoric acid. As an amino acid, OPS is found as a constituent of many proteins (such as phosphorylase, phosvitin, and caseins) as well as a constituent of biological membranes where it acts as a mediator in ion transport action [Renugopalakrishnan et al., 1986]. In caseins, for example, OPS acts as a metal ion chelator, in particular for Mg(II) and Ca(II), inhibiting the activity of phosphoserine phosphatase [Dickson e Perkins, 1974; Baummy et al., 1989; Bernos et al., 1997; Peeraer et al., 2004].

This characteristic of OPS has been exploited by its use as a chelating ligand in immobilized metal ion affinity chromatography (IMAC) for hard Lewis metal ions, such as Al(III), Ca(II), Fe(III), the borderline Cu(II), and the lanthanide Yb(III), when grafted to agarose gel aiming at the study of transferrin (Trf), α 2-macroglobulin, IgG and human serum albumin (HSA) binding characteristics and selectivity [Zachariou et al., 1993; Zachariou and Hearn, 2000]. More recently, Karas [2010] used OPS-Cu(II) grafted onto a dextran support for the separation of peptides and proteins isolated from string bean extracts, indicating that the use of OPS as a chelating agent can be recommended for analysis of plant peptides. The capacity of OPS to chelate metal ions is due to the participation of the phosphate and carboxyl groups and the nitrogen of the amine, characterizing it as a tridentate chelating ligand [Zachariou et al., 1993; Zachariou et al., 1996; Zachariou and Hearn, 2000].

The choice of chelating ligand is of paramount relevance in IMAC. Due to the presence of electron donor atoms in their structures, chelating ligands are responsible for binding metal ions and forming the metal chelates [Sulkowski, 1985]. As a group-specific affinity technique for separating biomolecules, the IMAC principle is based on the reversible interaction via coordination bonds between various amino acid side chains available in the biomolecule and the immobilized metal ions [Porath et al., 1975]. Among the chelating ligands used in IMAC, the tridentate iminodiacetic acid (IDA) and the tetradentates carboxymethylated aspartic acid (CM-Asp) and nitrilotriacetic acid (NTA) have been highlighted with

Lewis borderline metal ions, such as Cu(II), Ni(II), Zn(II), and Co(II) [Boden et al., 1995; Sharma and Agarwal, 2001; Sharma and Agarwal, 2002; Serpa et al., 2005; Ribeiro et al., 2008; Bresolin et al., 2010]. These metal ions are used in the purification of proteins with residues of histidine, tryptophan and cysteine, in which metal ions interact with the aromatic nitrogen of the imidazole groups, indole, and the sulfur of thiol grouping of each amino acid, respectively [Sulkowski, 1989; Chaga, 2001; Gaberc-Porekar and Menart, 2001; Ueda et al., 2003; Gutiérrez et al., 2007; Bresolin et al., 2009].

However, it has been shown that besides the affinity interaction between the chelated metal ion and the biomolecule, interactions of a different nature, including electrostatic, hydrophobic, and van der Waals forces could be involved, being not always possible to determine their relative contributions [Sharma and Agarwal, 2002; Gutiérrez et al., 2007; Bresolin et al., 2009]. In some cases, when combined appropriately, these interactions could be responsible for high selectivity downstream processing and, in some cases, could also solve intractable purification problems [Majors et al., 2009; Zhao et al., 2009].

Zachariou and coworkers [Zachariou et al., 1993; Zachariou and Hearn, 2000] showed the potential of using OPS as a chelating ligand in IMAC as it has shown high selectivity for human Trf and α 2-macroglobulin when hard Lewis metal ions were immobilized, providing attractive alternatives for selective fractionation of human serum proteins. When the borderline Cu(II) and the hard Lewis metal ion Al(III) were chelated with OPS, the IMAC adsorbents did not show any significant affinity for the human IgG in the studied conditions. Although OPS has been used as a chelating ligand for protein isolation and purification by IMAC with hard Lewis metal ions, and the borderline Cu(II) [Zachariou et al., 1993; Zachariou and Hearn, 2000; Karas, 2010], the use of classical Lewis borderline metal ions, such as Ni(II) and Co(II), had not been reported in the literature.

Due to the presence of accessible histidine residues on the surface of IgG [Vançan et al., 2002], IMAC has shown promising results in the purification of human IgG [Ribeiro et al., 2008; Tishchenko et al., 2002; Todorova-Balvay et al., 2004] and different species and subclasses of IgG from animal and vegetal

sources when chelating ligands such as IDA, CM-Asp, and Tris(2-aminoethyl)amine (TREN) complexed with Cu(II), Zn(II), or Ni(II) are used [Hale and Beidler, 1994; Serpa et al., 2005; Das et al., 2005; Zimmerman et al., 2006; Platis et al., 2008; Ramessar et al., 2008; Bresolin et al., 2010].

In this work we studied the effect of the tridentate OPS chelating ligand grafted onto agarose gel beads on the selectivity and capacity for adsorption of human IgG using Cu(II), Ni(II), Co(II), and Al(III) as the metals ions. The objective is to contribute to a better evaluation of the potential of this chelating ligand in the purification of IgG from human serum compared with that of conventional chelating ligands IDA and CM-Asp. Besides differences in coordination number, they also have different numbers and proportions of N, O, and P atoms involved in the formation of the chelating rings. Their performances were compared with the performance of OPS not complexed with metal ions (as a mixed-mode adsorbent for serum protein). Due to the presence of negatively charged phosphate and carboxyl groups, OPS can also be exploited for its ability to adsorb proteins using the ion exchange principle.

The human IgG was adsorbed using sodium phosphate, Tris-HCl, and Hepes buffers (25 mmol L⁻¹, without NaCl) in the pH range of 6.0 to 8.5 in order to find the best experimental condition for IgG capture. The use of low ionic strength adsorption buffers (without NaCl) represents a departure from traditional IMAC by exploiting electrostatic interaction away from the coordination bonds, and it has been shown to be effective in the purification of monoclonal antibodies and polyclonal human IgG [Serpa et al., 2005; Ribeiro et al., 2008; Bresolin et al., 2010]. The dynamic adsorption capacity of IgG was also evaluated by determination of the breakthrough curve. Adsorption equilibrium data on IgG at different initial concentrations was obtained in batch experiments and the experimental data were analyzed in terms of the Langmuir and Langmuir-Freundlich adsorption isotherm models. Parameters pertinent to the adsorption processes such as the apparent dissociation constant (K_d), the maximum binding capacity (Q_m), and the cooperativity were analyzed and discussed.

2. Experimental

2.1. Materials

Crystalline bovine serum albumin (BSA), N-(2-hydroxyethyl)piperazine-N'(2-ethanesulfonic acid) (Hepes), and aluminum nitrate were purchased from Sigma (USA). Sodium phosphate monobasic, sodium phosphate dibasic, Tris(hydroxyethyl amino methane) (Tris), disodium ethylenediamine-tetraacetic acid (EDTA), copper, nickel, and cobalt sulfate were purchased from Merck (Germany). For SDS-PAGE analysis, acrylamide, bis-acrylamide, sodium dodecyl sulphate (SDS), and dithiotrietol were purchased from Bio-Rad (USA). The electrophoresis calibration kit for determination of molecular mass (myosine, 212 kDa; α_2 -macroglobulin, 170 kDa; β -galactosidase, 116 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) was provided by GE Healthcare (USA). Human immunoglobulin G (containing 98.3% IgG according to the nephelometric analysis of IgG, IgM, IgA, HSA, and Trf done in our laboratory) was provided by CSL Behring (Germany). The nephelometric reagents were purchased from Beckman Coulter (USA). The water used for buffer and solution preparation was ultrapure (Milli-Q System, Millipore, USA). All other chemicals were of analytical reagent grade.

2.2. Affinity gels

The o-phospho-L-serine (OPS) immobilized on cross-linked 4% beaded agarose (CNBr-activated gel) was purchased from Sigma (USA) and the agarose (Sepharose-4B) was purchased from GE Healthcare (USA). Agarose activation with epichlorohydrin and iminodiacetic acid (IDA) coupling were carried out as described in the literature [Porath and Olin, 1983]. The synthesis of agarose-CM-Asp gel followed two steps: first, the coupling of L-aspartic acid to epichlorohydrin-activated gel and then the carboxymethylation reaction via nitrogen of Asp [Mantovaara et al., 1991].

2.3. Human serum

Fresh frozen serum samples were defrosted at 37°C. To ensure that proper ionic strength and pH were maintained for optimal binding, it was necessary to dilute serum samples with the appropriated adsorption buffer.

2.4. Chromatographic experiments

All chromatographic procedures were carried out with an automated chromatography system (BioLogic LP Liquid Chromatography System, Bio-Rad, USA) at 25°C at a flow rate of 0.5 mL min⁻¹ (superficial velocity of 38.2 cm h⁻¹). For studies concerning the influence of the buffer system on human protein adsorption, the following loading buffers at 25 mmol L⁻¹ covering a pH range of 6.0 to 8.5 within their respective buffering ranges were used: sodium phosphate, Tris-HCl, and Hepes. The gel was suspended in the loading buffer, degassed, and packed into columns (10.0 cm x 1.0 cm I.D.) to give a bed volume of 1.0 mL. Metal ions were immobilized on IMAC gels that had been previously loaded with Ni(II), Cu(II), and Co(II) from 50 mmol L⁻¹ sulfate solutions and with Al(III) from 50 mmol L⁻¹ nitrate solution. Non specifically bound metal was removed by washing the columns with the loading and elution buffers used in chromatographic experiments. Thus, the columns were equilibrated with loading buffer when no further metal was detected in the outstream.

Human serum diluted 20 times with an appropriate loading buffer was fed into a column previously equilibrated with loading buffer. For all experiments, after protein loading, the column was washed with loading buffer until the absorbance values at 280 nm at the outlet reached the baseline. Elution was performed with the loading buffer containing 0.4 mol L⁻¹ NaCl when OPS-agarose gel not complexed with metal ions was used and by the addition of a competitive agent (Tris or imidazole) when IMAC gels were used.

During all chromatographic steps absorbance at 280 nm was monitored and fractions of 1.0 mL were collected. The Bradford method [Bradford, 1976], nephelometric analysis, and SDS-PAGE were used to analyze the proteins in both nonretained and retained fractions. After each experiment, when IMAC was used, the column was regenerated first with 50 mmol L⁻¹ EDTA at pH 7.0 and then with 50 mmol L⁻¹ NaOH, followed by Milli-Q water and the loading buffer to restore it to its initial condition for a new experiment. When the gels were used without immobilized metal ions, the EDTA step was not used.

2.5. Adsorption breakthrough curves

In order to determine the IgG dynamic capacity of OPS-agarose, adsorption breakthrough curves were obtained at 25°C with an automated chromatography system (BioLogic LP Liquid Chromatography System, Bio-Rad, USA). After equilibrating the gel with the loading buffer (sodium phosphate 25 mmol L⁻¹, pH 6.5), human serum diluted 20 times with loading buffer was pumped through the column at a flow rate of 0.5 mL min⁻¹ (superficial velocity of 38.2 cm h⁻¹). The column outlet absorbance at 280 nm was continuously monitored. The loading of protein solution was stopped when absorbance at 280 nm at the column outlet became constant after an initial increase. The nonretained protein was washed out of the column with loading buffer. The adsorbed proteins were eluted with the adsorption buffer containing 0.4 mol L⁻¹ NaCl. The effluents were monitored as described previously (measurement of absorbance at 280 nm). After elution had been completed, the column was regenerated by washing sequentially with 25 mmol L⁻¹ NaOH, followed by Milli-Q water and the loading buffer.

Protein concentration in the nonretained and retained fractions was determined by the Bradford method [Bradford, 1976] and nephelometry and analyzed by SDS-PAGE under nonreducing conditions.

2.6. Batch adsorption of human IgG on OPS-agarose gel

Experiments aiming at determining the isotherms of IgG adsorption on OPS-agarose gel were carried out (in duplicate) at 25°C in stirred tanks (Eppendorf tubes) containing 50 µL of gel. Prior to adding 1.0 mL aliquots of IgG solutions (0.5 to 20.0 mg mL⁻¹) to the tubes, the gel was equilibrated with degassed 25 mmol L⁻¹ sodium phosphate buffer at pH 6.5. The tubes were agitated for 3 h to establish equilibrium. After this period, protein equilibrium concentration in the liquid phase (C*) was determined by dividing the absorbance at 280 nm (measured with an UV-vis spectrophotometer, Beckman DU 650, USA) by the specific absorptivity of 1.36 mL cm mg⁻¹ (according to the experimental analytical curve). The difference in unbound protein concentration between the experiments did not exceed 2.7%. The mass of protein adsorbed per volume of gel (mg mL⁻¹ of gel), Q*, was calculated as the difference between the amount of protein added and that remaining in the liquid phase after equilibrium divided by the volume of the adsorbent. The plot of Q* versus C* is the equilibrium isotherm. The parameters of the Langmuir (eq. 01) and the Langmuir-Freundlich (eq. 02) models were fitted to the experimental data employing the Levenberg-Marquardt iterative fitting method using Origin® software (Microcal, USA).

$$Q^* = \frac{Q_m C^*}{K_d + C^*} \quad \text{eq. (1)}$$

$$Q^* = \frac{Q_m (C^*)^n}{K_{dLF} + (C^*)^n} \quad \text{eq. (2)}$$

where Q_m is the maximum protein binding capacity; K_d is the dissociation constant, which represents the affinity between protein and adsorbent; K_{dLF} is the apparent dissociation constant; and n is the Langmuir-Freundlich coefficient, which indicates the presence or absence of cooperativity.

2.7. Analytical methods

2.7.1. Protein quantification

Total protein content was determined by the Bradford method using BSA as reference protein [Bradford, 1976]. Concentrations of IgG, IgA, IgM, HSA, and Trf were determined by nephelometry analysis using an Array Protein System (Beckman Coulter, USA) in accordance with the methods provided by the manufacturer. The lowest concentrations detected by the nephelometer are 0.93 mg dL⁻¹ for IgG, 0.62 mg dL⁻¹ for HSA, 1.11 mg dL⁻¹ for IgA, 0.69 mg dL⁻¹ for IgM, and 0.35 mg dL⁻¹ for Trf.

2.7.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of protein samples was performed in 7.5% polyacrylamide gels under nonreducing conditions [Laemmli, 1970] using a Mini-Protean III System (Bio-Rad, USA). The gels were stained with silver nitrate in accordance with Morrissey and coworkers [Morrissey et al., 1981].

2.7.3. Isoelectric focusing (IEF)

To determine the pI of retained and nonretained IgG samples, an IEF analysis was carried out using a PhastSystem (Pharmacia, Sweden) in a pH 3-9 gradient polyacrylamide gel (PhastGel IEF 3-9, GE Healthcare, USA). The gel was stained with silver nitrate in accordance with the methods provided by the manufacturer.

2.7.4. Native PAGE

Aiming to evaluate the homogeneity of retained and nonretained IgG samples, a native PAGE was carried out in a PhastSystem (Pharmacia, Sweden) in a 7.5% polyacrylamide gel (PhastGel Homogeneous 7.5, GE Healthcare, USA). The gel was silver stained in accordance with the methods provided by the manufacturer.

2.7.5. Determination of the amount of immobilized metal

The 1.0 mL metal-loaded columns were washed with 10 column volumes of Milli-Q water, 10 column volumes of sodium acetate buffer 25 mmol L⁻¹, at pH 4.0, 10 column volumes of Milli-Q water followed by elution with EDTA 50 mmol L⁻¹ at pH 6.5. The total amount of Ni(II), Co(II), and Cu(II) in the eluate was determined by reading the absorbance in triplicate on a UV-Vis spectrophotometer (DU 640 Beckman, USA) at 384 nm, 466 nm, and 730 nm for Ni(II), Co(II), and Cu(II), respectively.

3. Results and discussion

3.1. Adsorption of IgG from human serum on IMAC gels

In this section, the chromatography results of human serum solution on IMAC gels in mixed coordination-electrostatic mode are presented (low ionic strength of adsorption buffer). The metal ions Cu(II), Ni(II), Co(II), and Al(III) were immobilized on OPS-agarose and the experimental data of adsorption and purification of IgG from human serum were compared to those in the literature.

3.1.1. Agarose-OPS chelated with borderline Lewis metal ions as an adsorbent for IgG adsorption

Although OPS has been used as a chelating ligand for protein isolation and purification by IMAC with hard Lewis metal ions, such as immobilized Al(III), Ca(II), Fe(III), the lanthanide Yb(III), and the borderline Cu(II) [Zachariou et al., 1993; Zachariou and Hearn, 2000; Karas, 2010], the use of classical Lewis borderline metal ions, such as Ni(II), Zn(II), and Co(II), had not been reported in the literature. Cu(II), Ni(II), and Co(II) ion capacities were evaluated in this work for the chelating

OPS. Experiments with agarose-IDA and agarose-CM-Asp were also performed, and the results were compared to the data in the literature, as summarized in Table 1.

Table 1. Metal ion density ($\mu\text{mol g}^{-1}$ dry gel) for different chelating ligands immobilized on agarose gels.

Metal ion Chelator	Cu(II)	Ni(II)	Co(II)	Reference
OPS	52.0 ± 0.9	24.1 ± 0.2	49.2 ± 0.5	This work ^a
	39.2 ± 0.7	-	-	Zachariou et al., 1993 ^b
IDA	161.3 ± 1.7	121.7 ± 1.1	86.0 ± 0.7	This work ^a
	169.2 ± 1.3	114.1 ± 1.0	-	Ribeiro et al., 2008 ^a
CM-Asp	120.4 ± 1.0	109.5 ± 1.7	22.6 ± 0.3	This work ^a
	115.1 ± 1.3	110.6 ± 1.1	-	Bresolin et al., 2010 ^c

^a using UV-Vis Spectrometry (Beckman, USA) at 730 nm, 466 nm, and 384 nm for Cu(II), Co(II), and Ni(II), respectively.

^b using atomic absorption spectroscopy (CSL, Australia)

^c using atomic absorption spectroscopy (Perkin Elmer, USA)

As can be seen in Table 1, OPS metal ion density followed the order Cu(II) > Co(II) > Ni(II) and was found to be significantly lower than IDA and CM-Asp densities for all metal ions evaluated, except for CM-Asp-Co(II) which is lower than OPS-Co(II). The presence of the phosphate group in OPS results in an energetically less favorable ligand to achieve chelation than the IDA and the CM-Asp which have only carboxyl groups [Zachariou et al., 1996]. According to Lewis principle, the phosphate group is a harder base than a carboxyl group [Pearson, 1968a; Pearson 1968b], therefore the lower affinity of OPS for borderline metal ions than IDA or CM-Asp. However, the order of metal ion density OPS-Co(II) > CM-Asp-Co(II) possibly is due to others factor than Lewis acid-base properties.

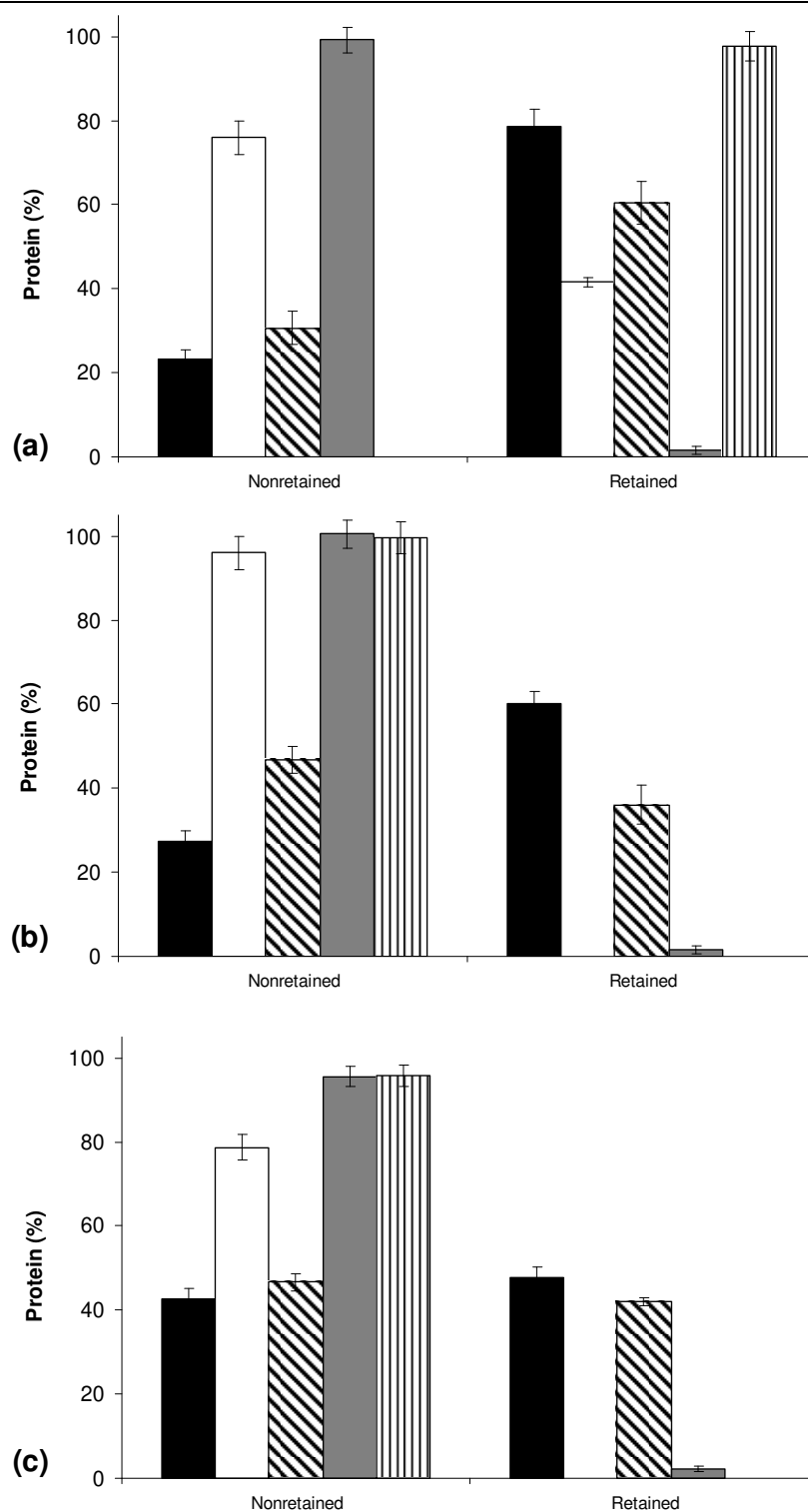


Figure 1. Effect of metal ion (a) Cu(II), (b) Co(II), and (c) Ni(II) on retention and nonretention of human ■ IgG, □ IgA, ■ HSA, ▨ IgM, and ▤ Trf by agarose-OPS-metal ions adsorbent. The retained and nonretained fractions were analyzed by nephelometry.

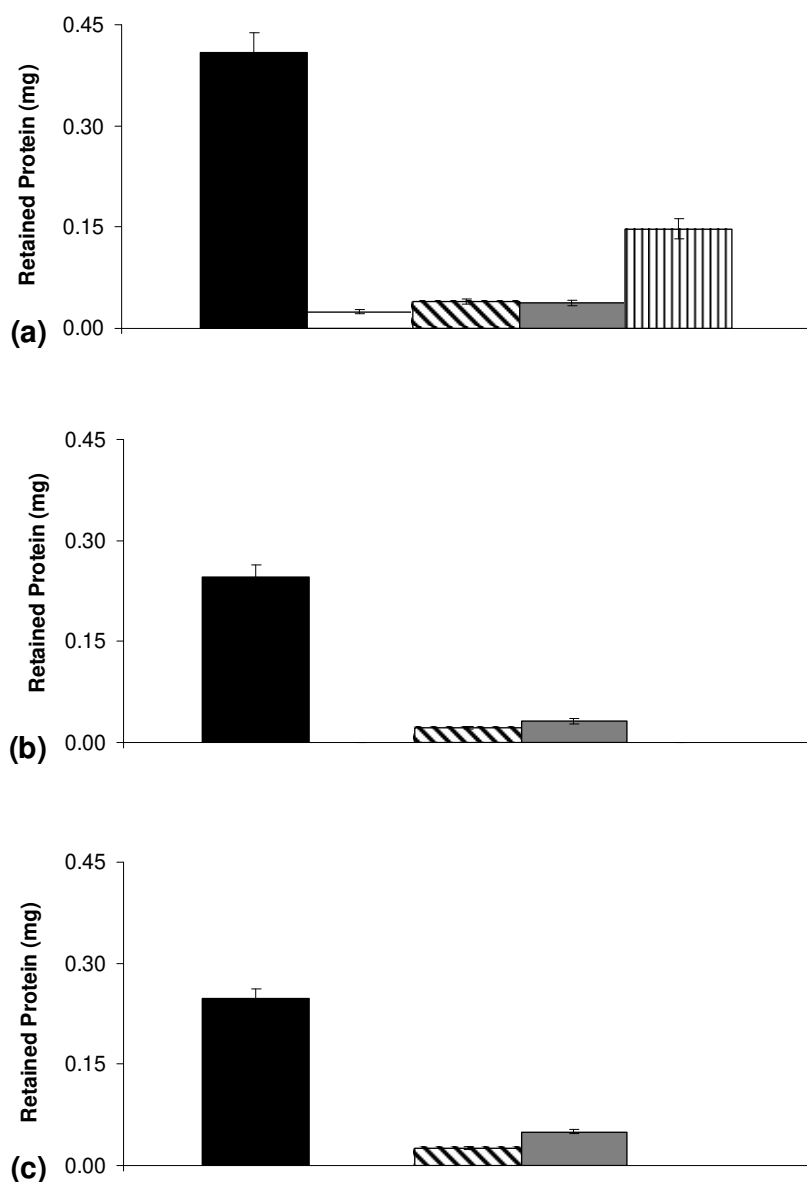


Figure 2. Retention of human ■ IgG, □ IgA, ■ HSA, ▨ IgM, and ▤ Trf by agarose-OPS-metal ions adsorbent (analyzed by nephelometry): (a) Cu(II), (b) Co(II), and (c) Ni(II)

In order to evaluate the selectivity of OPS-metal ions adsorbents in terms of IgG adsorption, chromatographic runs were carried out loading 50 μL of untreated human serum diluted 20 times (1.0 mL of serum solution; around 3 mg of total protein) in a low ionic strength buffer covering a pH range of 6.0 to 8.5. Under low ionic strength buffer condition (25 mmol L^{-1} sodium phosphate, 25 mmol L^{-1} Tris-HCl, and 25 mmol L^{-1} Hepes), these metal-ion chelate adsorbents behave as

pseudocation exchangers [Zachariou and Hearn, 2000]. Using sodium phosphate buffer at pH 6.5, higher IgG recoveries in retained fractions were achieved, among sodium phosphate, Tris-HCl, and Hepes. Figures 1 and 2 indicates the behavior in terms of the percentage of retained and nonretained serum proteins (IgG, IgM, IgA, HSA, and Trf) and mass of these proteins adsorbed, respectively. The order of total protein adsorption capacity was found to be $\text{Cu(II)} > \text{Ni(II)} \cong \text{Co(II)}$.

The nephelometric analysis shows that IgG was adsorbed with IgM and traces of HSA for Co(II) and Ni(II), indicating that Co(II)- and Ni(II)-chelate complexes show higher selectivities for the IgG than OPS-Cu(II) (Figure 1). For Cu(II), as expected, a larger amount of serum protein remained adsorbed. In this buffer condition, the OPS-Cu(II) adsorbed all Trf fed as well as IgG, IgM, and traces of HSA, in contrast the one observed by Zachariou and Hearn, 2000 under high ionic strength conditions at pH 5.5, in which substantial purification of Trf was achieved. At high ionic strength, the electrostatic interactions between the protein and the chelating complex are suppressed and the paired electron type of coordination bonding are promoted [Zachariou and Hearn, 2000].

Sulkowski [1989] showed that Cu(II) (when chelated to IDA) has the ability to adsorb larger amounts of peptides and protein that contain at least one or two accessible residues of histidine. Aromatic amino acids, such as tryptophan, are also important in the retention of proteins in IMAC. However, the contribution of a tryptophan residue can be considered small compared to the effect of histidine on the retention of proteins in IMAC. Cysteine also affects the retention of proteins in IMAC (but only when in reduced form). According to SDS-PAGE analysis, Vançan and coworkers [2002] also observed that both HSA and IgG were adsorbed in agarose-IDA-Cu(II) when a solution of human plasma (in a Mops-imidazole containing 1.0 mol L^{-1} NaCl buffer system) was chromatographed. Following the same analysis, Boden and colleagues [1995] observed the adsorption of both albumin and IgG when a solution of goat serum (in a Mops-acetate containing 1.0 mol L^{-1} NaCl buffer system at pH 7.0) was fed into a Novarose®-TREN-Cu(II) column.

On the other hand, when the nickel and cobalt metal ions were chelated with OPS, these adsorbents showed a higher selectivity for IgG than the IDA-Ni(II), CM-Asp-Ni(II) or IDA-Co(II) adsorbents (IgG, IgM, Trf, and HSA were adsorbed) [Vançan, 1999]. The IgG adsorbed in OPS-Ni(II) and OPS-Co(II) reached purity values of 91% and 92%, respectively, in the elution step (based on IgG, IgM, IgA, HSA, and Trf nephelometric analysis). This higher purity perhaps is due to the lower metal ion content of OPS-Ni(II) and OPS-Co(II) than IDA-Ni(II), CM-Asp-Ni(II), and IDA-Co(II), respectively.

3.1.2. Hard Lewis metal ion Al(III) immobilized on OPS, IDA, and CM-Asp as adsorbents for IgG adsorption

The use of OPS as a chelating ligand with several metal ions, especially the hard Lewis metal ion Al(III) was reported in the literature [Zachariou et al., 1993; Zachariou and Hearn, 2000]. Due to their higher affinity for OPS, Al(III) complexes were found to be more stable than OPS-Cu(II) [Zachariou et al., 1996]. In order to evaluate the selectivity of OPS-Al(III) in the adsorption of human serum proteins at pH values different from those used by Zachariou and Hearn, 2000, chromatographic experiments were performed using the adsorption buffers Tris-HCl, sodium phosphate, and Hepes, covering a pH range of 6.0 to 8.5. However, under all experimental conditions evaluated, IgG was recovered with the main impurities HSA and Trf. The presence of these impurities indicates that the adsorbent was not selective for IgG purification, showing a SDS-PAGE profile similar to that reported in Figure 3(a) and 3(b) corresponding to the use of Tris-HCl as adsorption buffer at 25 mmol L⁻¹ at pH 8.0 and 8.5, respectively.

In order to compare the adsorption of protein from human serum on IMAC gels with immobilized Al(III), we evaluated the performance of the chelating ligands IDA and CM-Asp. Although widely used as chelating ligands in IMAC, the literature contains no work using Al(III) immobilized on IDA and CM-Asp aiming at the purification of human IgG (except the work of Zachariou and Hearn [2000], using IDA). Due to its tendency to bind to phosphate and primary phosphoester, Al(III) is more frequently used in phosphoproteins separation, as reported by Andersson and Porath [1986] and Sulkowski [1988].

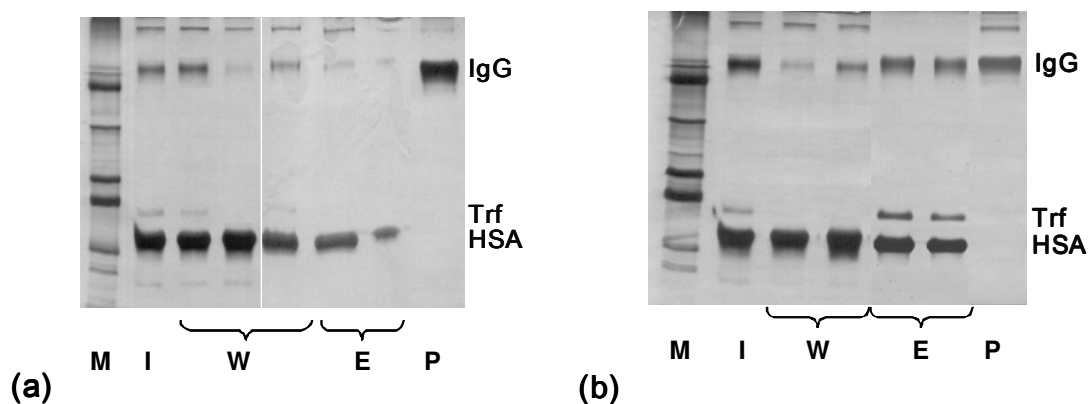


Figure 3. Nonreducing SDS-PAGE analysis of fractions from chromatography on agarose-OPS-Al(III) using Tris-HCl 25 mmol L⁻¹ (a) pH 8.0 and (b) pH 8.5: M, molecular mass protein marker; I, injected human serum solution; W, washing fractions; E, eluted fractions (with the adsorption buffer containing 0.4 mol L⁻¹ NaCl); P, human IgG standard (CSL Behring).

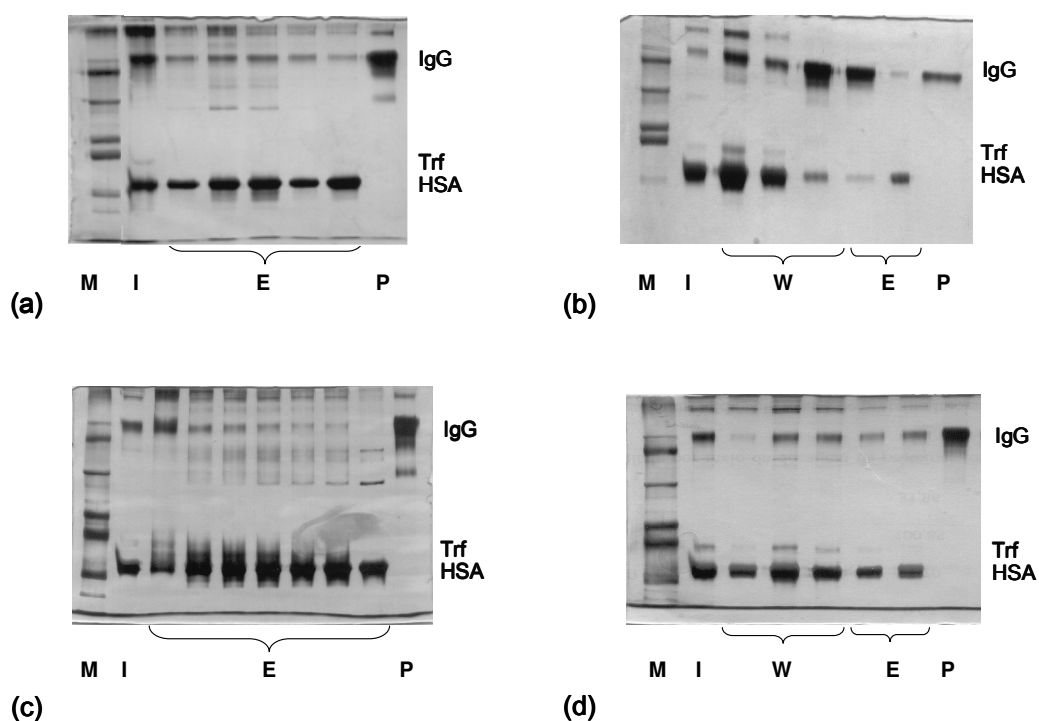


Figure 4. Nonreducing SDS-PAGE analysis of fractions from chromatography on agarose gel using Tris-HCl 25 mmol L⁻¹ (a) pH 7.0 on IDA-Al(III), (b) pH 8.5 on IDA-Al(III), (c) pH 7.0 on CM-Asp-Al(III), (d) pH 8.5 on CM-Asp-Al(III): M, molecular mass protein marker; I, injected human serum solution; W, washing fractions; E, eluted fractions (with Tris-HCl 300 mmol L⁻¹); P, human IgG standard (CSL Behring)

For these experiments, about 6 mg of total serum protein diluted in the adsorption buffer was fed into the column. According to SDS-PAGE, shown in Figures 4(a)-(d), neither IDA-Al(III) nor CM-Asp-Al(III) was a selective adsorbent for adsorption of human IgG when Tris-HCl 25 mmol L⁻¹ adsorption buffer (at pH 7.0 and 8.5) was used as adsorption buffer. The buffers sodium phosphate, and Hepes, covering a pH range of 6.0 to 8.0, were also evaluated (SDS-PAGE not shown) and the selectivities achieved were similar to those achieved with Tris-HCl.

According to the mass balance, at pH 7.0, 71.5% (4.3 mg) and 30.6% (1.8 mg) of the loaded protein was adsorbed on IDA-Al(III) and CM-Asp-Al(III), respectively. When the pH was increased to 8.5, the percentage of protein adsorbed was significantly lower: 7.0% and 11.5% for IDA-Al(III) and CM-Asp-Al(III), respectively. It is important to note that at pH 8.5, part of the IgG fed was recovered in the elution, but together with HSA, showing that they were not selective adsorbents for the purification of IgG.

Zachariou and Hearn [2000] presented a comparison for the adsorption of human serum proteins (5 mg) on IDA and OPS complexed with Al(III) and the metal ion free adsorbents using as adsorption buffer 30 mmol L⁻¹ Mes or Hepes containing 30 mmol L⁻¹ imidazole at pH 5.5 and 8.0. The amount of total protein adsorbed for IDA complexed with Al(III) and metal ion free adsorbent were similar and decreased from 42% to 6% when the pH adsorption buffer was increased from 5.5 to 8.0. For OPS, the higher amount of serum protein bound to OPS metal ion free than OPS-Al(III) indicating that the Al(III) had not contributed significantly to the increase in the amount of total protein adsorbed (quantified at 54.1 $\mu\text{mol g}^{-1}$ dry gel using atomic absorption spectroscopy).

3.2. Adsorption of IgG from human serum on OPS not complexed with metal ions

Due to the presence of phosphate and carboxyl groups, and the nitrogen of the amine, OPS can also be exploited for its ability to adsorb proteins using the ion exchange principle. In this section, the effect of pH on IgG recovery and selectivity as well as the results of the dynamic capacity from the breakthrough curve experiments will be shown.

3.2.1. Effect of pH on IgG recovery and the selectivity of the adsorbent

To evaluate the selectivity of the agarose-OPS adsorbent, the purity of the adsorbed IgG, and the percentages of total protein and IgG, unbound and eluted, were analyzed.

The pH and the nature of the buffer in the mobile phase had a significant impact on IgG adsorption. The charged state of OPS ionizable groups (phosphate, carboxyl, and amine) and the net charge of each serum protein are affected by the pH in the range studied [Roque et al., 2005]. The range of pH considered (6.0 to 8.5) is within the pH values commonly employed in chromatography by maintaining the protein in an environment similar to that of biological fluids.

The adsorption process using agarose-OPS was investigated loading 50 μL of untreated human serum diluted 20 times (1.0 mL of serum solution; around 3 mg of total protein) with three different low ionic strength buffers (25 mmol L^{-1} sodium phosphate, 25 mmol L^{-1} Tris-HCl, and 25 mmol L^{-1} Hepes), covering a pH range of 6.0 to 8.5 in order to determine the most favorable condition aiming at the selective adsorption of IgG. Retained and nonretained fractions were analyzed by the Bradford method [1976], nephelometry, and SDS-PAGE.

When sodium phosphate buffer was used (Table 2), a decrease in adsorbed IgG was observed when the pH was increased from 6.0 to 7.5. HSA, Trf, and IgA were not detected in retained fractions, but were recovered in nonretained fractions. However, when Tris and Hepes were used, besides IgG and IgM, traces of HSA and Trf were also detected in retained fractions (data not shown).

Table 2. Effect of the pH of sodium phosphate buffers in column chromatography on human serum in agarose-OPS. Feed: 50 μL of human serum diluted 20 times (total 1.0 mL). Loading buffer: Sodium phosphate 25 mmol L^{-1} . Elution: Sodium phosphate 25 mmol L^{-1} , NaCl 0.4 mol L^{-1} .

pH		6.0	6.5	7.0	7.5
Injection (mg)	IgG	0.47	0.50	0.42	0.37
	IgM	0.06	0.06	0.06	0.05
	IgA	0.06	0.06	0.06	0.04
	HSA	2.59	2.17	2.61	1.73
	Trf	0.14	0.15	0.13	0.11
Washing (mg)	IgG	0.07	0.18	0.21	0.29
	IgM	0.03	0.03	0.03	0.03
	IgA	0.06	0.06	0.06	0.04
	HSA	2.44	2.07	2.47	1.64
	Trf	0.13	0.15	0.13	0.11
Elution (mg) ^a	IgG	0.36	0.29	0.19	0.04
	IgM	0.03	0.03	0.03	0.02
IgG recovery (%) ^b		90	93	89	90
IgG purity in elution step (%) ^c		92	91	86	67

^a In elution, the amounts of IgA, HSA, and Trf were lower than the detectable range in the Array Protein System.

^b IgG recovery: mass ratio of the sum of IgG in the washing and elution steps to IgG mass in the injection.

^c IgG purity: mass ratio of IgG to the sum of proteins (IgG, HSA, Trf, IgA, and IgM) in the elution step.

Zachariou and Hearn [2000] compared the adsorption of a solution of human serum proteins (5 mg) to Al(III), Ca(II), Fe(III), Cu(II), and Yb(III) immobilized on agarose-OPS (Sephacrose 4B), using agarose-OPS metal ion free as a control. In all experiments, these authors used a low ionic strength (30 mmol L^{-1} Mes or Hepes, 30 mmol L^{-1} imidazole) and a high ionic strength (30 mmol L^{-1}

Mes or Hepes, 30 mmol L⁻¹ imidazole, 0.44 mol L⁻¹ NaCl) adsorption buffer covering a pH range of 5.5 to 8.0. In the experiments performed with OPS not complexed with metal ions at the low ionic strength, the authors observed a decrease from 34% to 16% in total retained protein (determined in accordance with Bradford [1976]) when pH was increased from 5.5 to 8.0. However, these fractions had not been quantified in terms of individual serum protein content. On the other hand, when the high ionic strength adsorption buffer was used, solely minimal amounts of HSA were retained only at a pH of 5.5, according to SDS-PAGE analysis.

This reduction in the amount of adsorbed protein can be explained in terms of the existence of electrostatic interactions between proteins in solution and the ligand OPS. According Zachariou et al., [1996] corroborated by Smiechowsky [2010], OPS has four titratable groups with pK_{a1} not determined, $pK_{a2} = 2.19 \pm 0.01$, $pK_{a3} = 5.78 \pm 0.01$, and $pK_{a4} = 9.85 \pm 0.01$ and is negatively charged at pH values higher than 5.78 (with the intensity of these negative charges increasing as pH is increased). HSA (pI of 4.9, Putnam, 1984), Trf (5.2 < pI < 5.9; Putnam, 1984) and IgA (4.7 < pI < 5.9; Prin et al., 1995) are also negatively charged in the buffer range studied. IgM and IgG, for instance, have a wider range of pI (IgM: 4.0 < pI < 9.1; IgG: 4.3 < pI < 9.9; Prin et al., 1995) consisting of neutral and positively and negatively charged molecules. Due to these pI distributions, the adsorption of both IgG and IgM molecules with positive charges can occur in the immobilized OPS ligand metal ion free.

According to the results presented in this topic (Table 2), loading 50 μ L of untreated human serum diluted 20 times (1.0 mL of serum solution) in sodium phosphate buffer 25 mmol L⁻¹ at pH of both 6.0 and 6.5, allowed the respective recovery of 90 and 93% of the IgG fed, reaching purity values higher than 90% in the elution step (based on IgG, IgM, IgA, HSA, and Trf nephelometric analysis).

The OPS metal ion free had not exhibited a decrease in selectivity when compared with OPS-Ni(II) and OPS-Co(II). The behavior of the OPS metal ion free adsorbent was very similar to the borderline metal ions OPS-Ni(II) and OPS-Co(II) adsorbents, with an identical protein binding capacity and IgG purity values. These

results highlight the selective nature of agarose-OPS metal ion free adsorbent for IgG purification from human serum solutions.

3.2.2. Determination of dynamic adsorption capacity of agarose-OPS metal ion free

As phosphate buffer experiments with human serum diluted 20 times had shown a possible purification of human IgG, we performed human serum adsorption experiments in a dynamic mode in order to determine the dynamic adsorption capacity of agarose-OPS. This capacity was estimated by a dynamic chromatography overloading of a 1.0 mL bed volume column with a 25.0 mL solution of human serum (1.25 mL of serum diluted 20 times) in sodium phosphate 25 mmol L⁻¹ pH 6.5 (75.45 mg of total protein).

Table 3. Nephelometric analysis of the breakthrough for agarose-OPS. Feed: 25.0 mL of human serum diluted 20 times. Loading buffer: Sodium phosphate 25 mmol L⁻¹, pH 6.5. Elution: Sodium phosphate 25 mmol L⁻¹, NaCl 0.4 mol L⁻¹, pH 6.5.

Steps	Proteins (mg)					IgG Purification	
	IgG	HSA	Trf	IgA	IgM	Purity ^a (%)	PF ^b
Human serum diluted 20 times	12.09	52.75	3.50	1.33	1.75	16.9	1.0
Flowthrough (fractions 1-25)	6.18	48.25	3.25	1.02	0.97	10.3	0.6
Washing (fractions 26-35)	1.33	6.24	0.44	0.26	0.14	15.8	0.9
Elution (fractions 48-55)	4.38	n.d. ^c	n.d.	n.d.	0.58	88.4	5.2
Regeneration	-	-	-	-	-	-	-
Total recovery	11.88	54.49	3.69	1.28	1.68	-	-

^a Purity: mass ratio of IgG to the sum of proteins IgG, HSA, Trf, IgA, and IgM.

^b PF: Purification factor

^c n.d.: Values lower than the detectable range in the Array Protein System

Bed volume: 1.0 mL

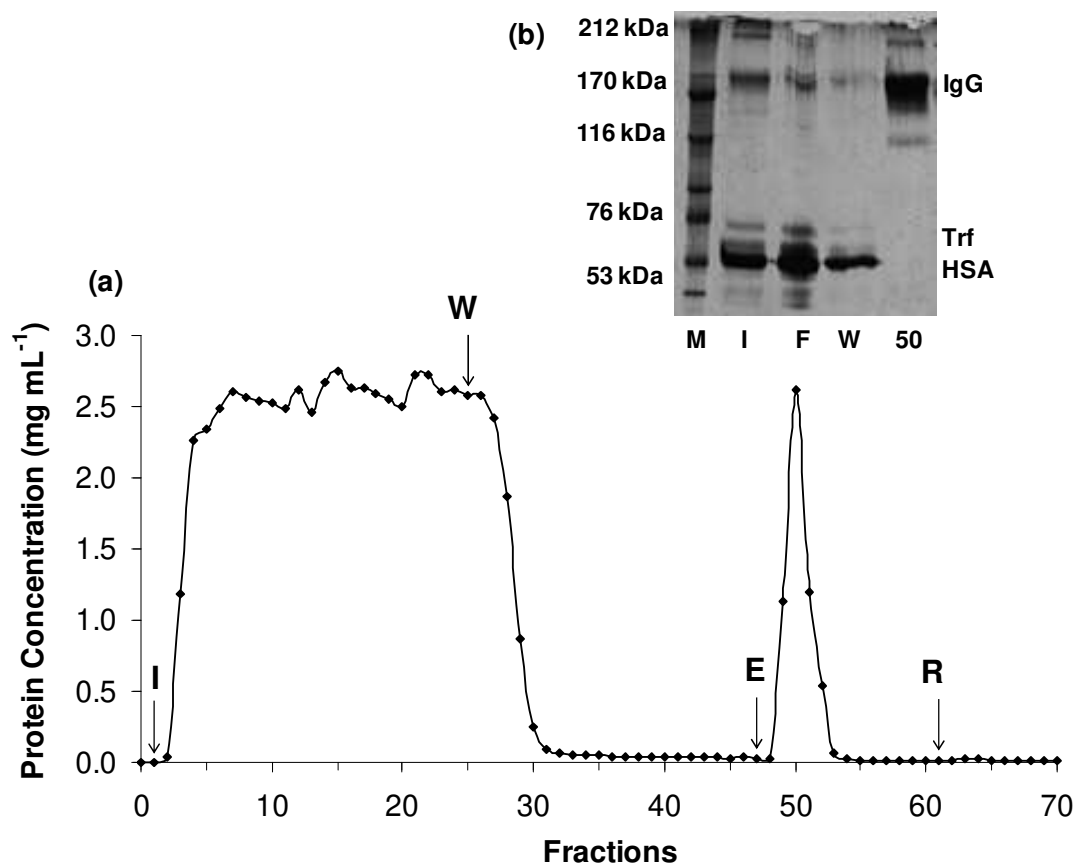


Figure 5. (a) Chromatogram and (b) SDS-PAGE analysis (under nonreducing conditions) of fractions of the breakthrough curve of human serum diluted 20 times in sodium phosphate 25 mmol L⁻¹ at pH 6.5 on agarose-OPS gel. Flow rate: 0.5 mL min⁻¹; I: injection, 25.0 mL at 3.02 mg of total protein mL⁻¹; W: washing, sodium phosphate buffer at pH 6.5; E: elution, sodium phosphate buffer at pH 6.5 containing 0.4 mol L⁻¹ of NaCl; R: regeneration, 25 mmol L⁻¹ of NaOH. In SDS-PAGE, M, molecular mass protein marker; I, initial solution; F, pool of flowthrough fractions (1-25); W, pool of washing fractions (26-35); 50, elution fraction #50.

The breakthrough curve and SDS-PAGE (Figure 5) and nephelometric analysis (Table 3) showed that both IgG and IgM were adsorbed, as shown in the experiments performed by loading 50 μ L of human serum diluted 20 times (1.0 mL of serum solution), reaching a dynamic capacity of 5.8 mg of total protein mL⁻¹ of gel. A purification factor of 5.2 was achieved for eluted IgG, but due to the presence of IgM, IgG purity (based on the mass ratio of IgG to the sum of proteins

IgG, HSA, Trf, IgA, and IgM) reached 88.4%, close to the 90.6% obtained in the experiments performed feeding only 50 μ L of diluted human serum (1.0 mL of total solution).

3.3. IgG adsorption from single protein solution in agarose-OPS adsorbent

3.3.1. IEF and Native PAGE

When human IgG is obtained from human serum, it has different subclasses (IgG₁, IgG₂, IgG₃, and IgG₄) with a broad range of isoelectric points (pI) due to its polyclonal origin: 4.3 to 9.9 [Prin et al., 1995]. If the predominant type of interactions between adsorbent and adsorbate is of an electrostatic nature, it is necessary to know the exact pI of retained and nonretained fractions of the chromatography.

An experiment was performed by feeding into a 1.0 mL bed of agarose-OPS an amount of 1.0 mg (nonsaturation condition) of commercial human IgG diluted in sodium phosphate 25 mmol L⁻¹, pH 6.5 (1.0 mg mL⁻¹) aiming at the subsequent analysis of IEF of the feed and retained and nonretained IgG molecules (Figure 6(a)).

Nonretained IgG (55%) had a pI range of 5.85 to 8.15, comprising positively charged, neutral, and slightly negative IgG molecules at pH 6.5. Retained IgG (45%) was positively charged at pH 6.5 with pI values ranging from 7.8 to 9.3, with more intensity between 8.15 and 8.45, indicating the predominance of interactions of an electrostatic nature between these molecules and the negatively charged adsorbent [Zachariou et al., 1996; Smiechowski, 2010]. However, other interactions could also occur to a lesser degree.

As the agarose-OPS gel was CNBr-activated, only one spacer atom was introduced, thus minimizing possible hydrophobic interactions. According Sigma-Aldrich certificate of analysis, the matrix attachment is promoted by the amino group of OPS, which favors the interaction of IgG (positively charged, as shown in the IEF of Figure 6a) and the phosphate and carboxyl groups (negatively charged).

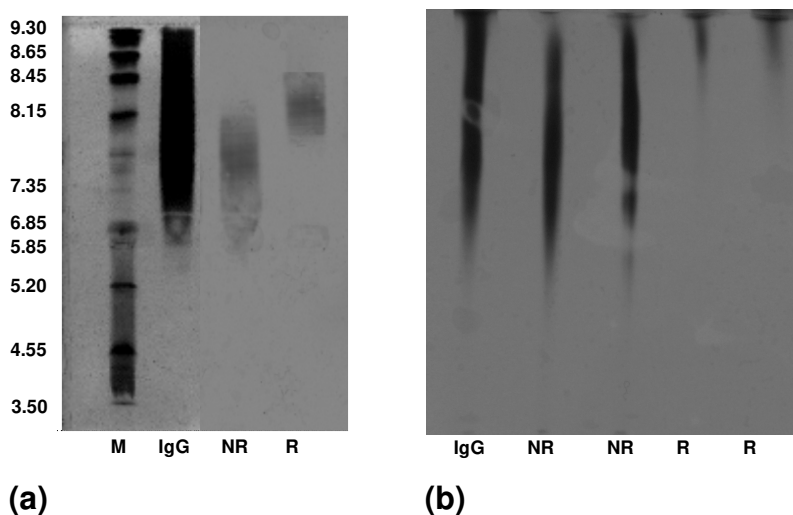


Figure 6. (a) Isoelectric focusing (IEF) and (b) Native PAGE of nonretained (NR) and retained (R) human IgG on agarose-OPS (loading and washing buffer: sodium phosphate 25 mmol L⁻¹, pH 6.5; elution buffer: sodium phosphate 25 mmol L⁻¹, NaCl 0.4 mol L⁻¹, pH 6.5). M: IEF pI 3-9 marker (GE Healthcare), IgG: human IgG (CSL Behring).

When analyzed together, IEF (Figure 6(a)) and native PAGE (Figure 6(b)) show that the procedure presented in this paper was able to capture human IgG, since native PAGE also has the potential for separating proteins of identical molecular weights which cannot be done with SDS-PAGE. As human IgG is polyclonal (containing the subclasses IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, and IgG₄) the retained IgG was not expected to be homogeneous, since it had a range of pI instead of only one pI in the IEF.

3.3.2. Determination of IgG adsorption isotherm

In order to evaluate the thermodynamic parameters, such as IgG maximum binding capacity (Q_m) the dissociation constant (K_d and K_{dLF}), and the cooperativity parameter (n), adsorption isotherms were determined from experimental data obtained in batch adsorption experiments at 25°C using agarose-OPS gel in sodium phosphate 25 mmol L⁻¹ at pH 6.5. The experimental data were analyzed

using the Langmuir and Langmuir-Freundlich models (Table 4). A comparison between experimental and theoretical profiles for human IgG adsorption onto agarose-OPS is shown in Figure 7.

Table 4. Parameters of the Langmuir and Langmuir-Freundlich isotherm models adjusted to experimental IgG adsorption data.

Parameters	Langmuir	Langmuir-Freundlich
Q_m (mg mL ⁻¹)	28.5 ± 1.7	24.2 ± 0.6
K_d (mol L ⁻¹)	$(1.6 \pm 0.7) \times 10^{-5}$	—
K_{dLF} (mol L ⁻¹)	—	$(1.7 \pm 0.2) \times 10^{-5}$
n	—	1.74 ± 0.15
R^2	0.960	0.994
χ^2	3.138	0.546

In accordance with Table 4, the Langmuir-Freundlich model described the IgG adsorption data more satisfactorily than the Langmuir model. The fitted cooperativity parameter of the Langmuir-Freundlich model (n) was found to be greater than one (1.74), indicating the presence of positive cooperativity probably due to the heterogeneous nature of the adsorption (multipoint interactions). In the case of multipoint interactions, one adsorbed IgG molecule tends to favor the adsorption of other IgG molecules [Johnson and Arnold, 1995].

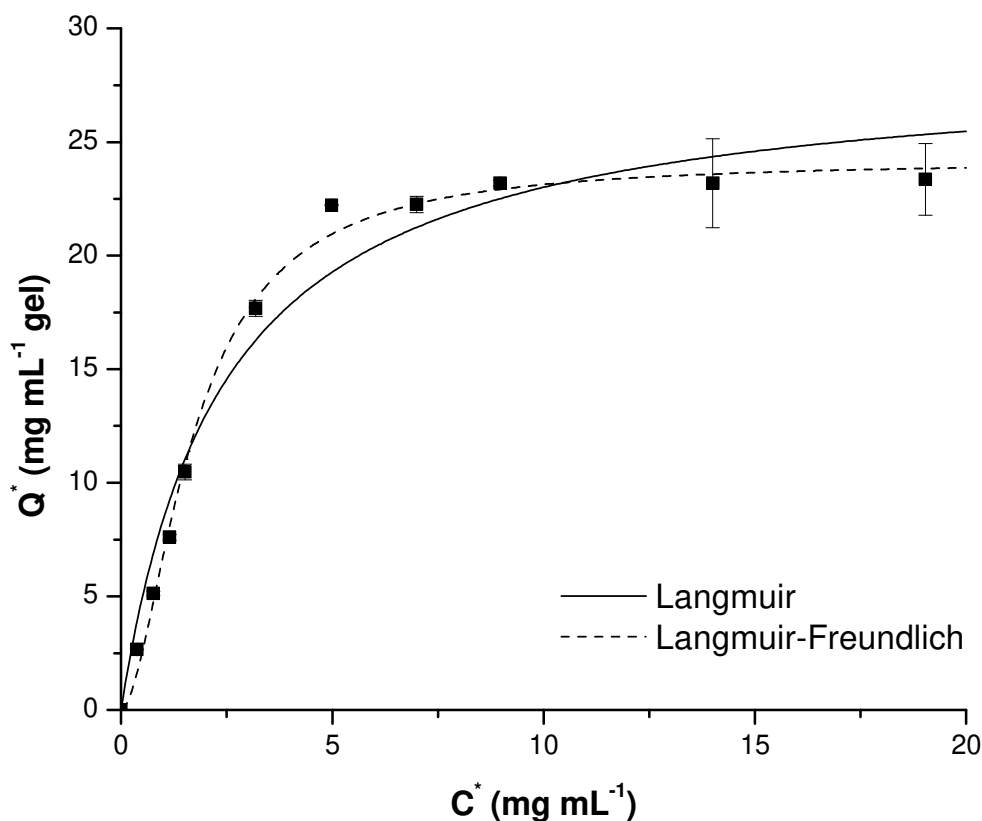


Figure 7. Experimental adsorption isotherms of human IgG on agarose-OPS gel (OPS not complexed with metal ions) with sodium phosphate 25 mmol L⁻¹ buffer pH 6.5 at 25°C. The solid and dashed lines correspond to fitting (nonlinear regression) of experimental data according to the Langmuir and Langmuir-Freundlich models, respectively.

According to both models, IgG maximum adsorption capacity (Q_m) values were in the range of 24.2 to 28.5 mg mL⁻¹. These values are also in the same order of magnitude of experimental data reported for human IgG adsorption onto the bioaffinity adsorbent protein A-Sepharose CL 4B (20-30 mg mL⁻¹ [Sigma-Aldrich and GE Healthcare product information]). For instance, the dissociation constant (K_d and K_{dLF} parameters) values were of the order of magnitude of 10⁻⁵ mol L⁻¹, which indicates that OPS (not complexed with metal ions) can be considered a low-affinity media for IgG adsorption [Vijayalakshmi, 1989].

4. Conclusions

In the present work, we demonstrated that under low ionic strength buffer condition, the selectivity and protein binding capacity of agarose-OPS-Ni(II) and agarose-OPS-Co(II) were very similar to agarose-OPS metal ion free adsorbent. The nature and the pH of the buffer influenced the adsorption of IgG and serum proteins (mainly IgM) due to the occurrence of electrostatic interactions (in the case of agarose-OPS) and mixed coordination-electrostatic interactions (in the case of agarose-OPS-metal ions) between charged molecules and the ligand. It was possible to use agarose-OPS as an adsorbent aiming at the capture of IgG with high pI values (7.8 to 9.3) from human serum in a single step. In accordance with the Langmuir-Freundlich model, a positive cooperativity effect was observed for agarose-OPS and the maximum adsorption capacity was found to be 28.5 mg mL⁻¹ near the value of the bioaffinity ligand protein A, but with a dissociation constant of the order of 10⁻⁵ mol L⁻¹, indicating that agarose-OPS can be considered a low-affinity media.

Thus, agarose-OPS constitutes an alternative adsorbent for the capture of human IgG with potential for integration into large-scale plasma fractionation processes.

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3.2. AGENTE QUELANTE TREN

Neste tópico estão apresentados os resultados experimentais do uso do agente quelante Tris(2-aminoetil)amina (TREN) sem metal imobilizado como ligante para purificação de IgG humana por cromatografia negativa, com o artigo intitulado “Adsorption of human serum proteins onto TREN-agarose: Purification of human IgG by negative chromatography”, publicado em *Journal of Chromatography B*, v. 877, p. 17-23, 2009. DOI: 10.1016/j.jchromb.2008.11.008.

O TREN é um agente quelante tetradentado utilizado em IMAC. Devido à quantidade de grupamentos amino disponíveis para interação com proteínas, avaliou-se o uso do TREN imobilizado em gel de agarose para a purificação de IgG a partir do soro humano.

Inicialmente foi avaliado o comportamento da adsorção de proteínas do soro humano utilizando quatro diferentes tampões de adsorção (Mops, Mes, Tris-HCl e Bis-Tris), na faixa de pH entre 5,5 e 8,0, visando a seleção de uma condição que permitisse a maior recuperação de IgG purificada nas frações não retidas (princípio da cromatografia negativa). Entretanto, quando foi usado o tampão Mes 25 mmol L⁻¹ em pH 6,5, 68% da IgG alimentada foi recuperada nas frações não retidas com pureza superior a 95%. A IgG remanescente foi adsorvida, provavelmente por interações eletrostáticas, uma vez que foi possível recuperá-la nas frações de eluição (pelo acréscimo de NaCl ao tampão de adsorção – aumento da força iônica,) juntamente com outras proteínas do soro humano (tais como HSA, IgM, IgA, Trf, entre outras).

Em uma segunda etapa, visando definir a diluição da solução de alimentação, experimentos cromatográficos foram realizados usando soro humano “in natura” ou diluído 5 e 10 vezes em tampão Mes 25 mmol L⁻¹ pH 6,5 e os resultados comparados com os experimentos que contaram com a alimentação de soro diluído 20 vezes. Quando foi utilizado soro diluído 10 vezes, 73,3% da IgG alimentada foi recuperada nas frações não-retidas apresentado pureza elevada (com base em SDS-PAGE e IgG, IgM, IgA, HSA e Trf), uma vez que um grau de pureza similar ao da IgG comercial foi alcançado.

Experimentos de curva de ruptura (com a alimentação de 34 mL de soro diluído 20 vezes em Mes 25 mmol L⁻¹ pH 6,5) mostraram que conseguiu-se recuperar 7,0 mg IgG não adsorvida por mL de gel, sendo que a HSA foi detectada apenas após a alimentação de 12 mL de soro, valor este que foi definido como sendo o ponto de ruptura.

A análise das isotermas de adsorção monocomponente (HSA ou IgG) e do soro humano, por meio do ajuste dos parâmetros do modelo de Langmuir, mostrou a elevada capacidade de adsorção do gel agarose-TREN (191,7 mg mL⁻¹ e 66,6 mg mL⁻¹, para HSA e proteínas totais do soro, respectivamente). A adsorção de HSA foi favorecida ($K_d = 4,97 \times 10^{-6}$ mol L⁻¹) quando comparada com a de IgG ($K_d = 2,11 \times 10^{-5}$ mol L⁻¹).

A análise conjunta de todos estes dados sugere que a cromatografia negativa em agarose-TREN é uma técnica potencial para a purificação de IgG do soro humano. A recuperação de IgG a partir soro humano pode ser alcançado sob condições brandas de pH, baixa força iônica, e temperatura ambiente. Do ponto de vista do processo em larga escala, a cromatografia negativa em gel agarose-TREN pode ser usado como uma das etapas em um processo de recuperação e purificação de IgG.

O artigo está apresentado na forma do manuscrito corrigido, de acordo com as normas da revista *Journal of Chromatography B*, sem a edição elaborada pela editora antes da publicação. Para facilitar o entendimento do leitor, as figuras e tabelas encontram-se inseridas no manuscrito.

**ADSORPTION OF HUMAN SERUM PROTEINS ONTO TREN-AGAROSE:
PURIFICATION OF HUMAN IgG BY NEGATIVE CHROMATOGRAPHY**

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Running title: Purification of human IgG by negative chromatography

Abstract

TREN (Tris(2-aminoethyl)amine) – a chelating agent used in IMAC - immobilized onto agarose gel was evaluated for the purification of IgG from human serum by negative chromatography. A one-step purification process allowed the recovery of 73.3% of the loaded IgG in the nonretained fractions with purity of 90-95% (based on total protein concentration and nephelometric analysis of albumin, transferrin, and immunoglobulins A, G, and M). The binding capacity was relatively high (66.63 mg of human serum protein/mL). These results suggest that this negative chromatography is a potential technique for purification of IgG from human serum.

Keywords: Human IgG; Purification; Negative chromatography; Human serum; TREN-agarose gel

1. Introduction

Human immunoglobulin G (IgG), the major class of serum glycoproteins, constitutes an important therapeutic protein for a number of malignancies [1] and is also required for immunodiagnostic and immunochromatographic (downstream-processing) purposes. Therefore, there is a high demand for high purity IgG, free from other serum proteins. Processes for the purification of human IgG typically involve precipitation with ethanol followed by chromatographic techniques (such as ion exchange chromatography, gel permeation chromatography, or affinity chromatography) [2-3].

The proteins A, G, and L are the most frequently biological specific ligands used in affinity adsorption for IgG. Due to their high affinity for the Fc antibody domain (proteins A and G) and the variable domain of the human kappa chain (protein L), these ligands can be employed in a one-step adsorption process for IgG purification or extracorporeal removal of autoimmune IgG from biological fluids [4-9]. However, protein A, G, and L adsorbents are expensive and the desorption of IgG involves drastic and denaturing elution conditions and cannot withstand the harsh conditions of cleaning procedures. In addition, these proteins are susceptible to degradation and leakage after some purification cycles [1, 10]. These drawbacks have stimulated the development of more stable and less expensive adsorbents in recent years.

Pseudospecific affinity ligands such as nonbiological molecules (metal chelates, thiophilic, and dye ligands) and biological molecules (like the amino acid histidine) have been studied for human IgG purification by many research groups [11-16]. Generally, the pseudobiospecific ligands are low cost and have high stability, capacity, simplicity, and selectivity [17]. Among these ligands, the immobilized amino acid histidine is an interesting alternative for human IgG purification [18-19]. As an example, histidine grafted aminohexyl-Sepharose 4B was used as a negative affinity adsorbent for purification of IgG from human plasma diluted twenty times in Mops buffer at pH 7.2 [20]. The negative

chromatographic mode (as cited by many authors [21-25]) aims at allowing the product to pass through the column, retaining only the contaminants or impurities [26]. The adsorption of serum proteins in histidine grafted aminohexyl-Sepharose seems to be due to the electrostatic interaction of albumin and other proteins of human serum by the free remaining cationic $\alpha\text{-NH}_3^+$ of histidine. The unprotonated imidazole group of histidine is also involved in the retention of serum protein around neutral pH [20].

The nonbiological ligand TREN (Tris(2-aminoethyl)amine) is a quadridentate chelating ligand used in IMAC with four nitrogen atoms, three of which are primary in nature and the fourth one is tertiary. TREN chelated with copper and nickel ions has been employed in protein purification [16, 27-29]. Due to its high amine residue content, TREN (without chelated metal ion) can serve as an anion exchanger. At a pH lower than 10.0, TREN is positively charged (according to the manufacturer, Sigma-Aldrich) and can adsorb negatively charged molecules, so, this ligand could be an excellent candidate for the purification of IgG from serum proteins. However, in the literature there is no study describing TREN as a ligand for IgG purification.

Therefore, the purpose of this study is to evaluate the feasibility of using the ligand TREN immobilized onto agarose through an ether linkage (epichlorohydrin-activated gel) for the purification of IgG from human serum by negative chromatography. The main advantages of this unconventional proposed process is the use of nonbiological ligand that costs less and is more stable than ligands traditionally used in affinity chromatographies. Experimental studies were conducted aiming to find the least favorable conditions – pH and buffer system (phosphate, Tris-HCl, Bis-Tris, Mes, and Mops) – for human IgG adsorption. The breakthrough curve and dynamic capacity of serum proteins were determined since they are the basis for process design, scale-up, and optimization of large-scale negative chromatographic mode separation processes.

2. Experimental

2.1. Materials

The TREN-agarose gel (cross-linked 4% beaded agarose, activated with epichlorohydrin), 3-(N-morpholino)propanesulfonic acid (Mops), bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris), crystalline bovine serum albumin (BSA), and human serum albumin (HSA, 98-99% purity) were obtained from Sigma (USA). The electrophoresis calibration kit for molecular mass determination (myosine, 212 kDa; α_2 -macroglobulin, 170 kDa; β -galactosidase, 113 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) was provided by GE Healthcare (USA). Tris(hydroxymethyl amino methane) and 2-(N-morpholino)ethanesulfonic acid (Mes) were purchased from Merck (Germany). Purified human immunoglobulin G (containing 98.3% IgG according to the nephelometric analysis of IgG, IgM, IgA, albumin (HSA), and transferrin done in our laboratory) was provided by Aventis Behring (Germany). The nephelometric reagents were purchased from Beckman Coulter (USA). The water used in all experiments was ultrapure water obtained using a Milli-Q System (Millipore, USA). All other chemicals were of analytical reagent grade.

2.2. Preparation of human serum

Blood samples from a healthy donor were collected without anticoagulant. These samples were centrifuged at 4°C for 5 min at 1,000 g and the supernatant was used without further treatment. To ensure that proper ionic strength and pH were maintained for optimal binding, it was necessary to dilute serum samples with the proper adsorption buffer.

2.3. Analysis of serum proteins

Total protein concentration was determined by the Bradford method [30] using BSA as reference protein. The concentrations of IgG, IgA, IgM, HSA, and transferrin in the fractions collected in the chromatographic experiments were determined nephelometrically using an Array Protein System (Beckman Coulter, USA), in accordance with the method provided by the manufacturer.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectrofocusing (IEF)

The chromatographic fractions were analyzed by SDS-PAGE (7.5% polyacrylamide gels) under nonreducing conditions using a Mini-Protean III System (Bio-Rad, USA) in accordance with Laemmli [31] and stained with silver nitrate in accordance with Morrissey [32]. The PhastSystem (Pharmacia, Sweden) and pH 3 to 9 gradient gels (GE Healthcare, USA) were used for IEF and stained with silver nitrate in accordance with the method provided by the manufacturer.

2.5. Chromatographic experiments

All chromatographic procedures were carried out with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA) at 25°C at a flow rate of 0.5 mL min⁻¹. For studies concerning the influence of the buffer on human serum protein adsorption, the following loading buffers at 25 mM covering a pH range from 5.5 to 9.0 within their respective buffering ranges were used: Mops, Mes, Tris-HCl, Bis-Tris-HCl, and sodium phosphate.

The TREN-agarose gel was suspended in the loading buffer described above, degassed, and packed into columns (10.0 cm x 1.0 cm I.D., GE Healthcare, USA) to give bed volumes of approximately 1.0 or 3.0 mL.

Human serum (0.1 or 0.3 mL) and its solutions diluted five, ten, or twenty times with an appropriate loading buffer were injected into the column, which had

been previously equilibrated with loading buffer. For the experiments with prepurified human IgG, protein samples containing 2.0 mg of IgG diluted in 2.0 mL of equilibration buffer was loaded into the column (bed volume of 3.0 mL). For both experiments, after protein injection, the column was washed with loading buffer until the absorbance values at 280 nm of eluate were close to zero. Elution was performed with the loading buffer containing 0.4 M NaCl.

During the wash steps and elution, absorbance at 280 nm was monitored and fractions of 1.0 and 2.0 mL, respectively, were collected. The Bradford method [30], nephelometric analysis, SDS-PAGE, and IEF were used to analyze the proteins in nonretained and retained fractions. After each experiment, the column was washed with 50 mM NaOH, followed by water, and finally by the loading buffer to restore it to its initial conditions (regeneration) for carrying out the next experiment.

2.6. Adsorption breakthrough curves

These experiments were carried out at 25°C with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA). After equilibration of TREN-agarose with the loading buffer (25 mM Mes buffer, pH 6.5), human serum diluted twenty times with the loading buffer was pumped through the column at a flow rate of 0.5 mL min⁻¹ (residence time, t_R of 36 s, calculated by dividing the bed interstitial volume by the flow rate). The column outlet absorbance at 280 nm was continuously monitored. The loading of the protein solution was stopped when absorbance at 280 nm at the column outlet became constant after an initial increase. The unabsorbed protein was washed out of the column with loading buffer. The adsorbed proteins were eluted with a 25 mM Mes buffer, pH 6.5, containing 0.4 M NaCl. The effluents were monitored as described previously (measurement of absorbance at 280 nm). After elution had been completed, the column was regenerated by sequentially washing with 50 mM NaOH, followed by water and the loading buffer.

Protein concentrations in the retained and nonretained fractions were determined by the Bradford method [30] and nephelometric analysis and analyzed by SDS-PAGE under nonreducing conditions. Breakthrough curves were plotted as the ratio of the total protein concentration at the outlet (C) to that in the feed stream (C_0) as a function of the volume of protein solution throughput.

2.7. Batch adsorption of human serum proteins, HSA, and human IgG on TREN-agarose gel

Experiments for the determination of human serum proteins, HSA, and human IgG adsorption isotherms on TREN-agarose gel at 25°C were carried out (in duplicates) in Eppendorf tubes (as stirred tanks) with 50 μ L of gel. The gel was previously equilibrated with degassed 25 mM Mes buffer at pH 6.5 and the aliquots of 1.0 mL of human serum, HSA, or IgG solutions were added to the tubes. The initial protein concentrations of HSA, IgG, and human serum were in the range of 0.5 to 50.0 mg/mL, 0.5 to 20.0 mg/mL, and 0.5 to 12.2 mg/mL, respectively. The tubes were agitated for 3 h to allow equilibrium to be established. After this, protein equilibrium concentration in the liquid phase (C^*) was quantified. For single systems (HSA and IgG), the concentration of protein was measured based on absorbance at 280 nm (UV-vis spectrophotometer, Beckman DU 650, USA). For human serum proteins, the total protein concentration was determined with the Bradford method [30]. The mass of protein adsorbed per volume of gel (mg/mL), Q , was calculated as the difference between the amount of protein added and the one remaining in the liquid phase after equilibrium divided by the volume of the adsorbent. Plotting Q as a function C^* yielded the equilibrium isotherm. Langmuir isotherm model [33] (Equation 1) was used to fit the data:

$$Q = \frac{Q_m C^*}{K_d + C^*} \quad (1)$$

in which Q_m is the maximum protein binding capacity, and K_d is the dissociation constant, which represents the affinity between protein and adsorbent. Values of K_d and Q_m were estimated from a nonlinear least-squares (Levenberg-Marquardt) fit of Langmuir model to the data.

To estimate the global experimental error for this method, the difference in the unbound protein concentrations between the experiments did not exceed 2.0%, 0.5%, 1.5% for IgG, HSA, and human serum, respectively.

3. Results and discussion

3.1. Effect of buffer system on serum protein adsorption

In order to select the best loading buffer for IgG purification by negative chromatography, chromatographic experiments were carried out by loading 0.1 mL of human serum diluted twenty times with four loading buffers at different pHs (Mops, Mes, Tris-HCl, and Bis-Tris) onto 1.0 mL of TREN-agarose column. After washing the nonretained proteins, the buffer was changed to loading buffer containing 0.4 M NaCl. Retained and nonretained fractions were analyzed by the Bradford method [30], nephelometry, and SDS-PAGE. It was expected that the impurities would adsorb onto the matrix, while IgG would be collected in the nonretained (flow-through and washing) fractions. The results were evaluated in terms of IgG recovery in the flow-through and washing streams and of selectivity for IgG in order to determine the one that is least favorable for IgG adsorption.

3.1.1. IgG recovery in nonretained fractions

IgG recovery in the flow-through and washing streams was found to depend on pH and the nature of the buffer ions (Figure 1). Nonretention of IgG was

observed between pH 5.5 and 8.0 irrespective of the buffer used. For the case of Tris buffer at pH 9.0, around 99% of the loaded IgG was retained under this condition. The pH range most favorable for separation of IgG by negative chromatography was between 5.5 and 7.5, but were different for each buffer; for example, approximately the same amount of IgG was nonretained in Mops buffer at pH 7.5 (0.44 mg) as in Bis-Tris at pH 6.5 (0.39 mg).

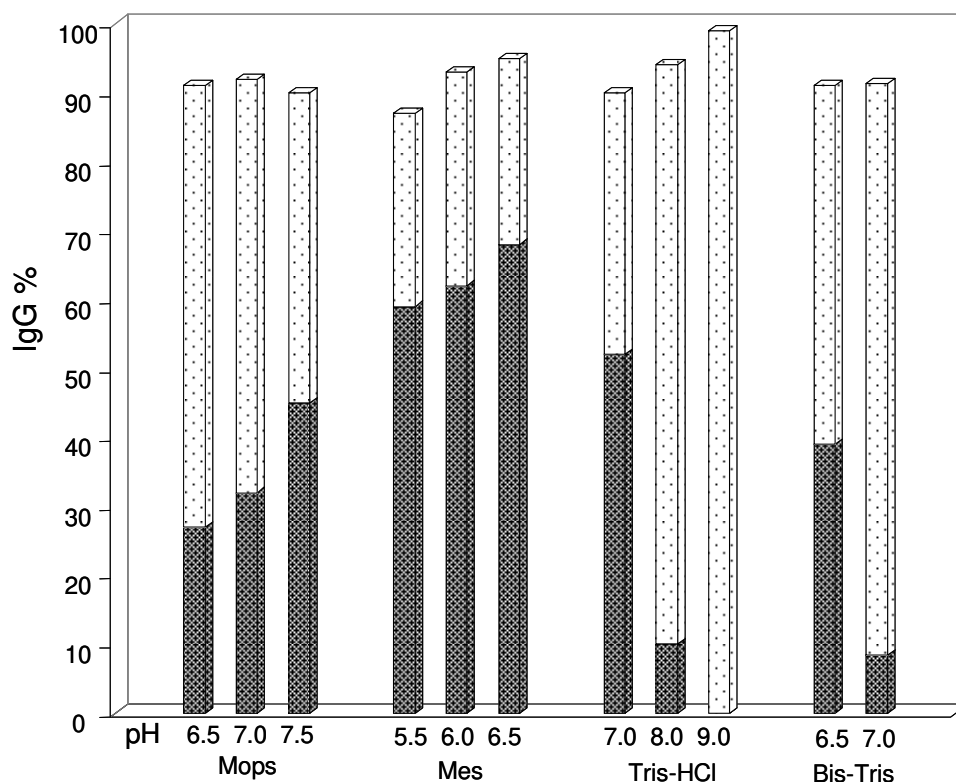


Figure 1: Effect of buffer system and pH on retention and nonretention of human IgG by TREN-agarose adsorbent. The retained and nonretained fractions were analyzed for IgG by nephelometry. (■) % of nonretained IgG; (▨) % of retained IgG.

The interactions between the adsorbent and human IgG seemed to be mainly electrostatic interactions between IgG and amino charged groups of immobilized TREN. According to the manufacturer, the charge on the TREN-agarose is zero at pH values around 10.5 and fully charged at pH values around 4.5. The gel is probably positively charged at around pH 9.0. Then, in the pH range

of 5.5 to 7.5, the TREN-agarose had a strong positive charge which created a repulsion for the IgG molecules (pI in the range of 5.0 to 9.0). As the pH was increased from 7.5 to 9.0, the IgG molecules gradually lost their positive charge, resulting in a higher IgG adsorption.

Thus, the conditions least favorable for IgG adsorption of each buffer studied (Mes pH 6.5, Mops pH 7.5, Tris-HCl pH 7.0, and Bis-Tris pH 6.5) were chosen to evaluate the selectivity of the adsorbent.

3.1.2. Selectivity of the adsorbent

The effect of the nature of the buffer ions on IgG purification by negative chromatography was examined (Figure 2 and Table 1). For all buffers studied, the serum proteins were efficiently captured with the TREN-agarose adsorbent. The SDS-PAGE showed that the purified IgG from human serum obtained in unbound fractions had a purity similar to that of the commercial IgG product (Aventis Behring). Under all experimental conditions tested, the amount of nonretained HSA, IgM, and IgA was lower than the Array Protein System detection range. The purity of IgG in the nonretained fractions was in the range of 90-95%, based on IgG and total protein concentrations determined with nephelometric and Bradford [30] methods, respectively. However, the highest IgG recovery in nonretained fractions was obtained with Mes buffer, pH 6.5 (68%).

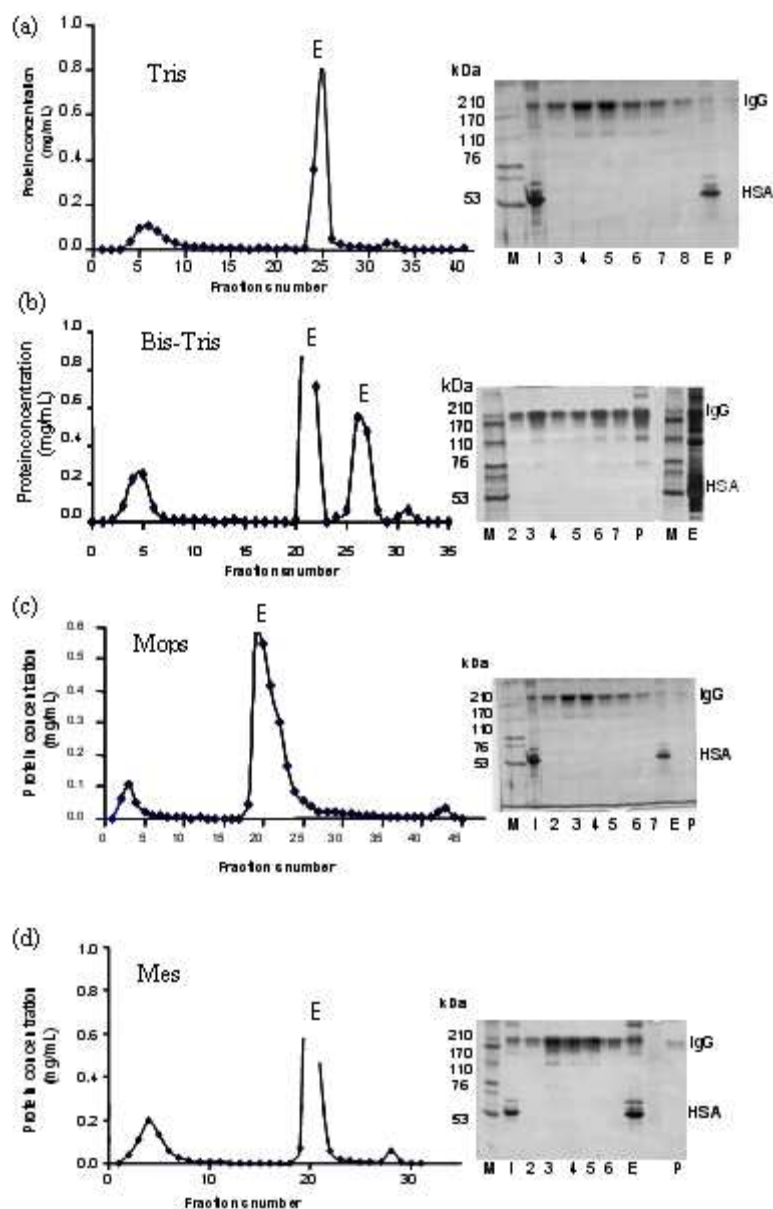


Figure 2: Effect of the nature of the buffer ions on purification of IgG from human serum diluted twenty times by negative chromatography. (a) Tris-HCl buffer, pH 7.0; (b) Bis-Tris buffer, pH 6.5; (c) Mops buffer, pH 7.5, and (d) Mes buffer, pH 6.5. Nonreducing SDS-PAGE analysis of fractions from chromatography on TREN-agarose gel: M, molecular mass protein marker; I, injected human serum solution; Numbers in SDS-PAGE correspond to the nonretained fractions indicated in chromatograms; E, pool of retained fractions (eluted with the adsorption buffer containing 0,4 M NaCl).

The optimum combination of yield and purity of the IgG recovered from human serum with TREN-agarose adsorbent was similar to the results for a biomimetic ligand immobilized in agarose reported by Teng and colleagues [34]. They used this synthetic bifunctional ligand to purify IgG from human plasma. The retained human IgG was eluted with glycine-HCl buffer pH 2.7-2.9 with recovery of 60-69% of recovery and purity of 92.5-99.2% [34].

The adsorption behavior of serum proteins varied in TREN-agarose, depending on the buffer system. When phosphate buffer was tested at pH 6.0 and 8.0 for IgG purification by negative chromatography, the serum proteins were partially adsorbed and IgG was obtained in nonretained fractions contaminated with several serum proteins (data not shown). This may be the result of the electrostatic interaction of phosphate for the immobilized TREN (phosphate buffer has a negative charge and TREN has a positive charge), masking the binding site of the ligand molecule.

The nature of the interaction between TREN-agarose and human IgG has yet to be established. IgG adsorption seemed to be governed by electrostatic forces since elution was possible with salt (NaCl). In order to verify this hypothesis the isoelectric points (pI) of the retained and nonretained IgG fractions were determined by isoelectrofocussing (electrophoresis not shown). The results showed pI from 5.8 to 9.0 for the prepurified IgG used for the experiments, a presence of molecules of pI in the range of 5.8 to 7.3 in the retained fraction and proteins with pI from 6.3 to 9.0 in the nonretained pool. These results are in accordance with the electrostatic mode of interaction proposed above. However, the present data alone does not allow a full understanding of the interactions between the TREN-agarose gel and the other serum proteins. A precise understanding is made difficult by the heterogeneous nature of the system.

Table 1: IgA, IgG, IgM and HSA recovery after chromatographic purification of human serum on TREN-agarose gel (human serum diluted twenty times)

Buffer	pH	Injection				Unbound fractions				Bound fractions				Unbound	Bound
		(mg)				(mg)				(mg)				(%)	(%)
		IgG	IgA	IgM	HSA	IgG	IgA	IgM	HSA	IgG	IgA	IgM	HSA	IgG	IgG
Mes	6.5	1.23	0.21	0.15	3.54	0.84	0.00	0.00	0.00	0.33	0.20	0.13	3.38	68	27
Mops	7.5	0.98	0.06	0.08	3.36	0.44	0.00	0.00	0.00	0.33	0.00	0.06	3.27	45	34
Tris-HCl	7.0	1.12	0.07	0.11	3.42	0.59	0.00	0.00	0.00	0.39	0.06	0.07	3.80	52	35
Bis-Tris	6.5	1.50	0.15	0.18	4.48	0.59	0.00	0.00	0.00	0.32	0.15	0.08	3.01	39	21

Protein adsorption depends very much on nature of the adsorbent and the protein containing in sample; the target protein can be partially or completely nonretained in charged adsorbent. Qi and colleagues [35] studied a chromatographic method tandem system (coupled DEAE anion-exchange and Protein G affinity columns) for simultaneous separation and purification of IgG and albumin from mouse serum. Under neutral conditions, DEAE column adsorption most of the acidic proteins; mouse IgG was completely unretained on the column (with few contaminants), was subsequently captured at the Protein G column, and eluted with high purity [35].

Although human serum proteins in Mes buffer at pH 6.5 showed effective binding to TREN-agarose, an optimization of the feedstream dilution is required in order to establish a simple one-step procedure for the purification of IgG from human serum by negative chromatography.

3.2. Effect of feedstream dilution

In order to define feedstream dilution, chromatographic experiments were performed on TREN-agarose equilibrated with 25 mM Mes buffer pH 6.5 (conductivity of 1130 $\mu\text{S}/\text{cm}$). Conductivity and pH values decreased as the dilution rate was increased: human serum “in natura” (conductivity of 9.21 mS/cm, pH 7.4) and diluted five (conductivity of 3.02 mS/cm, pH 6.9), ten times (conductivity of 2.10 mS/cm, pH 6.7), and 20 times (conductivity of 1595 $\mu\text{S}/\text{cm}$, pH 6.5) with 25 mM Mes buffer pH 6.5.

Retained and nonretained fractions were analyzed with the Bradford method for protein concentration and with SDS-PAGE for purity (Table 2, Figure 3). The results were compared with experiments carried out with human serum diluted twenty times, shown in Figure 2d. Serum proteins retention on TREN-agarose was affected by conductivity and pH of the medium. TREN-agarose gel adsorbed considerable amounts of HSA and other serum proteins (Figure 3), but high purity

IgG was obtained only when the serum was diluted at least ten times. The binding capacity of the gel for serum proteins increased with feedstream dilution: the protein capture was more efficient at lower conductivity and pH values closer to 6.5. These results are in accordance with the electrostatic mode of interaction of classical ion-exchangers, in which dilution of serum or plasma is necessary for high adsorption due to high conductivity of these fluids.

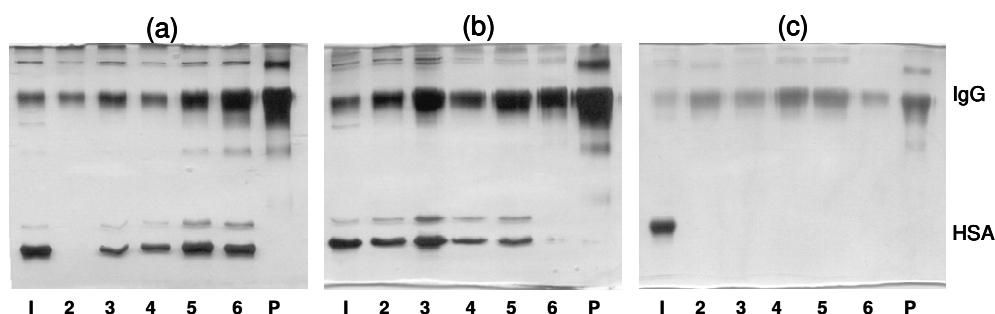


Figure 3: Effect of dilution factor of human serum in IgG purification by negative chromatography in TREN-agarose. Nonreducing SDS-PAGE of (a) human serum “in natura”; (b) human serum diluted five times; (c) human serum diluted ten times. I, injected human serum solution; Numbers in SDS-PAGE correspond to the nonretained fractions; P, human IgG standard (Aventis, Behring).

73.3% of the loaded IgG was obtained in nonretained fractions when a 3.0 mL bed volume column of TREN-agarose was loaded with 0.3 mL of human serum diluted ten times (Table 3). It was shown that nonretained fractions have high purity (based on SDS-PAGE analysis and IgG, IgM, IgA, HSA, and transferrin nephelometric analysis), since a purity similar to that of the commercial IgG product was achieved (Aventis Behring).

Table 2: Total protein recovery after chromatographic purification of human serum on TREN-agarose gel

Pool fractions	Dilution factor							
	0 times		5 times		10 times		20 times	
	mg	%	mg	%	mg	%	mg	%
Injection	15.93	100.0	11.25	100.0	16.50	100.0	15.14	100.0
Unbound	7.82	49.1	2.09	18.6	1.84	11.1	2.79	18.4
Bound	9.78	61.4	7.66	68.2	14.37	87.0	14.92	98.6
Regeneration	0.00	0.0	0.04	0.4	0.22	1.3	0.06	0.4
Recovery	17.61	110.6	9.79	87.1	16.44	99.6	19.04	117.0

Table 3: Nephelometric analysis of IgA, IgG, IgM, HSA, and transferrin after chromatographic purification of human serum on TREN-agarose (human serum diluted ten times)

Fractions	IgA		IgG		IgM		HSA		Transferrin	
	mg	%	mg	%	mg	%	mg	%	mg	%
Injection	1.12	100.0	4.32	100.0	0.44	100.0	12.90	100.0	0.77	100.0
Unbound	0.00	0.0	3.17	73.3	0.00	0.00	0.00	0.00	0.00	0.00
Bound	1.05	94.3	1.09	25.2	0.44	100.6	13.66	105.9	0.74	96.3
Recovery	1.05	94.3	4.25	98.4	0.44	100.6	13.66	105.9	0.74	96.3

3.3. Adsorption breakthrough curve

The dynamic capacity of TREN-agarose was estimated by overloading a 1.0 mL bed volume column with human serum diluted twenty times in Mes buffer, pH 6.5 (3.50 mg mL^{-1} (119.00 mg) of total protein and 0.58 mg mL^{-1} (19.72 mg) of IgG) at a flow rate of 0.5 mL min^{-1} . Figure 4a displays the breakthrough curves quantified by the Bradford method (the unbound fractions were analyzed with SDS-PAGE (Figure 4b)). Initially, the IgG band (molecular mass of approximately 150 kDa) was detected by SDS-PAGE, leading to the conclusion that the other concentration of proteins at the outlet was zero, reflecting complete adsorption of the serum molecules onto the TREN-agarose. As the loading step proceeded and binding sites became occupied, HSA was detected at the outlet (after fraction #12). Defining the breakthrough point as the fraction in which HSA is detected by SDS-PAGE, a volume of 12.0 mL of human serum solution was loaded prior to breakthrough (approximately 7 mg of IgG unbound mL^{-1} of matrix). Similar results were obtained by Pitiot et al. [20] for purification of IgG from human plasma on histidine grafted aminohexyl-Sepharose 4B used as a negative affinity adsorbent. The authors obtained 6.46 mg IgG (corresponding to 72.6 % of the injected IgG) in nonretained fractions when 1.0 mL of human plasma diluted twenty times in Mops buffer at pH 7.2 was fed into the column.

In a large-scale purification process, it is desirable to minimize product loss during the loading step. Consequently, the loading of feed is allowed to proceed until breakthrough occurs, at which point the loading step is terminated [36] (in this case, loading 12.0 mL of human serum solution).

The contaminant proteins that were observed in the pass-through fractions after 12.0 mL could be removed by reprocessing of the nonretained fractions. SDS-PAGE analysis showed that pure IgG fractions could be obtained after the second run (data not shown).

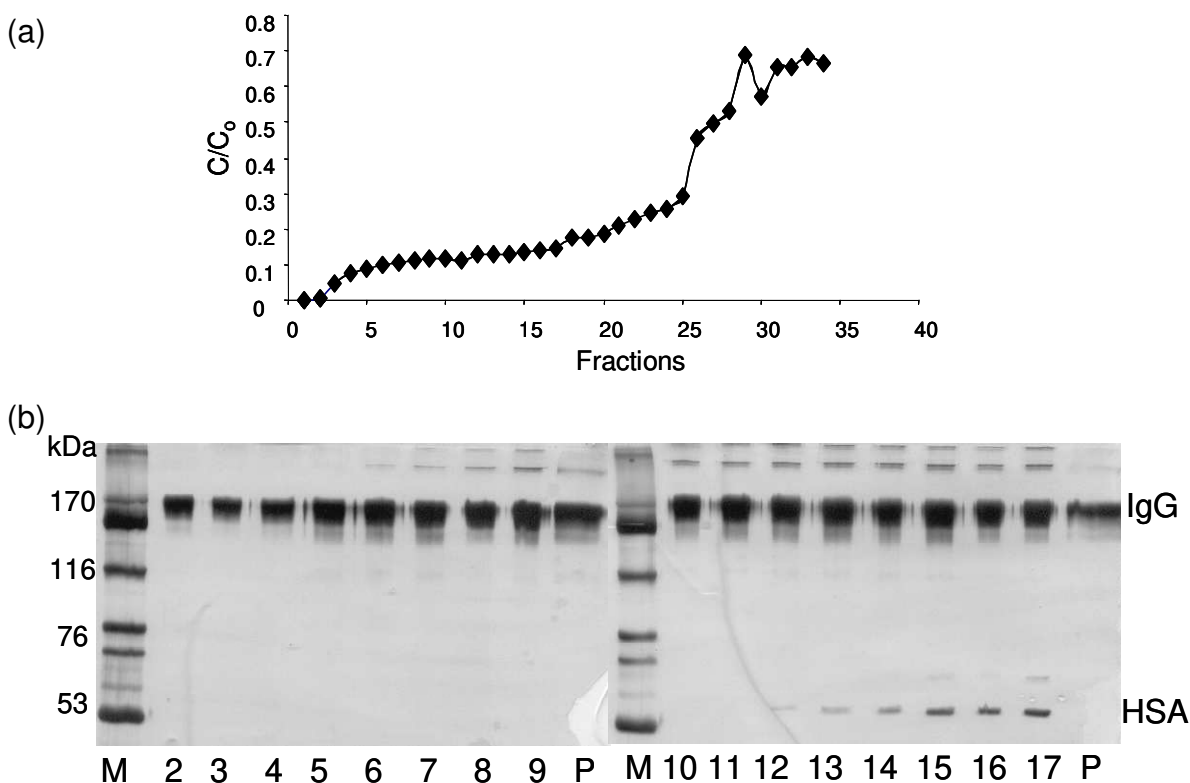


Figure 4: (a) Breakthrough curve of human serum solution (diluted twenty times) for TREN-agarose gel. (b) SDS-PAGE analysis under nonreducing conditions of fractions of breakthrough curve: M, molecular mass protein marker; 2-17, nonretained fractions; P, human IgG standard (Aventis, Behring).

After washing, adsorbed proteins were eluted with Mes buffer, pH 6.5 containing 0.4 M NaCl and protein concentration in the eluted fractions was determined by the Bradford method and analyzed by SDS-PAGE. The dynamic capacity of TREN-agarose under this condition was 31.65 mg of serum proteins/mL of gel. The protein profile given by SDS-PAGE analysis of eluted fractions from the breakthrough experiment was similar to the one verified for eluted samples from negative chromatography experiments carried out by loading 0.1 mL of human serum diluted twenty times (data not shown).

3.4. HSA, human IgG, and human serum proteins adsorption isotherms

Single component adsorption isotherms at 25°C for HSA and IgG are shown in Figure 5b and 5c. An excellent fit with experimental data was achieved using Langmuir model (Table 4). Adsorption favored HSA ($K_d = 4.97 \times 10^{-6}$ mol/L) when compared to IgG ($K_d = 2.11 \times 10^{-5}$ mol/L). Also, the IgG binding capacity of 38.52 mg/mL of gel was five times lower than the value of 191.70 mg/mL of gel for HSA. The lower capacity for IgG is in accordance with the electrostatic mode of interaction as discussed in section 3.1.

Table 4: Langmuir parameters for the adsorption of HSA, IgG and human serum proteins onto TREN-agarose gel

Proteins	Q_m (mg/mL)	K_d (mol/L)	R^2
HSA	191.70 ± 6.18	$4.97 \pm 0.87 \times 10^{-6}$	0.98
IgG	38.25 ± 1.24	$2.11 \pm 0.26 \times 10^{-5}$	0.99
Human serum	66.63 ± 2.34	-----	0.97

The maximum capacity for the HSA obtained for TREN-agarose was approximately two times higher than the maximum capacity for BSA and HSA onto two different adsorbents: 89.5 ± 2.3 mg BSA/mL of DEAE-cellulose [37] and 93.3 mg HSA/mL of histidine grafted aminohexyl-Sepharose [38].

The isotherm at 25°C for human serum and the related Langmuir parameters are presented in Figure 5a and Table 4, respectively. There was a decrease in the maximum binding capacity of protein when compared with the value for HSA (66.63 and 191.70 mg/mL of gel, respectively).

Although we did not investigate why this difference occurred we can speculate about possible reasons. The serum has a higher conductivity and slightly higher pH than its solutions. Except HSA, other proteins in serum may adsorb with negative cooperativity or cause steric hindrance. Also protein-protein interaction may form complexes that do not adsorb to the gel. A true dissociation constant (K_d) could not be determined since adsorption isotherm was constructed using human serum protein, a complex mixture.

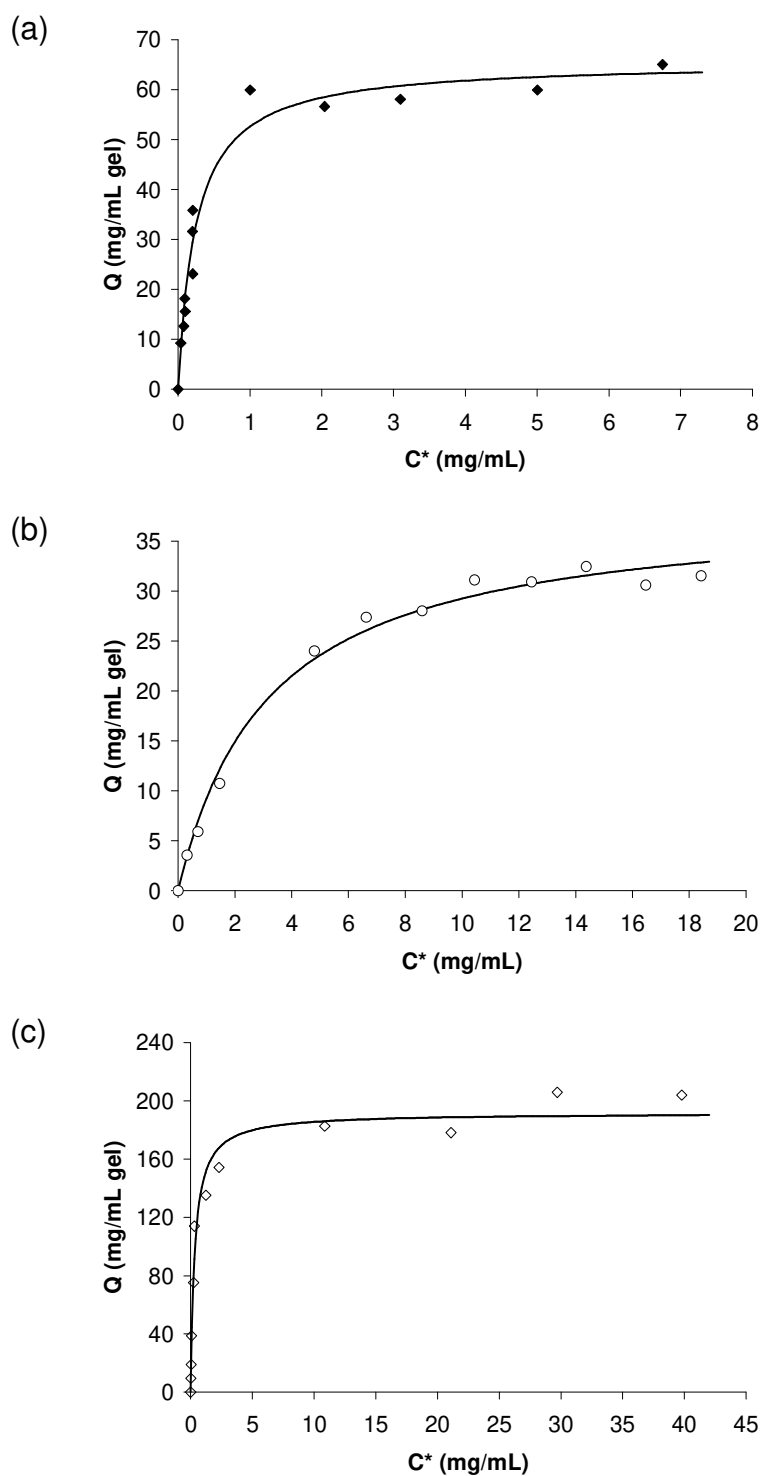


Figure 5: Experimental adsorption isotherms (symbols) for (a) human serum, (b) IgG and (c) HSA on TREN-agarose gel with Mes buffer, pH 6.5, at 25°C. The solid lines correspond to fitting (non-linear regression) of experimental data in accordance with Langmuir model.

4. Conclusion

We have demonstrated that negative chromatography using the nonbiological ligand TREN immobilized onto agarose gel beads is a simple and efficient method for the purification of IgG from human serum in a simple step. The adsorption of IgG and serum proteins was influenced by the nature of the buffer and pH. The TREN-agarose adsorbent had a capacity and selectivity for negative IgG purification similar to that of histidine grafted aminohexyl-Sepharose. IgG recovery from human serum could be achieved under mild conditions of pH, low ionic strength, and room temperature. From a large-scale process point of view, negative chromatography using TREN-agarose gel can be used as the basis for a new, more robust (nonbiological ligand) and lower cost process.

Acknowledgments

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3.3. UTILIZAÇÃO DE LIGANTES L-LISINA E POLI-L-LISINA

Neste tópico estão apresentados os resultados experimentais relativos ao uso de ligantes contendo grupamentos aminas visando a purificação de IgG humana por cromatografia negativa, com o artigo intitulado “A comparison of IgG purification by negative chromatography in amine-based ligands: effect of L-lysine and poly-L-lysine”, a ser submetido para publicação.

O aminoácido L-Lisina e seu polímero poli-L-Lisina (PLL) são muito usados como ligantes em cromatografia de afinidade visando a separação de biomoléculas como plasminogênio, ativador do plasminogênio, apolipoproteína A, angiostatina, entre outras. Como tais ligantes possuem grupamentos amina em suas estruturas e o grupo de pesquisa da Profa. Dra. Sônia Maria Alves Bueno vem utilizando poliaminas lineares (ω -aminohexil e ω -aminodecil) e ramificadas (como o agente quelante de IMAC Tris(2-aminoetil)amina – TREN) na adsorção de proteínas do soro humano e consequente purificação de IgG por cromatografia negativa, este trabalho buscou avaliar se os ligantes L-Lisina e PLL também apresentam este potencial.

Inicialmente foi avaliado o comportamento da adsorção de proteínas do soro humano em L-Lisina e PLL imobilizadas em géis de agarose, mediante o uso de diferentes tampões de adsorção (Mops, Mes, Tris-HCl, Bis-Tris, Hepes e fosfato de sódio) em uma faixa tamponante de 5,5 a 9,0 para diluir 20 vezes um volume de 50 μ L de soro, visando a seleção de uma condição que permitisse maior recuperação de IgG o mais pura possível nas frações não retidas (princípio da cromatografia negativa). Em termos de seletividade, a condição experimental que mais favoreceu a purificação de IgG humana por cromatografia negativa foi alcançada quando o tampão Bis-Tris-HCl foi usado em pH 7,0 para a L-Lisina e em pH 6,0 para a PLL.

A diferença entre aminas lineares estudadas anteriormente (aminohexil e aminodecil) e aminoácidos (como a lisina) é que após a imobilização pelo grupamento NH_2 , no caso de aminoácidos, o grupo carboxila fica livre para

interação com as proteínas. Dessa forma, a carga negativa da carboxila tem um efeito muito grande na adsorção das biomoléculas (dificultando a adsorção das proteínas do soro como a IgM, por exemplo). No caso da PLL, isso não acontece, uma vez que a carboxila está ligada covalentemente a outra molécula de lisina por meio de ligação peptídica.

Em uma segunda etapa, foram realizados experimentos de curva de ruptura (com a alimentação de uma quantidade de 15 a 25 vezes maior de soro humano) visando avaliar o comportamento do adsorvente PLL-agarose e determinar sua capacidade dinâmica de adsorção de proteínas do soro humano. Comparando os melhores resultados obtidos com os ligantes PLL e ω -aminohexil (alcançados quando foram usados os tampões de adsorção Bis-Tris-HCl, pH 6,0 e Hepes, pH 6,8, respectivamente), com PLL houve uma recuperação de 79% IgG, com grau de pureza de 95%, enquanto que para o ω -aminohexil, a recuperação foi de 76% com pureza de 100%. Para alimentação de uma solução de 15,0 mL de soro humano diluído 20 vezes, a capacidade dinâmica de PLL-agarose encontrada foi de $12,7 \text{ mg mL}^{-1}$ de gel, inferior ao atingido pelo ligante ω -aminohexil ($13,7 \text{ mg mL}^{-1}$).

Os dados experimentais de adsorção monocomponente utilizando HSA ou IgG foram avaliados por meio do ajuste dos parâmetros dos modelos de Langmuir e Langmuir-Freundlich. O modelo de Langmuir descreveu os dados de adsorção de HSA satisfatoriamente com o valor estimado de Q_m de $76,4 \text{ mg de HSA mL}^{-1}$, na mesma ordem de grandeza de dados apresentados para a adsorção de HSA em histidina ($93,3 \text{ mg mL}^{-1}$) e em ω -aminodecyl ($81,0 \text{ mg mL}^{-1}$). A constante de dissociação (K_d) foi da ordem de grandeza de $10^{-5} \text{ mol L}^{-1}$, mostrando que PLL pode ser considerada um ligante de fraca afinidade para adsorção de HSA.

Para IgG, por sua vez, detectou-se a presença de efeito de cooperatividade, uma vez que o parâmetro n do modelo de Langmuir-Freundlich foi ajustado para 1,60. A adsorção de IgG em adsorventes que contém grupamentos amina é promovida, principalmente, por fenômenos de troca iônica e interação hidrofóbica uma vez que a solução de IgG inicial continha moléculas com cargas positivas, negativas e nulas.

A análise conjunta de todos estes dados mostra que PLL agarose pode ser considerado como um adsorvente alternativo para o isolamento de IgG a partir do soro humano, apresentando potencial para ser integrado a processos de purificação em larga escala.

O artigo está apresentado na forma do manuscrito a ser enviado a revista *Separation Science and Technology* contendo, entretanto, as figuras e tabelas inseridas no manuscrito visando facilitar o entendimento do leitor.

A COMPARISON OF IgG PURIFICATION BY NEGATIVE CHROMATOGRAPHY IN AMINE-BASED LIGANDS: EFFECT OF L-LYSINE AND POLY-L-LYSINE

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Abstract

The amino acid L-lysine (Lys) and Poly-L-lysine (PLL) immobilized onto agarose gel were evaluated as ligands aiming at the purification of immunoglobulin G (IgG) from human serum. Since these ligands have amine groups in their structures and polyamines (such as histidine-aminoethyl, Tris(2-aminoethyl)amine, ω -aminoethyl, and ω -aminodecyl) have been successfully used on protein adsorption from human serum, this study aimed to evaluate the potential use of Lys and PLL in this task. We have demonstrated that the most selective adsorbent was PLL, since the carboxyl group of one Lys molecule is covalently linked to another Lys via a peptide bond. When the adsorption buffer Bis-Tris was used, a 79% recovery of IgG was achieved with a purity of 95% (based on the mass ratio of IgG to the protein sum of IgG, HSA, Trf, IgA, and IgM by nephelometry). Human serum albumin (HSA) and IgG adsorption equilibrium studies showed that the first followed the Langmuir model whereas the second followed the Langmuir-Freundlich model, due to the presence of positive cooperativity effect ($n = 1.60$). The joint analysis of these data shows that PLL-agarose can be considered as an alternative adsorbent for the isolation of IgG from human serum with the potential to be integrated to a purification process on a large scale.

Keywords: IgG purification, human serum, lysine, poly-L-lysine, negative chromatography

1. Introduction

L-lysine (Lys) is the levogire optical isomer of the amino acid lysine, whereas Poly-L-lysine (PLL) is a small polypeptide formed by L-lysine units. Due to the presence of the amino groups, Lys and PLL have the ability to interact with proteins, nucleic acids, and phospholipids, being candidates to be used as ligands [Houen, 2001]. To be used as chromatography ligands, Lys and PLL are grafted via its α -amino group, which leaves, for Lys, both the ϵ -amino and carboxyl groups free to interact with the biomolecules during the chromatography. The binding mechanism involved seems to be governed by electrostatic and stereo-specific (affinity) interactions [Okuda et al., 2003].

Several studies have indicated that the use of amines as ligands in chromatography can lead to high selectivity in the purification of proteins with affinity for amino groups, such as plasminogen, plasminogen activator (tPA) and apolipoprotein A [Houen, 2001]. The plasminogen purification from human plasma can be performed on Lys-Sepharose due to the presence of lysine-binding kringles in plasminogen molecules which remain adsorbed, being eluted with aminocaproic and aminohexanoic acid solution [Deutsch and Mertz, 1970; Chibber et al., 1974]. Angiostatin, an angiogenesis inhibitor, for example, was produced by controlled plasminogen proteolysis and purified by adsorption of the lysine-binding fragments by affinity chromatography on Lys-Sepharose [O'Reilly et al., 1996; Cao et al., 1996]. Apolipoprotein A, a homologous of plasminogen, has also being purified by affinity chromatography on Lys-Sepharose [Radcliffe et al., 1978, McLean et al., 1986; Mooser et al., 1996; Xia et al., 2000]. Belczewski et al. (2005), for instance, compared the binding affinities of baboon and human apolipoprotein A kringle IV type for Lys-agarose.

As examples of using PLL as ligand, Cheng and coworkers [Cheng et al., 2000] used a sequence of chromatographies on calmodulin-Sepharose 4B followed by PLL-agarose, in order to identify and isolate four different phosphatases from human brain. Di Sabato (1982) used PLL-agarose affinity chromatography as a step in the purification of rat interleukin 2 (IL2). This step was

found to improve the efficiency of removal of contaminating proteins present in the preparation (IL2 from spleen cells of rats prepared in RPMI 1640 medium supplemented with 2 mmol L⁻¹ glutamine and 0.1 mmol L⁻¹ alanine). Ding and coworkers (2009) presented the downstream processing of a 46 kDa mitogenactivated protein kinase from maize leaves. Protein eluted from PLL-agarose presented an increase of 5 times in its specific activity.

Ramified diamines like Tris(2-aminoethyl)amine (TREN, a polyamine with four amino groups) were able to adsorb human serum proteins probably due to electrostatic interactions, permitting the purification of human IgG by negative chromatography [Bresolin et al., 2009]. Negative chromatography aims at obtaining the product in the flowthrough and washing steps, while the contaminants or impurities remain adsorbed and can be recovered in elution steps [Anspach, 2000, Bresolin et al., 2009].

Non-ramified diamines such as aminohexyl (1,6 diaminohexane) and aminodecyl (1,10 diaminodecane) grafted on agarose gel are shown to be feasible for the purification of IgG from human serum or plasma solutions by negative chromatography with recoveries around 75%. In both cases, due to the long carbonic chain, effects of hydrophobic nature also collaborate in the adsorption process [Souza et al., 2010; Bresolin et al. 2010].

The way the ligand is immobilized in the support is of paramount relevance, as it can allow the accessibility of a particular group over another. As an example of IgG purification from human serum/plasma using immobilized histidine, when the ligand is immobilized by its amino group, IgG is adsorbed and chromatography is positive [Bueno et al., 1995] while when the ligand is immobilized by the carboxyl group, IgG is not adsorbed and the chromatography is negative [Pitiot et al., 2001].

Although both Lys and PLL have been used successfully for purification of different proteins, it is relevant to evaluate their behavior in IgG purification, since their structure presents amine groups. The main difference between linear diamines previously studied (such as ω -aminohexyl and ω -aminodecyl [Souza et al., 2010; Bresolin et al. 2010]) and Lys is that, in the case of the amino acid, the carboxyl

group is able to interact with the proteins, due to formation of a dipole after the immobilization. So, the negative charge of carboxyl has a great effect on the adsorption of biomolecules.

Thus, this work aims to evaluate the use of Lys and PLL as ligands for IgG purification from human serum. The effects of operational conditions (pH, and buffer system) on the capacity and selectivity of the system were also investigated for Lys and PLL immobilized on agarose gel. Adsorption isotherms, breakthrough curves and dynamic capacity of serum proteins were determined in order to obtain data useful for the development of large scale processes for purification of human IgG.

2. Experimental

2.1. Materials

L-Lysine and poly-L-Lysine immobilized in cross-linked 4% beaded agarose (CNBr activated), crystalline bovine serum albumin (BSA), 3-(N-morpholino)propanesulfonic acid (Mops), bis(2-hydroxyethyl)amino-tris(hydroxymethyl) methane (Bis-Tris), and N-(2-hydroxyethyl)piperazine-N'(2-ethanesulfonic acid) (Hepes) were purchased from Sigma (USA). Sodium phosphate monobasic, sodium phosphate dibasic, Tris(hydroxymethyl amino methane) (Tris), and 2-(N-morpholino)ethanesulfonic acid (Mes) were purchased from Merck (Germany). Acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), and dithiotrietol used in SDS-PAGE analysis were provided by Bio-Rad (USA). The electrophoresis calibration kit for determination of molecular mass (myosine, 212 kDa; α_2 -macroglobulin, 170 kDa; β -galactosidase, 116 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) was purchased from GE Healthcare (USA). Human immunoglobulin G (containing 98.3% IgG according to the nephelometric analysis of IgG, IgM, IgA, human serum albumin (HSA), and transferrin (Trf) done

in our laboratory) was acquired from CSL Behring (Germany). The nephelometric reagents were purchased from Beckman Coulter (USA). The water used for buffer and solution preparation was ultrapure (Milli-Q System, Millipore, USA). All other chemicals were of analytical reagent grade.

2.2. Human serum

Fresh frozen serum samples were defrosted at 37°C. To ensure that proper ionic strength and pH were maintained for optimal binding, it was necessary to dilute serum samples with the proper adsorption buffer.

2.3. Protein determination

The total protein content in the chromatographic samples was determined with the Bradford method using BSA as reference protein [Bradford, 1976]. The content of IgG, IgA, IgM, HSA, and Trf were determined by nephelometric procedure carried out in an Array Protein System (Beckman Coulter, USA) in accordance with the manufacturer's methods. The lower concentration detected by the nephelometer are 0.93 mg dL⁻¹ for IgG, 0.62 mg dL⁻¹ for HSA, 1.11 mg dL⁻¹ for IgA, 0.69 mg dL⁻¹ for IgM and 0.35 mg dL⁻¹ for Trf.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analyzed by SDS-PAGE in 7.5% polyacrylamide gels under nonreducing conditions [Laemmli, 1970] in a Mini-Protean III System (Bio-Rad, USA) and the gels were silver staining according Morrissey (1981).

2.5. Chromatographic runs

All chromatographic procedures were carried out with an automated chromatography system (BioLogic LP Liquid Chromatography System, Bio-Rad, USA) at 25°C at a flow rate of 0.5 mL min⁻¹ (superficial velocity of 38.2 cm h⁻¹). For studies concerning the influence of the buffer system on human protein adsorption, the following loading buffers at 25 mmol L⁻¹ covering a pH range from 5.5 to 8.0 within their respective buffering ranges were used: Mes, Bis-Tris, Mops, Tris, Hepes, and sodium phosphate. The gels were suspended in the loading buffer, degassed, and packed into columns (10.0 cm x 1.0 cm I.D.) to give bed volumes of 1.0 or 3.0 mL.

Human serum diluted 20 times with an appropriate loading buffer was fed into a column previously equilibrated with loading buffer. For all experiments, after protein loading, the column was washed with loading buffer until the absorbance values at 280 nm at the outlet reached the baseline. Elution was performed by the addition of 0.4 mol L⁻¹ NaCl in the loading buffer.

During all chromatographic steps absorbance at 280 nm was monitored and fractions of 1.0 mL were collected. The Bradford method [Bradford, 1976], nephelometric analysis, and SDS-PAGE were used to analyze the proteins in both nonretained and retained fractions. After each experiment, the column was regenerated with 25 mmol L⁻¹ NaOH, followed by Milli-Q water and the loading buffer to restore it to its initial condition for a new experiment.

2.6. Adsorption breakthrough curves

In order to determine the dynamic capacity of PLL-agarose, adsorption breakthrough curves were obtained at 25°C with an automated chromatography system (BioLogic LP Liquid Chromatography System, Bio-Rad, USA). After equilibrating the 3 mL gel bed with the loading buffer, human serum diluted 20 times with the loading buffer was pumped through the column at a flow rate of 0.5 mL min⁻¹ (superficial velocity of 38.2 cm h⁻¹). The column outlet absorbance at 280 nm was continuously monitored. The loading of protein solution was stopped when

absorbance at 280 nm at the column outlet became constant after an initial increase. The nonretained protein was washed out of the column with loading buffer. The adsorbed proteins were eluted with the loading buffer containing 0.4 mol L^{-1} NaCl. After elution had been completed, the column was regenerated by sequentially washing with 25 mmol L^{-1} NaOH, followed by Milli-Q water and the loading buffer.

Protein concentration in the nonretained and retained fractions was determined by the Bradford method [Bradford, 1976] and nephelometry and analyzed by SDS-PAGE under nonreducing conditions.

2.7. Batch adsorption of HSA and human IgG on PLL-agarose gel

Experiments for the determination of HSA and IgG adsorption isotherms on PLL-agarose gel at 25°C were carried out (in duplicates) in Eppendorf tubes (as stirred tanks) containing $50 \text{ }\mu\text{L}$ of gel. The gel was equilibrated with degassed 25 mmol L^{-1} Bis-Tris-HCl buffer at pH 6.0 prior to adding the aliquots of 1.0 mL of HSA or IgG solutions to the tubes, which initial concentrations were in the range of 0.5 to 50.0 mg mL^{-1} and 0.5 to 20.0 mg mL^{-1} , respectively. The tubes were agitated for 3 h to allow equilibrium to be established. After this, protein equilibrium concentration in the liquid phase (C^*) was quantified by measuring the absorbance at 280 nm (UV-vis spectrophotometer, Beckman DU 650, USA). The difference in unbound protein concentration between the experiments did not exceed 3.2% and 3.4% for HSA and IgG, respectively. The mass of protein adsorbed per volume of gel (mg mL^{-1} of gel), Q^* , was calculated as the difference between the amount of protein added and that remaining in the liquid phase after equilibrium divided by the volume of the adsorbent. Plotting Q^* against C^* yielded the equilibrium isotherm. The parameters of the Langmuir (eq. 01) and Langmuir-Freundlich (eq. 02) models were fitted to the experimental data employing the iterative fitting method of Levenberg-Marquardt using Origin® (Microcal, USA).

$$Q^* = \frac{Q_m C^*}{K_d + C^*} \quad \text{eq. (1)}$$

$$Q^* = \frac{Q_m (C^*)^n}{K_{dLF} + (C^*)^n} \quad \text{eq. (2)}$$

where Q_m is the maximum protein binding capacity; K_d is the dissociation constant, which represents the affinity between protein and adsorbent; K_{dLF} is the apparent dissociation constant; and n is the Langmuir-Freundlich coefficient.

3. Results and discussion

Since adsorbents containing amine groups as ligands (such as TREN-, ω -aminohexyl-, and ω -aminodecyl-agarose; Bresolin et al., 2009; Souza et al., 2010; Bresolin et al., 2010) are able to adsorb human serum protein, it was expected that, due to its amino content, Lysine and PLL grafted to agarose gel could also provide adsorption of human serum protein.

3.1. Selectivity of L-Lys as ligand for the IgG purification: Buffer screening

In order to select a buffer condition that would favor the adsorption of human serum protein, chromatographic experiments were carried out by loading 0.1 mL of human serum diluted 20 times with different loading buffers (covering a pH range of 5.5 to 9.0) onto a 1.0 mL bed of Lys-agarose (zero point charge of 9.7; [Greenstein, 1933]). After the washing step, the buffer was changed to loading buffer containing 0.4 mol L^{-1} NaCl. Retained and nonretained fractions were analyzed by the Bradford method [Bradford, 1976] and SDS-PAGE. In the studies concerning the influence of the buffer system on human serum protein adsorption,

experiments were carried with Mes, Bis-Tris, Mops, Tris, Hepes, and sodium phosphate buffers.

The results depicted in Figure 1 showed that, for Mes and Bis-Tris, when the pH was increased, the amount of adsorbed protein also increased. However, opposite profiles were observed for Mops, Hepes, and sodium phosphate. The unique exception occurred when Tris-HCl buffer was used: the percentage of adsorbed protein maintained constant around 6.5% irrespective of the pH.

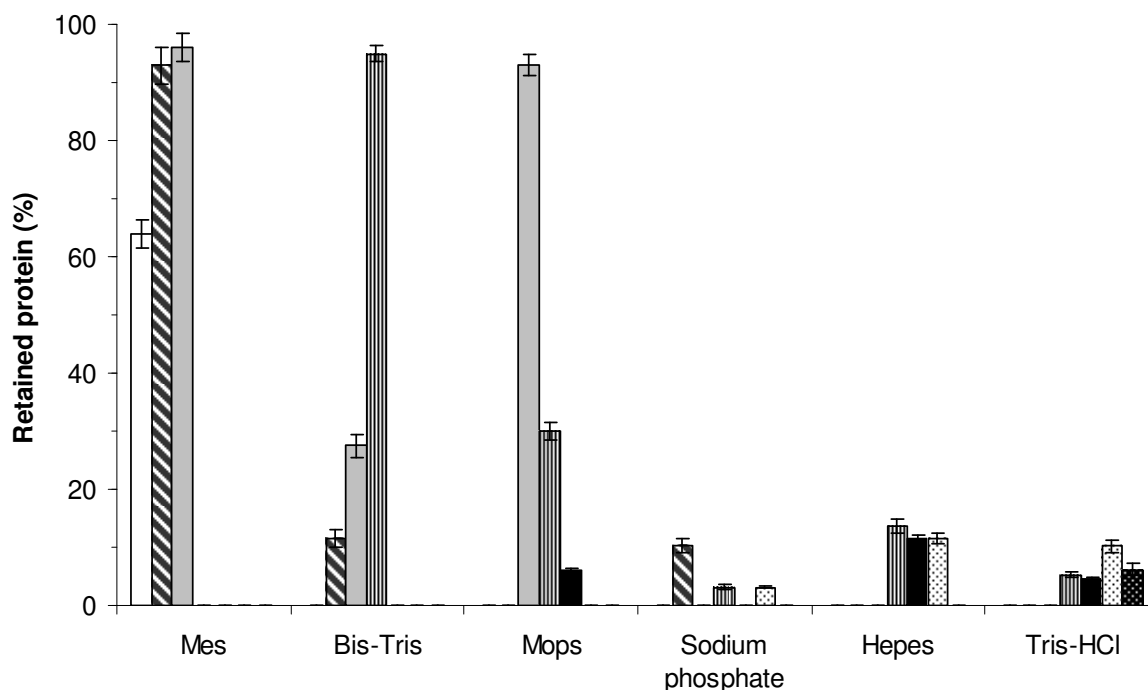


Figure 1. Effect of buffer system and pH (□ 5.5; ▨ 6.0; ▩ 6.5; ▪ 7.0; ■ 7.5; ▤ 8.0; ▥ 9.0) on the retention of human serum protein by Lys-agarose adsorbent. The amount of retained protein was determined by Bradford method [Bradford, 1976] after the elution from column, using the buffers at 25 mmol L⁻¹ with 0.4 mol L⁻¹ NaCl.

For Mes and Bis-Tris buffer, the amount of total protein adsorbed increased as the pH was increased (Figure 2). However, in terms of selectivity, the unique experimental condition that favored the purification of human IgG by negative chromatography was reached when Bis-Tris-HCl buffer was used at pH 7.0. In this condition, 3.9 mg of total protein was injected and Lys-agarose adsorbed 3.7 mg (95% of total protein fed). Considering that about 20% of the total protein fed is IgG

(0.8 mg) and that the amount of 0.2 mg of total protein recovered corresponds to electrophoretically high pure IgG, one can estimate that the IgG recovery would be around 25%.

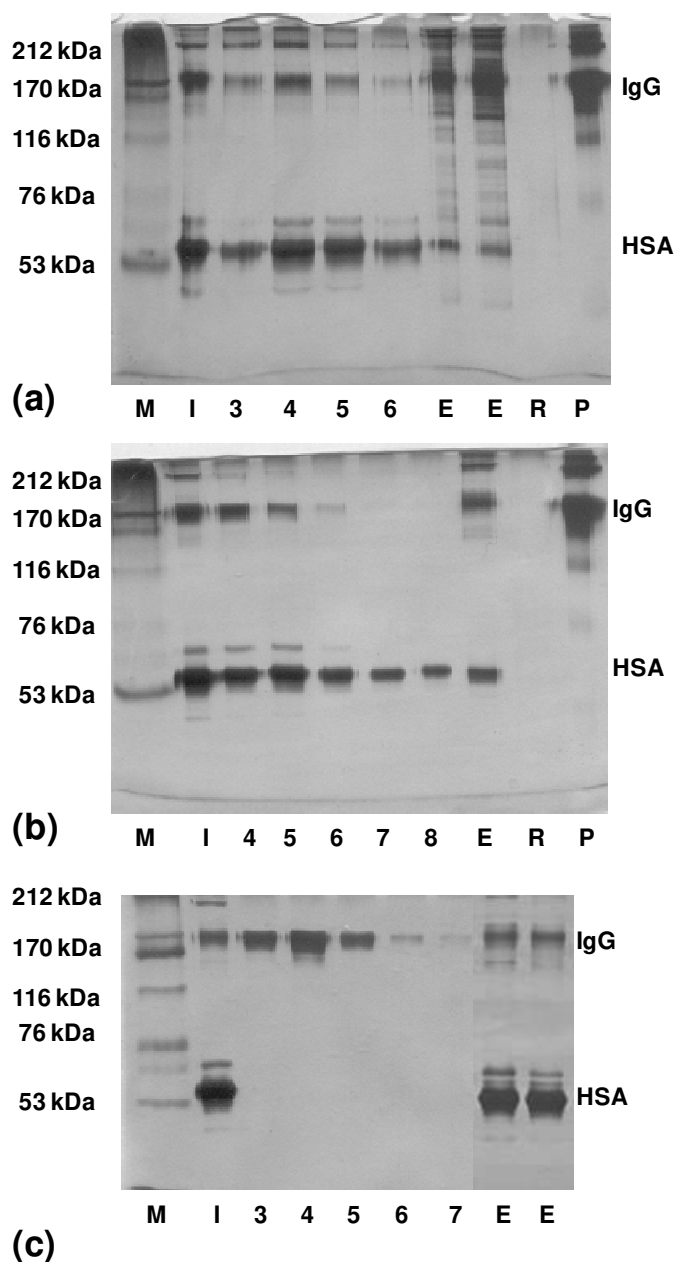


Figure 2. Effect of pH of Bis-Tris-HCl buffer in IgG purification by negative chromatography in Lys-agarose. Nonreducing SDS-PAGE of human serum diluted 20 times in Bis-Tris-HCl 25 mmol L⁻¹ at (a) pH 6.0; (b) pH 6.5; (c) pH 7.0 I, injected human serum solution; numbers in SDS-PAGE correspond to the nonretained fractions; E, elution performed by adding 0.4 mol L⁻¹ NaCl in loading buffer; P, human IgG standard (CSL Behring).

Similar percentages of adsorbed protein were observed when Mes at pH 6.0 and pH 6.5 and Mops at pH 6.5 were used as loading buffer. Therefore, the selectivity achieved in these experimental conditions was inferior to Bis-Tris-HCl at pH 7.0 due to the presence of HSA and Trf in nonretained fractions (SDS-PAGE of Mes and Mops buffers experiments not shown). For almost all conditions (pH and buffer), higher amounts of IgG are adsorbed together with other proteins, thus preventing its purification by negative chromatography. As this carboxyl group charge does not appear in PLL, it is important to do a similar evaluation of PLL-agarose behavior on human serum protein adsorption.

3.2. Selectivity of PLL as ligand for the IgG purification: Buffer screening

In order to evaluate the effect of the use of PLL as a ligand for the adsorption of human serum protein, chromatographic experiments were carried out in the same condition as those performed with Lys (except for Hepes buffer). Retained and nonretained fractions were analyzed by the Bradford method [Bradford, 1976] and SDS-PAGE. According to experimental data in Figure 3, except for Tris-HCl at pH 9.0, for all other experimental conditions, the amount of retained protein was higher than 90%.

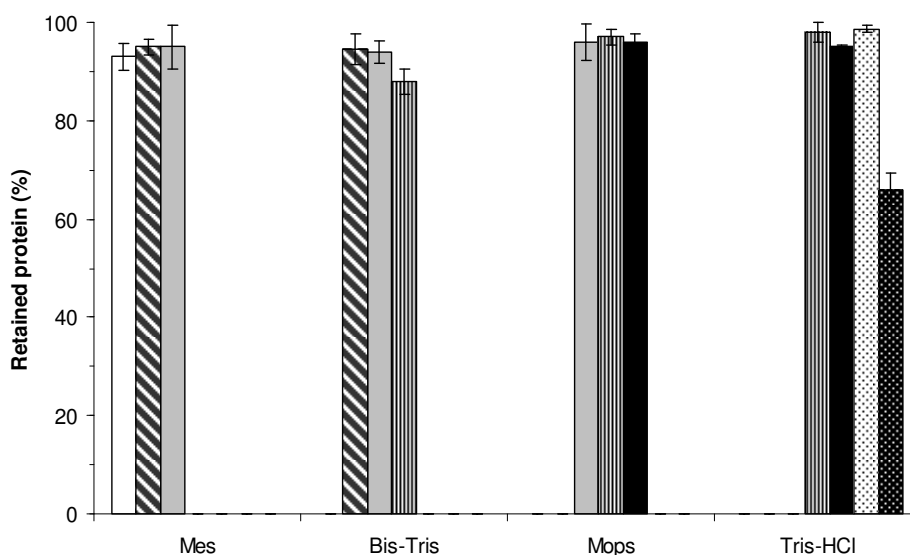


Figure 3. Effect of buffer system and pH (□ 5.5; ▨ 6.0; ▩ 6.5; ▪ 7.0; ■ 7.5; ▤ 8.0; ▥ 9.0) on the retention of human serum protein by PLL-agarose adsorbent. The amount of retained protein was determined by Bradford method [Bradford, 1976] after the elution from column, using the buffers at 25 mmol L⁻¹ with 0.4 mol L⁻¹ NaCl.

For all buffers studied, the serum proteins were efficiently adsorbed by the PLL-agarose adsorbent. The SDS-PAGE showed that the purified IgG from human serum obtained in unbound fractions had purity similar to that of the commercial IgG product (IgG marker, prepared with commercial IgG from CSL Behring). In terms of selectivity, the most promising results aiming at the purification of IgG by negative chromatography were achieved when Mops pH 6.5, and 7.0, Bis-Tris pH 6.0 and Tris-HCl pH 7.5 were used as adsorption buffers, according to the SDS-PAGE shown in Figure 4a-d. It also can be observed that the selectivity achieved was higher when Mops and Bis-Tris were used instead of Tris-HCl (Figure 4d).

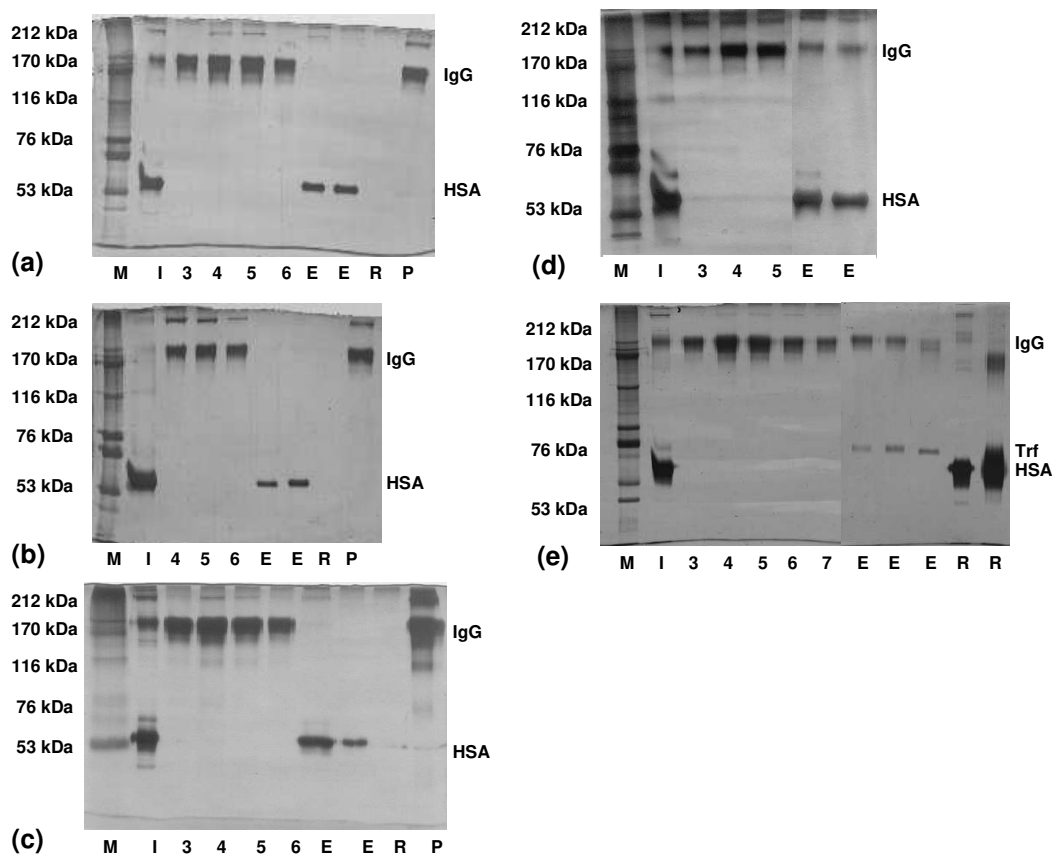


Figure 4. Effect of buffer system and pH in IgG purification by negative chromatography in PLL-agarose. Nonreducing SDS-PAGE of human serum diluted 20 times in (a) Mops 25 mmol L⁻¹, pH 6.5; (b) Mops 25 mmol L⁻¹, pH 7.0; (c) Bis-Tris-HCl 25 mmol L⁻¹, pH 6.0; (d) Tris-HCl 25 mmol L⁻¹, pH 7.5; (e) Mes 25 mmol L⁻¹, pH 6.5; I, injected human serum solution; numbers in SDS-PAGE correspond to the nonretained fractions; E, elution performed by adding 0.4 mol L⁻¹ NaCl in loading buffer; R, regeneration performed with 25 mmol L⁻¹ NaOH; P, human IgG standard (CSL Behring).

In the pH range evaluated (5.5 to 9.0), the PLL was positively charged ($pK_a = 10.5-10.7$; [Dorris et al., 2008]) while HSA ($pI = 4.9$; Putnam, 1984) and Trf ($5.2 < pI < 5.9$; Putnam, 1984) are almost all negatively charged (HSA and Trf correspond to about 65% of serum protein [Putnam, 1984], being the major impurities for IgG purification). As Mes ($pK_a = 6.15$) and Mops ($pK_a = 7.2$) are zwitterionic Good's buffers [Good et al., 1966], both have neutral net charge at $pH < pK_a$ (5.5 and 6.0 for Mes and 6.5 and 7.0 for Mops) and the buffer charge does not have a capital importance in the adsorption. The neutral net charge only indicates that there is a balance in the amount of positive and negative charges of the buffer counter-ions. In the other hand, when $pH > pK_a$, the buffers were negative charged and, in this condition, their negative counter-ions possibly were being repulsed by negative charged proteins (such some molecules of IgG, since its pI ranges from 5.5 to 9.3 according isoelectrical focusing electrophoresis done in our lab [Bresolin et al., 2010]), promoting the adsorption of part of IgG in the ligand PLL (SDS-PAGE not shown of Mops pH 7.5).

When Mes buffer was used, at pH 6.5, it was observed a strong retention of HSA. In this condition, 95% of the retained protein was only recovered when the adsorbent was regenerated with NaOH, while 5% of retained protein was recovered using the elution buffer: Mes 25 mmol L⁻¹, NaCl 0,4 mol L⁻¹, pH 6.5. According the SDS-PAGE behavior (Figure 4e), high pure IgG was found in washing, a mixture of IgG and Trf was found in the elution, and HSA was observed only in regeneration (also with other proteins). Due to the use of NaOH as regeneration agent, it was not possible to quantify the HSA by nephelometry.

For Bis-Tris buffer ($pK_a = 6.5$), the most selective results were achieved at pH 6.5 ($pH = pK_a$), as can be seen in Figure 4c. In this condition, it was recovered IgG electrophoretically pure in the nonretained fractions similar of those achieved when Mops buffer was used at pH 6.5 and 7.0.

In general, according to the experimental results, we can observe that the selectivity achieved for the purification of IgG was higher when using PLL instead of Lys as ligand. In the same condition of buffer and pH, the negative charge of the carboxyl of Lys has a great effect, repulsing many negatively charged proteins that

could be adsorbed on PLL. In PLL the carboxyl group of one Lys molecule is covalently linked to another Lys (via a peptide bond) and the absence this negative charge may explain this more efficient adsorption of negatively charged protein.

The accessibility of carboxyl group was reported in literature when the histidine was used as ligand for IgG purification from human serum and plasma [Bueno et al., 1995; Pitiot et al., 2001]. When the ligand was immobilized by its amino group, the carboxyl group could interact with IgG, promoting its adsorption [Bueno et al., 1995] whereas when the ligand was immobilized by the carboxyl group, the amino group could interact with other serum protein, promoting their adsorption and IgG was not adsorbed, being purified in a negative mode [Pitiot et al., 2001].

3.3. Determination of dynamic adsorption capacity by breakthrough curves

As serum protein adsorption experiments had shown a possible purification of human IgG using Mops, pH 6.5 and Bis-Tris-HCl, pH 6.0 as adsorption buffers, we have performed human serum adsorption experiments in a dynamic mode. These experiments aimed at the determination of the breaking point and the dynamic adsorption capacity of this adsorbent in both situations. Figures 5 and 6 depict the breakthrough curves and SDS-PAGE, while Tables 1 and 2 show the protein mass balance for the experiments performed with Mops, pH 6.5, and Bis-Tris-HCl, pH 6.0, respectively.

According to the SDS-PAGE (Figure 5b) and nephelometric analysis (Table 1), when Mops buffer was used at pH 6.5, it can be observed that the nonretained fractions contain IgG, IgM, IgA, and HSA. A similar profile was obtained when Mops buffer was used at pH 7.0 (data and SDS-PAGE not shown), however with a lower recovery of IgG in nonretained fractions (58.7% at pH 7.0, and 70.2% at pH 6.5, according to mass balance). On the other hand, when Bis-Tris-HCl was used at pH 6.0 (Fig. 6), 95.2% purity IgG (based on the mass ratio of IgG to the protein sum of IgG, HSA, Trf, IgA, and IgM) could be recovered on flowthrough fractions 1-19, which contained only IgM as contaminant. Fractions 20-25 (flowthrough) and 26-28 (washing) were pooled together and the presence of HSA and IgM was

detected by nephelometry (Table 2; HSA bands could be clearly seen in SDS-PAGE of Figure 6b). A less expressive amount of IgG (11.1%) was recovered in fractions 29-33 containing only IgM according data in Table 2. When these results are analyzed together (nonretained fractions of flowthrough and washing), a 79.0% of the fed IgG could be recovered, achieving a purity degree of 91.9%. As an example, the commercial IgG (CSL Behring) used in this work to prepare the SDS-PAGE IgG marker contained IgG of 98.3% purity, according the same nephelometric analysis done in this work.

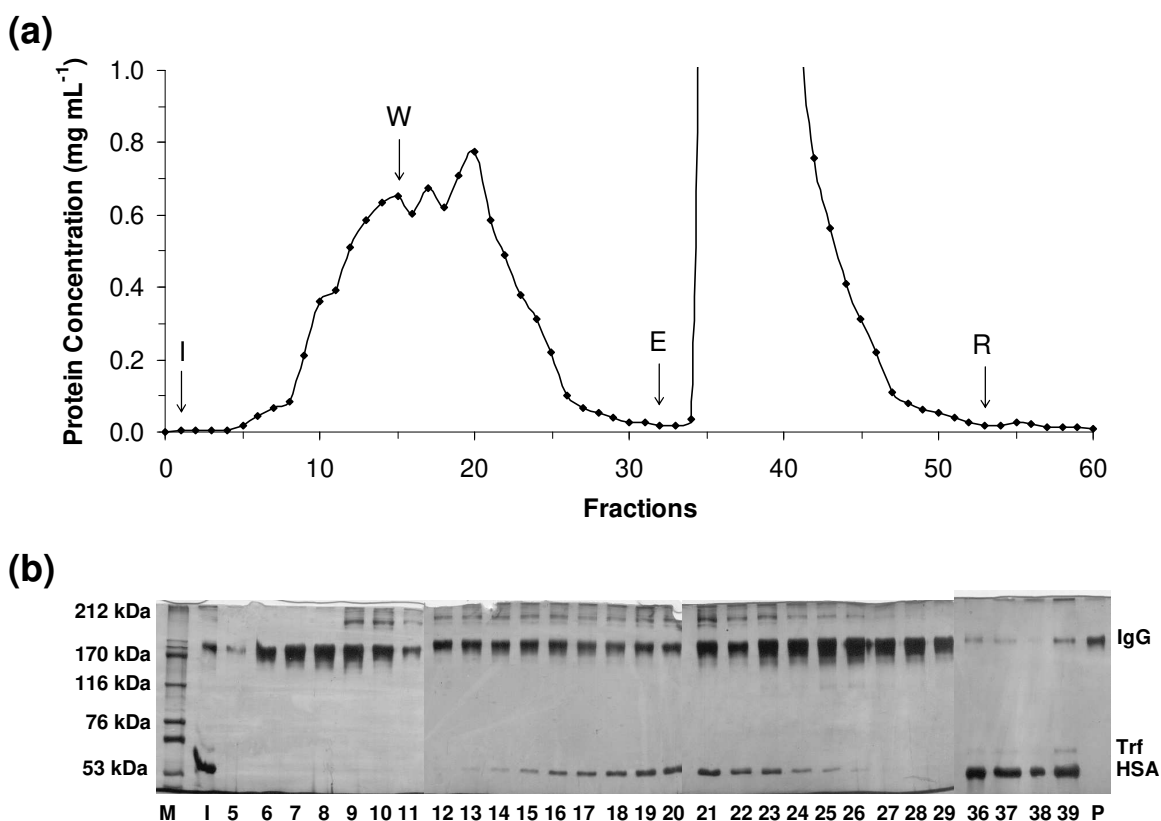


Figure 5. (a) Breakthrough curve for 15.0 mL of human serum solution diluted 20 times in Mops buffer 25 mmol L⁻¹ pH 6.5 for PLL-agarose gel (3.0 mL bed). I: Injection (42.2 mg of total protein determined using Bradford method [Bradford, 1976]), W: Washing, E: Elution, R: Regeneration; (b) SDS-PAGE analysis under nonreducing conditions of the breakthrough curve, M: molecular mass protein marker, I: injected human serum solution, 6-15: feed, 16-29: washing, 36-39: elution (performed with the loading buffer containing 0.4 mol L⁻¹ NaCl), P: human IgG standard (CSL Behring).

Table 1. Nephelometric analysis of the breakthrough in PLL-agarose. Feed: 750 μL of human serum diluted 20 times (15.0 mL). Loading buffer: Mops 25 mmol L^{-1} , pH 6.5. Elution: Mops 25 mmol L^{-1} , NaCl 0.4 mol L^{-1} , pH 6.5.

Steps	Proteins (mg)					IgG Purification		
	IgG	HSA	Trf	IgA	IgM	Σ^a	Purity ^b (%)	PF ^c
Human serum diluted 20 times	9.15	27.90	2.55	2.86	1.20	43.66	21.0	1.0
Pool of fractions 1-12	1.20	n.d. ^d	n.d.	0.08	0.08	1.36	88.2	4.2
Pool of fractions 13-31	5.22	0.60	n.d.	0.75	0.30	6.87	76.0	3.6
Pool of elution: fractions 35-50	3.36	29.12	2.59	2.31	0.91	38.29	8.8	0.4
Total recovery	9.78	29.72	2.59	3.14	1.29	46.52	-	-

^a Refers to the protein sum of IgG, HSA, Trf, IgA, and IgM

^b Purity: mass ratio of IgG to protein sum

^c PF: Purification factor

^d n.d.: Values lower than the detectable range of Array Protein System

Bed volume: 3.0 mL

Using the Bradford method [Bradford, 1976] to determine the total protein content, it was possible to express the dynamic adsorption capacity of PLL-agarose for human serum protein as 17.02 mg mL^{-1} gel and 19.84 mg mL^{-1} gel using Mops, pH 6.5 and Bis-Tris-HCl, pH 6.0, respectively. When a 15.0 mL of serum solution was used, for Mops, pH 6.5, HSA was detected in SDS-PAGE (Figure 5b) at the outlet after fraction #12 (breaking point), while for Bis-Tris-HCl, pH 6.0 this breaking point cannot be determined as no HSA could be detected in nonretained fractions, since HSA saturation was not reached (data not shown). Another breakthrough was performed, feeding 25.0 mL of serum solution and, in this case, HSA breaking point was detected in SDS-PAGE at the outlet after fraction #20 (Figure 6b).

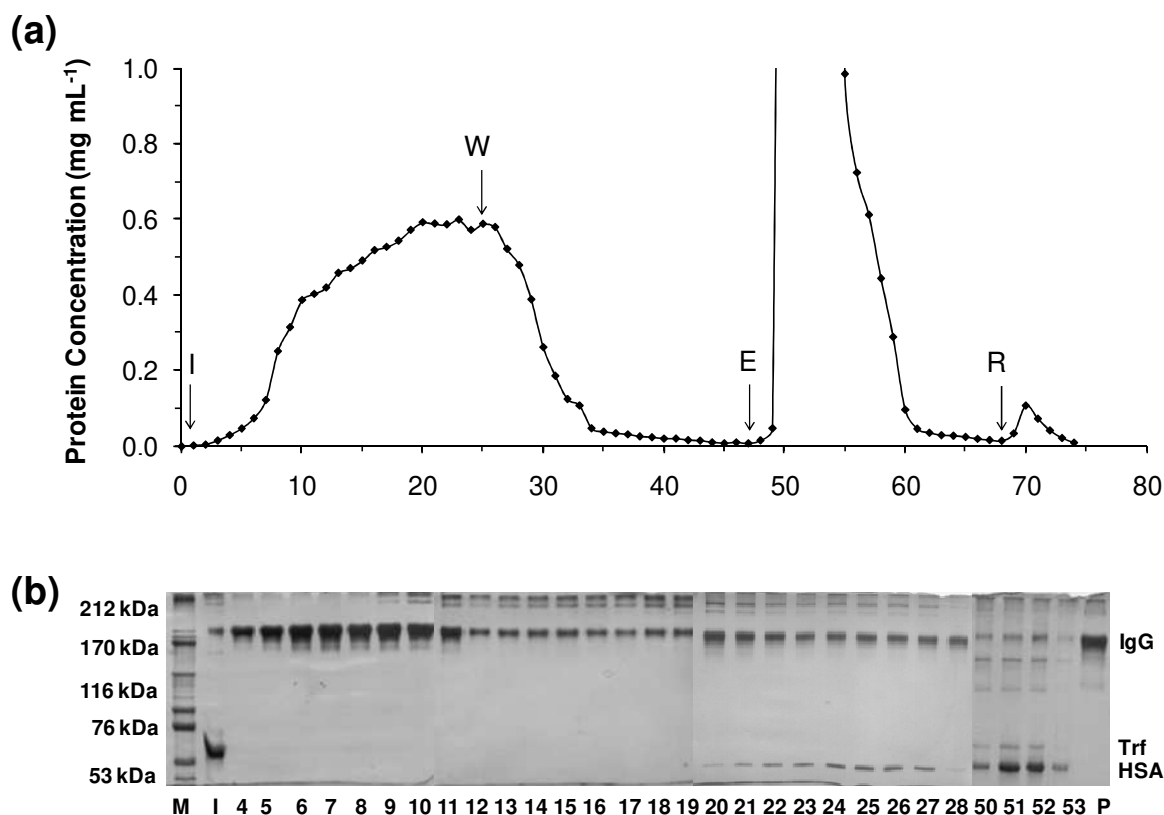


Figure 6. (a) Breakthrough curve for 25.0 mL of human serum solution diluted 20 times in Bis-Tris-HCl buffer 25 mmol L⁻¹ pH 6.0 for PLL-agarose gel (3.0 mL bed). I: Injection (47.7 mg of total protein determined using Bradford method [Bradford, 1976]), W: Washing, E: Elution, R: Regeneration; (b) SDS-PAGE analysis under nonreducing conditions of the breakthrough curve, M: molecular mass protein marker, I: injected human serum solution, 4-25: feed, 26-28: washing, 50-53: elution (performed with the loading buffer containing 0.4 mol L⁻¹ NaCl), P: human IgG standard (CSL Behring).

Souza et al. (2010) performed a comparison of the use of the ligand aminohexyl (1,6-diaminohexane) immobilized on two different activated agarose gel (CNBr and epoxy) aiming at the purification of human IgG by negative chromatography, investigating the capacity and selectivity of the system. IgG recovery in nonretained fractions in this work was similar to the data presented by Souza and coworkers (2010) that used 15.0 mL of human serum solution diluted 20 times in Hepes buffer pH 5.8: 69% for CNBr-activated gel (spacer arm of 1 atom), and 76% for epoxy-activated gel (spacer arm of 12 atoms). Due to a larger spacer arm, there was no detection of any major impurities (IgA, IgM, Trf, and

HSA) presented in human serum. In the present work, for 25.0 mL of human serum solution fed in the column, when the adsorption buffer Bis-Tris was used, a higher IgG recovery was achieved (79.0%) and a lower IgG purity (91.9%) while Souza et al. [2010], using Hepes as adsorption buffer reached a purity of 100% but a recovery of 76% of the IgG fed.

Table 2. Nephelometric analysis of the breakthrough in PLL-agarose. Feed: 1.25 mL of human serum diluted 20 times (25.0 mL). Loading buffer: Bis-Tris-HCl 25 mmol L⁻¹, pH 6.0. Elution: Bis-Tris-HCl 25 mmol L⁻¹, NaCl 0.4 mol L⁻¹, pH 6.0.

Steps	Proteins (mg)					IgG Purification		
	IgG	HSA	Trf	IgA	IgM	Σ^a	Purity ^b (%)	PF ^c
Human serum diluted 20 times	13.70	48.75	3.25	1.50	1.50	68.70	19.9	1.0
Pool of fractions 1-19	5.00	n.d. ^d	n.d.	n.d.	0.25	5.25	95.2	4.8
Pool of fractions 20-28	4.30	0.40	n.d.	n.d.	0.15	4.85	88.7	4.5
Pool of fractions 29-33	1.52	n.d.	n.d.	n.d.	0.16	1.68	90.5	4.5
Pool of elution: fractions 50-59	2.50	48.00	3.10	1.50	1.00	56.10	4.5	0.2
Total recovery	13.32	48.40	3.10	1.50	1.56	67.88	-	-

^a Refers to the protein sum of IgG, HSA, Trf, IgA, and IgM

^b Purity: mass ratio of IgG to protein sum

^c PF: Purification factor

^dn.d.: Values lower than the detectable range of Array Protein System

Bed volume: 3.0 mL

3.4. HSA and IgG adsorption from single protein solutions: Isotherm determination

The most abundant protein present in human serum is HSA, which comprises about half of the blood serum protein [Putnam, 1984]. Since HSA was efficiently adsorbed onto adsorbents containing amine groups (TREN-, ω -

aminohexyl-, and ω -aminodecyl- agarose; [Bresolin et al, 2009; Souza et al., 2010, Bresolin et al., 2010]), it was expected that PLL immobilized in agarose could also adsorb HSA efficiently.

The experimental adsorption data of HSA and IgG (obtained from batch adsorption experiments, performed at 25°C with Bis-Tris-HCl buffer, pH 6.0) and the fitted curves of the Langmuir and Langmuir-Freundlich models are presented in Figure 7. Table 3 presents the fitted parameters of the models.

Table 3. Parameters of the Langmuir and Langmuir-Freundlich isotherm models adjusted to experimental HSA and IgG adsorption data.

Parameters	HSA	IgG	
	Langmuir	Langmuir	Langmuir-Freundlich
Q_m (mg mL ⁻¹)	76.4 ± 2.0	29.1 ± 2.6	23.6 ± 1.6
K_d (mol L ⁻¹)	$(6.0 \pm 0.6) \times 10^{-5}$	$(3.0 \pm 1.6) \times 10^{-5}$	—
K_{dLF} (mol L ⁻¹)	—	—	$(3.8 \pm 0.8) \times 10^{-5}$
n	—	—	1.60 ± 0.25
R^2	0.991	0.963	0.982
χ^2	6.1	3.14	1.75

According to Table 3, the Langmuir model described the HSA adsorption data satisfactorily (correlation coefficient of 0.991) with the Q_m value estimated as 76.4 ± 2.0 mg of HSA mL⁻¹ of gel. The adjustment of isotherms parameters for HSA adsorption onto histidine grafted aminohexyl-Sepharose (93.3 mg HSA mL⁻¹ gel [Pitiot et al., 2001]), and ω -aminodecyl-agarose (81.0 mg HSA mL⁻¹ gel [Bresolin et al., 2010]) were in the same order of magnitude of experimental data reported in the present work (Table 3 and Figure 7a). The constant of dissociation (K_d) value was of the order of magnitude of 10^{-5} mol L⁻¹, showing that PLL can be considered a low-affinity media for HSA adsorption [Vijayalakshmi, 1989].

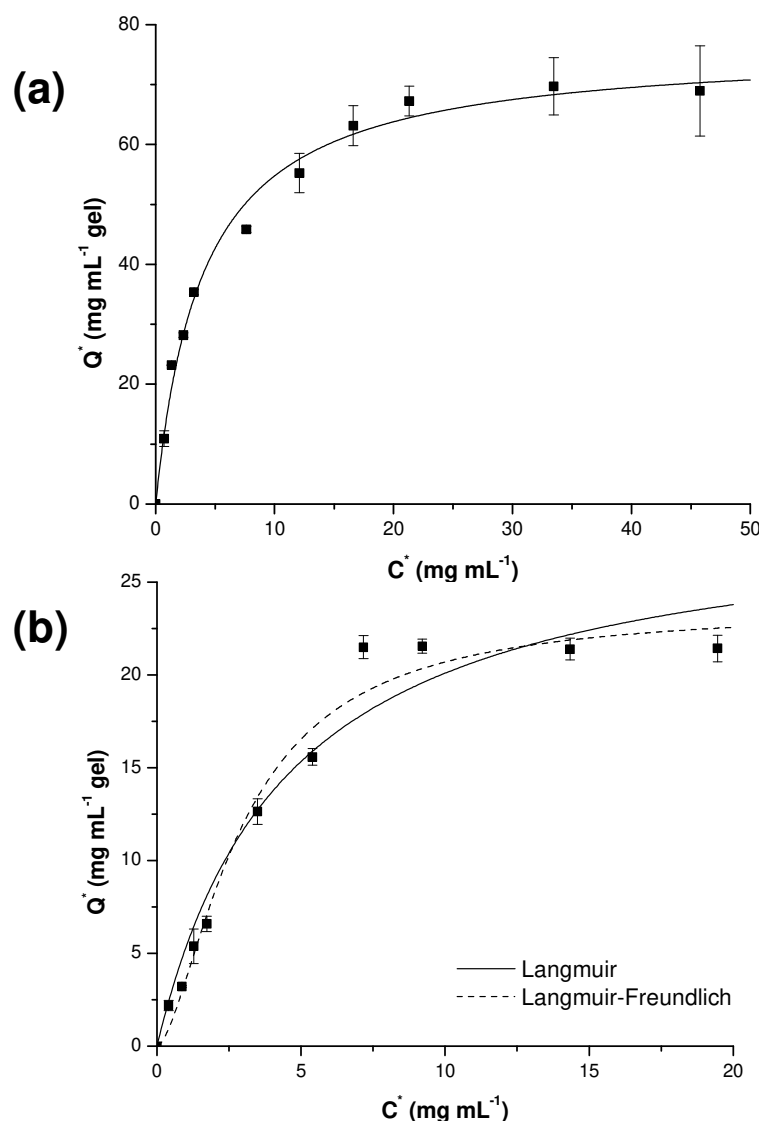


Figure 7. Experimental adsorption isotherms for (a) HSA and (b) IgG on PLL-agarose gel with Bis-Tris-HCl buffer pH 6.0 at 25°C. The solid and dashed lines correspond to fitting (nonlinear regression) of experimental data in accordance with the Langmuir and Langmuir-Freundlich models, respectively.

For IgG adsorption, good model fit was obtained for the Langmuir-Freundlich model (coefficient of correlation of 0.982; Figure 7b and Table 3). The Langmuir-Freundlich isotherm model gave $n = 1.60$, which indicates the presence of cooperative effects and the heterogeneous nature of the adsorption due to multipoint interactions. The presence of cooperatively effects was also observed for the adsorption of human IgG onto ω -aminohexyl ($n = 2.71$), and ω -aminodecyl ($n = 2.30$) grafted agarose.

The IgG maximum adsorption capacity (Q_m) values were estimated as 29.1 ± 2.6 and 23.6 ± 1.6 mg IgG mL⁻¹ gel, for the Langmuir and Langmuir-Freundlich models, respectively. These values are also in the same order of magnitude of experimental data reported for human IgG adsorption onto TREN-agarose (38.2 mg of IgG mL⁻¹) ω -aminodecyl-agarose (19.8 mg of IgG mL⁻¹) [Bresolin et al., 2009; Bresolin et al., 2010]. IgG adsorption on amino based adsorbents was mainly produced by ion exchange and hydrophobic interactions as the initial IgG solution contained molecules that had positive, negative, and zero charges [Bresolin et al., 2009; Souza et al., 2009; Bresolin et al., 2010].

4. Conclusions

In the present work we have demonstrated that negative chromatography on PLL-agarose was a successful method to isolate IgG from human serum, with 79.0% recovery in nonretained fractions reaching a purity degree of 91.9% (based on IgG, IgA, IgM, HSA, and Trf nephelometric and SDS-PAGE analysis). The adjustment of the Langmuir-Freundlich adsorption model to experimental IgG data showed the presence of cooperative effects between the IgG and the PLL ligand. The results achieved with this ligand are similar to other amine-based ligands presented in literature for IgG purification by negative chromatography, since the antibodies are recovered under mild conditions of pH, low ionic strength, and room temperature. Thus, PLL-agarose can be considered as an alternative adsorbent for the isolation of IgG from human serum, with potential for integration into large-scale IgG purification processes.

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CAPÍTULO 4: DISCUSSÃO GERAL

A pesquisa científica e o consequente desenvolvimento de novas tecnologias que visam à recuperação e purificação de biomoléculas (RPB ou “dowstream processing”) têm como principais objetivos maximizar parâmetros como seletividade, rendimento e pureza, ao menor custo possível. No caso específico de anticorpos, como a IgG humana, a cromatografia de afinidade continua sendo utilizada, uma vez que elevadas pureza e seletividade são atingidas em condições operacionais bem conhecidas. Entretanto, neste contexto, a busca de adsorventes alternativos, bem como a determinação das melhores condições experimentais para a purificação (tampões de adsorção, pH, força iônica, condições de eluição, velocidade superficial, etc) é vista com bons olhos por indústrias em seus processos de RPB.

Para a escolha de um ligante a ser imobilizado em uma matriz cromatográfica, de acordo com LABROU e CLONIS (1994) devem ser levados em conta três fatores principais: especificidade (apropriada para cada tipo de aplicação), reversibilidade (formação de um complexo reversível com a proteína a ser purificada) e estabilidade (ser estável nas condições utilizadas durante o processo de imobilização, purificação e sanitização). Além disso, o ligante deve apresentar grupos que possam se ligar covalentemente à matriz (tais como $-NH_2$, $-COOH$, $-CHO$, $-SH$ e $-OH$, entre outros) não sendo essenciais para a ligação com a proteína. No que diz respeito à afinidade, deve-se buscar ligantes cuja constante de dissociação K_d encontrem-se na faixa 10^{-8} mol L⁻¹ a 10^{-4} mol L⁻¹, de modo que a dissociação do complexo entre proteína e ligante possa ser feita por meio de uma combinação de fatores como pH, força iônica, concentração de ligante livre ou competidor, etc).

Para um melhor entendimento da discussão geral dos resultados deste trabalho, pode-se dividi-lo em duas grandes partes. A primeira diz respeito ao uso de agentes quelantes de IMAC como ligantes atuando como grupos ionogênicos (sem íon metal imobilizado) enquanto que a segunda trata da utilização de

poliaminas como ligantes, ambas visando a purificação de IgG a partir do soro humano.

Neste trabalho foram empregados dois agentes quelantes não-tradicionais de IMAC como ligantes, que apresentam características totalmente distintas: o Tris(2-aminoetil)amina (TREN) e a o-fosfoserina (OPS).

OPS possui em sua estrutura grupamentos fosfato, carboxila e amina, que apresentam a habilidade de quelatar íons metálicos e, na ausência destes, se comporta como um trocador de íons. De acordo com dados apresentados por ZACHARIOU et al. (1996) utilizando medidas potenciométricas corroborado por SMIECHOWSKI (2010) que utilizou cálculos de teoria da densidade funcional, OPS apresenta 3 valores de pKa: 2,19, 5,78 e 9,85. Para o ligante livre, em valores de pH abaixo de 2,19 há um predomínio da carga positiva do grupo amina, ao passo que, conforme o pH aumenta, há um aumento de predominância das cargas negativas dos grupos carboxila e fosfato. Neste sentido, avaliou-se o desempenho de OPS imobilizado em agarose (via grupamento amino, de acordo com dados do catálogo do fabricante – Sigma-Aldrich, produto P3578) sem metal imobilizado, aproveitando suas características que o permitem atuar como um trocador de íons.

Na faixa de pH avaliada (de 6,0 a 8,5), o tampão que proporcionou maior seletividade foi o fosfato de sódio. Com este tampão, houve a recuperação de IgG nas frações retidas juntamente com IgM (em todos os valores de pH avaliados), além de ter ocorrido uma diminuição na quantidade de IgG adsorvida quando o pH foi aumentado de 6,0 até 7,5. Esta redução na quantidade de IgG adsorvida com o aumento do pH pode ser explicada em termos da existência de interações eletrostáticas entre o ligante OPS (com carga líquida total negativa) e a IgG em solução (com maioria de moléculas positivamente carregada nesta faixa de pH, pois apresenta valores de pI entre 7,8 a 9,3).

Diferentemente do que ocorre com OPS, o TREN possui somente grupamentos amino para ser utilizado na quelação de íons metálicos em IMAC. Assim, na ausência de íons metálicos, na faixa de pH estudado, este ligante possui carga líquida total positiva, permitindo a adsorção dos principais

contaminantes (HSA e Trf, que se encontram carregadas negativamente). Assim, avaliou-se a condição experimental que proporcionasse maior seletividade e pureza de IgG utilizando cromatografia negativa. Para este fim, avaliaram-se, em um primeiro momento, diferentes tampões e, numa etapa seguinte, diferentes diluições do soro humano.

Para ambos adsorventes foram realizados experimentos de curva de ruptura (nas melhores condições operacionais para cada adsorvente, ou seja, tampão e pH) visando à determinação da capacidade dinâmica de adsorção. Para OPS-agarose, obteve-se 5,8 mg de proteínas totais mL^{-1} de gel, enquanto que para TREN-agarose este valor foi de 31,6 mg de proteínas totais mL^{-1} de gel. Para ambos os adsorventes, entretanto, nestes experimentos, recuperou-se cerca de 36% da IgG alimentada, apresentando elevada pureza (88 a 95%).

Foram determinadas também as isotermas de adsorção multicomponente (IgG e HSA) visando à determinação de parâmetros como capacidade máxima de adsorção (q_m), bem como a constante de dissociação proteína-ligante (K_d). No caso do TREN, o maior interesse está no valor de q_m da HSA, que é a proteína mais abundante a ser removida do soro humano, visando a purificação de IgG por cromatografia negativa. Neste sentido, o gel TREN-agarose apresentou um elevado valor de q_m ($191,7 \text{ mg mL}^{-1}$), cerca de 2 vezes superior a DEAE-celulose e histidina-aminohexil-agarose apresentados na literatura para a adsorção de BSA e HSA, respectivamente (ANSPACH et al., 1996; PITIOT et al., 2001a). Para IgG de alta pureza, o valor de q_m foi mais baixo ($38,2 \text{ mg mL}^{-1}$), enquanto que para soro total o valor de q_m atingiu a $66,6 \text{ mg mL}^{-1}$. Por sua vez, no caso do gel OPS-agarose, o valor de q_m variou entre $24,2$ e $28,5 \text{ mg mL}^{-1}$, valores muito parecidos com o gel de afinidade Proteína A-Sepharose (20 a 30 mg mL^{-1}). Entretanto, o K_d do TREN para a HSA esteve na ordem de $10^{-6} \text{ mol L}^{-1}$, enquanto que para IgG o gel OPS-agarose apresentou K_d na ordem de $10^{-5} \text{ mol L}^{-1}$. De acordo com a classificação apresentada por VIJAYALAKSHMI (1989), os valores de K_d caracterizam estes ligantes como sendo de média afinidade.

No que diz respeito ao uso de poliaminas como ligantes, comparações podem ser feitas entre os dados obtidos com o TREN (Figura 2-2a) e aqueles obtidos com o aminoácido L-Lisina (Figura 4-1a) e seu polímero poli-L-Lisina (PLL

– Figura 4-1b). Como tais ligantes possuem grupamentos amina que podem ser usadas na adsorção de proteínas do soro humano e consequente purificação de IgG por cromatografia negativa, este trabalho avaliou o potencial de uso destes ligantes e determinou as melhores condições operacionais para cada um deles.

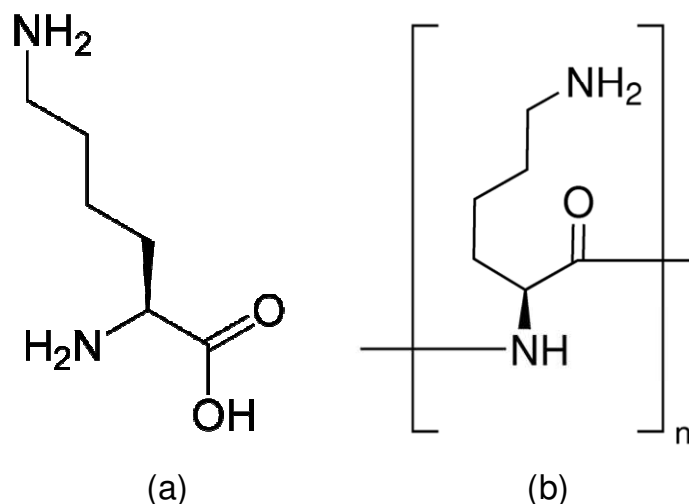


Figura 4-1. Estruturas químicas do aminoácido Lisina (a) e seu polímero poli-L-Lisina (b) utilizados como ligantes neste trabalho.

A faixa tamponante trabalhada variou entre 5,5 a 9,0, para se determinar uma condição que permitisse atingir uma maior recuperação de IgG o mais pura possível nas frações não retidas (princípio da cromatografia negativa). Em termos de seletividade, a condição experimental que favoreceu maior purificação de IgG humana por cromatografia negativa foi alcançada quando o tampão Bis-Tris-HCl foi usado em pH 7,0 para a Lisina e em pH 6,0 para a PLL.

A principal diferença entre a lisina e a PLL é que após a imobilização via grupamento NH₂, no primeiro caso, o grupo carboxila fica livre para interação com as proteínas. Dessa forma, a carga negativa da carboxila tem um efeito muito grande na adsorção das biomoléculas, podendo interferir na adsorção das proteínas do soro como a IgM, por exemplo. Tal fato não é observado na PLL, uma vez que a carboxila está ligada covalentemente a outra molécula de lisina por meio de ligação peptídica não havendo a disponibilidade dessa carga negativa.

Experimentos de curva de ruptura similares aos apresentados com os demais ligantes visaram avaliar somente o comportamento do adsorvente PLL-

agarose (uma vez que este se mostrou mais seletivo que lisina-agarose) e determinar sua capacidade dinâmica de adsorção de proteínas do soro humano. Quando o tampão de adsorção Bis-Tris foi utilizado, atingiu-se maior recuperação de IgG (79%) porém com grau de pureza de IgG (95%) similar ao TREN (recuperação de 36% de IgG). Para alimentação de uma solução de 15,0 mL de soro humano diluído 20 vezes, a capacidade dinâmica de PLL-agarose encontrada foi de 12,7 mg mL⁻¹ de gel, cerca de 2,5 vezes inferior ao atingido pelo ligante TREN (31,6 mg mL⁻¹), quando se alimentou uma quantidade de proteína total cerca de 72% superior (119 mg para o TREN e 69 mg para a PLL).

Similarmente ao realizado com o TREN, foram levantadas as isotermas de adsorção monocomponente utilizando soluções de HSA ou IgG, ajustando os parâmetros dos modelos de Langmuir e Langmuir-Freundlich aos dados experimentais. O modelo de Langmuir descreveu os dados de adsorção de HSA satisfatoriamente atingindo Q_m de 76,4 mg de HSA mL⁻¹, na mesma ordem de grandeza de dados apresentados para a adsorção de HSA em histidina (93,3 mg mL⁻¹; PITIOT et al., 2001a), ω -aminodecyl (81,0 mg mL⁻¹; BRESOLIN et al., 2010b), porém 2,5 vezes inferior ao TREN (191,7 mg mL⁻¹). Tais resultados podem ser explicados pela acessibilidade à interação com a HSA de três grupamentos amino do TREN, ao passo que para a histidina há um grupamento amina e o anel imidazol e para PLL e o ω -aminodecyl há apenas um grupamento amina, quando os ligantes estão imobilizados. A constante de dissociação (K_d) foi da ordem de grandeza de 10⁻⁵ mol L⁻¹, mostrando que PLL pode ser considerada um ligante de média afinidade para adsorção de HSA.

A adsorção de IgG em adsorventes que contém grupamentos amina, por sua vez, é promovida, principalmente, por uma combinação de fenômenos de troca iônica e interação hidrofóbica. Por esta razão, detectou-se a presença de efeito de cooperatividade positiva, uma vez que o parâmetro n do modelo de Langmuir-Freundlich foi ajustado para 1,60. Na cooperatividade positiva, a adsorção de uma molécula tende a auxiliar a adsorção de outra molécula. De acordo com JOHNSON e ARNOLD (1995), a presença de cooperatividade positiva é um indicativo da natureza heterogênea da adsorção, muito provavelmente, devido

à presença de interações multiponto, que são favorecidas pela ocorrência simultânea de interações eletrostáticas e hidrofóbicas.

A análise conjunta de todos estes resultados experimentais sugere que, quando se deseja IgG com valores mais elevados de pureza, medidas complementares são necessárias no processo de purificação quando se utilizarem os ligantes OPS e PLL. Para o TREN, a pureza de IgG obtida foi elevada baseado em análises de IgG, IgM, albumina, transferrina e IgA (devido a ausência das principais proteínas que compõem o soro humano). Porém, ao se pensar globalmente em um processo de RPB de IgG a partir do soro humano, deve-se buscar aumentar ao máximo a recuperação de IgG para todos os ligantes avaliados. A grande vantagem de todos os ligantes estudados neste trabalho está no fato de se atingir elevados teores de pureza fazendo-se uso de tampões de baixa força iônica, temperatura ambiente e condições de pH próximas à neutralidade, que não causam significativa degradação ou perda de atividade biológica na molécula de IgG. Assim, todos estes adsorventes podem ser considerados como alternativos para o isolamento de IgG a partir do soro humano, pois apresentam potencial para serem integrados a processos de RPB em larga escala.

CAPÍTULO 5: CONCLUSÕES

A realização deste trabalho nos permite as seguintes conclusões:

Ligantes contendo grupamentos amina como o TREN e a PLL permitiram que a IgG alimentada foi recuperada nas frações não-retidas (cromatografia negativa), apresentado pureza superior a 90% e recuperação de 73% e 86%, respectivamente, indicando que esta IgG poderia ser utilizada em aplicações intramusculares e intravenosas devido a ausência de IgA.

Por sua vez, quando o ligante OPS foi utilizado, a IgG foi recuperada somente com IgM nas frações retidas, sendo eluídas com aumento da força iônica pela adição NaCl, atingindo uma pureza de 88%, com um fator de purificação de 4.9. Este ligante se mostrou eficiente quando se deseja purificar IgG humana que possui pontos isoelétricos na faixa de 7,8 a 8,5.

Para todos os ligantes, a recuperação de IgG a partir soro humano pode ser alcançada sob condições brandas de pH, baixa força iônica, e temperatura ambiente. De um ponto de vista de processo em larga escala, todos os ligantes estudados neste trabalho apresentam potencial para serem usados como uma das etapas em um processo industrial de RPB de IgG a partir do soro humano.

CAPÍTULO 6: SUGESTÕES PARA TRABALHOS FUTUROS

A realização da presente tese de doutorado, bem como as conclusões oriundas dos dados experimentos e a discussão e comparação com resultados da literatura, abre algumas possibilidades que podem vir a ser exploradas em eventuais trabalhos futuros de pesquisa.

- 1) Nos ligantes utilizados neste trabalho (OPS, TREN, Lisina e PLL), avaliar se ocorre adsorção e/ou não-adsorção de subclasses de IgG humana (IgG₁, IgG_{2a}, IgG_{2b}, IgG₃ e IgG₄), uma vez que as subclasses de IgG possuem valores de pI distintos: IgG₁ e IgG₃ possuem pI mais altos (de 7,8 a 9,0) que os da IgG₂ e IgG₄ (de 6,3 a 8,0).
- 2) Avaliar se estes ligantes também se apresentam seletivos para a purificação de anticorpos monoclonais produzidos pelo cultivo de hibridomas, uma vez que os anticorpos encontram-se em um meio complexo contendo aminoácidos e proteínas (como a albumina oriunda do soro fetal bovino utilizado no meio de cultura)
- 3) Imobilizar estes ligantes em membranas, avaliando seu comportamento na purificação de IgG a partir do soro humano, comparando com os resultados deste trabalho onde os mesmos ligantes foram utilizados imobilizados em géis de agarose.
- 4) Avaliar os parâmetros pureza, seletividade e fator de purificação de IgG humana (a partir do soro) em um sistema com duas colunas em série, contendo, na primeira o ligante TREN ou PLL, e na segunda o ligante OPS.
- 5) Realizar a representação matemática do sistema de separação por cromatografia visando avaliar os fenômenos físico-químicos presentes no interior da coluna, de modo a manter a coluna operando nas melhores condições possíveis.
- 6) Determinar as condições de operação para o isolamento e purificação de IgG humana, seguindo três etapas distintas: modelagem

matemática do leito de gel, estimação dos parâmetros de equilíbrio de adsorção e transferência de massa e otimização numérica das condições de operação. O equilíbrio da adsorção entre a IgG e demais componentes (contaminantes) e o adsorvente pode ser avaliado experimentalmente e incorporado a um modelo matemático baseado no balanço de massa transiente no leito para a simulação dos perfis de concentração de proteína total.

- 7) Realizar uma avaliação técnico-econômica criteriosa da viabilidade de uso destes ligantes em um processo em maior escala.

CAPÍTULO 7: REFERÊNCIAS

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CAPÍTULO 8: ANEXOS

8.1. ANEXO I – INFORMAÇÃO CCPG/01/2008

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG nº 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.

II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.

III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.

IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).

V. Sumário.

VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.

VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.

VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.

IX. Todas as páginas com numeração “ímpar” serão impressas como “frente” e todas as páginas com numeração “par” serão impressas como “verso”.

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2º desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em

formato alternativo, desde que observados os incisos I, II, III, IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Uma versão definitiva da dissertação ou tese gravada em arquivo PDF, deve ser encaminhada à gráfica da Unicamp acompanhada do formulário “Requisição de Serviços Gráficos”, onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

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§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

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§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 09 de abril de 2008

Profa. Dra. Teresa Dib Zambon Atvars

Presidente

Comissão Central de Pós-Graduação

8.2. ANEXO III – AUTORIZAÇÃO ELSEVIER (ARTIGO DA REVISTA JOURNAL OF CHROMATOGRAPHY B)

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