UNIVERSITI PUTRA MALAYSIA

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By

RATTANA WONGCHUPHAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

October 2010

DEDICATED TO

MEMORY OF MY PARENTS

WHO REMINDED THEIR CHILDREN ALWAYS
ON KNOWLEDGE EMPHASIS

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

DEVELOPMENT OF DYE AFFINITY ADSORBENTS FOR RECOVERY OF POLYCLONAL ANTI-HEPATITIS B CORE ANTIGEN IMMUNOGLOBULIN G

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October 2010

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Faculty: Engineering

Antibodies such as immunoglobulin G (IgG) have been used extensively for therapeutic and diagnostic purposes. Protein A affinity chromatography which is highly specific towards IgG is a standard method to purify it. However, using expensive and unstable protein A in large-scale production has increased the antibody production cost accordingly. Affinity dye-ligands which are widely used for protein purification has demonstrated their high binding capacity as 40 mg/mL comparable to protein A. Moreover, their widespread availability, ease and speed of preparation, chemical stability, and ease of storage, render them an attractive alternative choice. Especially, their economy is also a major consideration in replacement of expensive protein A. Thus, the development of selective recovery of polyclonal anti-hepatitis B core antigen immunoglobulin G (anti-HBcAg IgG) from rabbit sera has been investigated.

Four different reactive dye-ligands; Cibacron Blue 3GA (CB), Reactive Brown 10 (RB 10), Reactive Red 120 (RR 120) and Reactive Green 5 (RG 5) were covalently

attached on the Streamline quartz base matrix via triazine linkage under alkali condition. Essentially at start, IgG antibody's binding capacity screening of these immobilized dyes was required. Similar amount of dye-ligands attached on the bare matrix, determined by mass balance method was attributed relatively in comparison of adsorption capacities for different dye-ligands. From the simulating adsorption study in single protein system, the immobilized RG 5 was chosen as its capacity for fewer albumins and more IgG adsorbed at pH 7.0, compared to other immobilized dye-ligands possessing similar ligand density. The content of RG 5 immobilized on the matrix was 17.4 μmol/mL adsorbent. About 64% of rabbit IgG was bound on the immobilized RG 5 at pH 7.0 in binary protein binding system with similar ratio of both albumin and rabbit IgG. The maximum adsorption capacity (q_m) of RG-5 immobilized adsorbent for rabbit IgG was 49.0 mg/mL adsorbent and the dissociation constant (K_d) value was found to be 3.33×10^{-6} M. The phenomenon of reversible IgG adsorption on the adsorbent appeared to follow the Langmuir-Freundlich isotherm model. Serum from the immunized rabbits against hepatitis B core antigen (HBcAg) was used as a feedstock containing polyclonal anti-HBcAg IgG solely for batch antibody purification study. Highly abundant albumin and other serum proteins which constitute about 80% of total serum protein are a major interference in dye-ligand affinity chromatographic studies. This leads to the strategy of removing contaminant proteins before subjecting to dye-ligand immobilized system. Anion exchange adsorbents like the Streamline DEAE and Streamline Q XL were introduced as their high capacity available for albumin. Although both anion exchangers were capable of removing most of albumin and other contaminants greater than 90%, the loss of IgG was higher in the presence of Q XL. As a result, the removal of albumin was accomplished in high efficiency via a strong adsorption on

DEAE under optimized conditions as followed: 0.5 mg/mL initial protein concentration, pH 8.0; 0.25 mL settled bed volume of Streamline DEAE. Consequently, 80% of polyclonal anti-HBcAg IgG was recovered. A two step procedure using Streamline DEAE anion exchanger and RG-5 immobilized adsorbent was performed for removing albumin and capturing IgG, respectively, under the optimized conditions. After antibody adsorption, bound IgG was eluted in elution medium, pH 8.0 containing 1.0 M NaCl, resulting about 53% IgG recovered with 86% purity and a purification factor of 6.

As exhibited in the current study, DEAE anion exchanger is credited for high efficacy to remove most contaminant proteins from rabbit serum. The purified antibodies can be a useful reagent in diagnosis of chronically infected hepatitis B carriers. Moreover, synthetic dye-ligands can be a potential alternative possessing a tendency of binding to biomolecules for several biological purposes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN DYE AFFINITY BAHAN PENJERAP UNTUK POLIKLONAL ANTI-HEPATITIS B ANTIGEN CORE IMMUNOGLOBULIN G

Oleh

RATTANA WONGCHUPHAN

Oktober 2010

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Antibodi seperti imunoglobulin G (IgG) secara umumnya telah banyak digunakan dalam tujuan rawatan dan diagnostik. Kromatografi afiniti Protein A yang sangat khusus terhadap IgG adalah kaedah piawai untuk penulenan antibodi. Walaubagaimanapun, penggunaan protein A yang mahal dan tidak stabil dalam kapasiti pengeluaran yang tinggi telah meningkatkan kos pengeluaran antibodi dengan setara. Ligand bahan penjerap yang digunakan secara berleluasa untuk tujuan penulenan protein telah menujukkan kapasiti pengikatan yang tinggi iaitu 40 mg/mL setanding dengan protein A. Selain itu, ketersediaannya yang tersebar luas, mudah dan kepantasan persediaannya, kestabilan kimia, dan kemudahan penyimpanan, menjadikan protein ini suatu pilihan yang menarik. Paling utama factor ekonominya juga merupakah suatu pertimbangan yang utama sebagai pengganti untuk protein A yang mahal. Dengan demikian, perkembangan penemuan teras poliklonal anti-antigen hepatitis B imunoglobulin G yang terpilih dari sera arnab telah diselidiki.

Terdapat empat reaktif dye-ligan yang berbeza; iaitu Cibacron Biru 3GA (CB), reaktif Brown 10 (RB 10), reaktif Red 120 (RR 120) dan reaktif Green 5 (RG 5) adalah terikat secara kovalen pada dasar Streamline berasaskan matriks dan digunakan sebagai penjerap pegun untuk penjerapan protein melalui sambungan triazine dalam keadaan alkali. Pada permulaannya, penyaringan kapasiti pengikatan antibodi IgG dye pegun ini adalah diperlukan. Jumlah dye ligan yang sama juga terdapat pada matriks di mana ia ditentukan dengan kaedah keseimbangan jisim relatif berbanding kapasiti jerapan untuk dye ligan. Dari kajian simulasi dalam sistem jerapan protein tunggal, RG 5 yang pegun untuk dipilih kapasitinya untuk albumin sedikit dan lebih IgG diserap pada pH 7.0, dibandingkan dengan ligan dye ligan pegun mempunyai ketumpatan yang sama. Kandungan RG 5 pegun ialah pada 17.4 μmol/mL penjerapan. Sebanyak 64% daripada IgG arnab terikat dalam sistem yang mengikat RG 5 pegun pada pH 7.0 dalam protein sistem binari dengan nisbah yang sama dari kedua-dua albumin dan IgG arnab. Penjerapan yang maksimum bagi (q_m) RG 5 pegun untuk IgG arnab adalah 49.0 mg/mL dan nilai pemalar perceraian (K_d) itu dijumpai dalam 3.33×10^{-6} M. Fenomena pembalikan penjerapan IgG pada bahan penjerap muncul berpandukan model isoterm Langmuir-Freundlich. Sera daripada arnab terimun terhadap antigen core hepatitis B (HBcAg) digunakan sebagai bahan asas yang mengandungi poliklonal anti-HBcAg IgG untuk kajian berkelompok penukaran antibodi. Kelimpahan albumin dan serum protein yang tinggi dimana terdiri daripada 80% jumlah serum protein merupakan gangguan utama dalam kajian afiniti dye ligan kromatografi. Ini menyebabkan kepada strategi penyingkiran bahan pencemar protein sebelum tumpuan terhadap sistem dye ligan pegun. Pertukaran penjerap anion DEAE Streamline dan Q XL Streamline diperkenalkan sebagai berkapasiti tinggi yang sedia ada untuk albumin. Walaupun kedua-dua penukar anion

mampu menghilangkan sebahagian besar daripada albumin dan pencemar lain lebih dari 90%, kehilangan IgG adalah lebih tinggi pada Q XL. Akibatnya, pembuangan albumin tercapai pada kecekapan yang tinggi melalui perjerapan yang kuat pada DEAE Streamline dalam keadaan dioptimumkan sebagai berikut: 0.5 mg/mL kepekatan serum protein, pH 8.0; 0.25 mL untuk DEAE Streamline. Akibatnya, 80% daripada IgG anti-HBcAg poliklonal telah ditemui. Terdapat dua langkah untuk menggunakan penukar anion DEAE Streamline dan RG 5 pegun adsorben dilakukan untuk menyingkirkan albumin dan mendapaan IgG, masing-masing, pad keadaan optimum. Selepas penjerapan antibodi, batas IgG terilusi terikat dalam medium elusi, pH 8.0 yang mengandungi 1.0 M NaCl, IgG sebanyak 53% ditemui dengan ketulenan sebanyak 86% dan penulenan faktor sebanyak 6.

Menurut kajian terkini, penukaran anion DEAE dikreditkan untuk keberkesanan yang tinggi bagi menghapuskan sebahagian besar bahan pencemar protein percemaran dari pada serum arnab. Antibodi yang ditulenkan boleh menjadi alat yang berguna dalam diagnosis bagi pembawa jangkitan kronik hepatitis B. Tambahan pula, pewarna sintetik-ligan dapat menjadi sumber alternatif yang berpotensi untuk mengikat biomolekul bagi beberapa tujuan biologi.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

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RATTANA WONCHUPHAN

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