Journal of Chromatography A, 1245 (2012) 55–64

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Alkaline-stable peptide ligand affinity adsorbents for the purification of biomolecules

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ARTICLE INFO

Article history:
Received 22 February 2012
Received in revised form 24 April 2012
Accepted 26 April 2012
Available online 4 May 2012

Keywords:
Peptide ligand
Affinity adsorbent
NaOH stability
Modification of surface chemistry
Solid-phase peptide synthesis
Cleaning-in-place

ABSTRACT

A strategy of modification of resin surface chemistry is presented to produce hydrophilic peptide-based alkaline-stable affinity adsorbents for the purification of biopharmaceuticals from complex media. In this work, the peptide-based affinity adsorbent HWRGWV-Toyopearl resin for the purification of IgG is presented as an example. When prepared by direct peptide synthesis on the chromatographic matrix, the peptide-based resin showed lability under alkaline conditions. In fact, the regeneration with aqueous 0.1 M NaOH caused the leaching of 40% of the peptide ligand, resulting in a decrease of IgG yield from 85% to 23%. It was found that the ligand leaching was caused by the coupling of a significant amount of peptide by alkaline-labile ester bonds. A method was designed to prevent the formation of ester bonds and allow the synthesis of the ligand exclusively on alkaline-stable bonds. The method consists in activating the hydrophilic base resin, blocking the hydroxyl groups responsible for alkaline lability and performing the peptide synthesis exclusively via alkaline-stable amide bonds. Repeated cycles of IgG purification from a cell culture medium were performed, each followed by cleaning with aqueous NaOH (0.1 M, 0.5 M and 1 M). The IgG yield decreased from 91% to 85% after 200 purification cycles with 0.1 M NaOH. However, the IgG purity remained almost constant at around 95% based on SDS-PAGE analysis. The procedure presented is rapid, efficient and inexpensive and does not require any equipment other than the conventional instrumentation for peptide synthesis. The method also has a broad application since it is valid for any peptide ligand identified for the purification of a biopharmaceutical target.

1. Introduction

Biopharmaceuticals have emerged as a very important class of therapeutics for the treatment of a vast array of diseases. However, these products are very expensive to consumers and have not been able to reach a large segment of the world's population. The high cost is mainly imputed to the purification processes (downstream processing), which account for over 50–80% of the overall manufacturing costs [1]. Chromatography is a central figure in downstream processing, being the only scalable technique that has the ability to achieve the high purity standards required by the regulatory authorities for commercial biologicals [2]. Among the different types of chromatography, affinity chromatography has the highest potential for the selective purification of therapeutic proteins. In order to be applied on the industrial scale, an affinity adsorbent should exhibit high binding capacity and specificity, low cost and good chemical resistance toward the treatments of cleaning-in-place (CIP) and sanitization-in-place (SIP) periodically performed to ensure its safe reusability [3]. The industrial standard for CIP and SIP of chromatographic media is aqueous NaOH (0.1–0.5 M), as it is the most effective agent in eliminating bacteria, endotoxins, and viruses and does not present any serious environmental disposal problem [4]. The widely used protein ligands for IgG purification, Protein A and Protein G, are expensive ($ 8000–15,000 per liter of resin) and can release immunogenic fragments into the process stream [5–10]. To overcome these problems, attempts have been made over the last two decades to develop specific, chemically robust and cost-effective synthetic ligands [11–13]. In particular, peptides are of great interest due to their high specificity and stability and low cost compared to biological ligands. Our group has identified several peptide ligands for the purification of biomolecules from complex media [14–18]. Recently, three linear hexapeptide ligands HWRGWV, HYFKFD and HFRRHL were discovered that bind human IgG through its Fc portion, thus mimicking the binding mechanism of Protein A [19,20]. These hexapeptide ligands successfully capture monoclonal antibodies from CHO cell culture supernatants and exhibit yield, purity, host cell protein removal and DNA removal that are comparable to the results obtained with Protein A and Protein G [21].

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http://dx.doi.org/10.1016/j.chroma.2012.04.072

The method adopted by our group for the preparation of peptide-based affinity adsorbents consists in building the peptide directly on polymethacrylate-based Toyopearl AF-Amino-650M resin via conventional Fluorenylmethyloxycarbonyl (Fmoc)-based coupling chemistry [22]. The resin was chosen as it is suited for both peptide synthesis and chromatographic applications, due to its chemical resistance to organic solvents and reagents, mechanical stability, high hydrophilicity and low non-specific binding [23]. However, experimental work has shown that these adsorbents do not withstand the alkaline conditions (0.1–0.5 M NaOH) used in CIP and SIP treatments. In fact, upon exposure to alkaline wash the peptide ligand is leached from the adsorbent in one cycle, resulting in a significant loss of binding capacity. To the best of our knowledge, the alkaline lability of affinity adsorbents produced by direct peptide synthesis on chromatographic media has not yet been described in literature.

At first we tested the stability of the peptide itself in alkaline conditions. The peptide was found to be stable in aqueous 1 M NaOH for up to 1 h. This indicated that the cause of ligand loss was likely due to the chemical linkage between the peptide and the base matrix. Based on the evaluation of the properties of the functional groups present on the hydrophilic amino resins, it was determined that the peptide synthesis chemistry could yield two different populations of peptides, one coupled onto amino groups and the other onto hydroxyl groups, via amide and ester bonds respectively. While the former are highly chemically stable, the latter are labile in alkaline conditions and could be responsible for the ligand leakage during the NaOH wash. As described in this paper, the fraction of peptides coupled through ester bond was found to be around 40%. Since the amino groups are much stronger nucleophiles than hydroxyl groups and their ratio on the resin is not disclosed, such high percentage of ester linkages were not expected.

As a result, a new strategy of resin surface modification was developed for synthesizing alkaline stable peptide adsorbents. The strategy consists of following steps: (i) activating the base matrix, (ii) selectively blocking the hydroxyl groups, and (iii) performing the peptide synthesis exclusively on amino groups through alkaline-stable amide bonds.

This work presents as a case study the on-resin synthesis of the affinity adsorbent HWRGWV-Toyopearl for the purification of IgG from cell culture medium. First, it is shown that a CIP step performed with 0.1 M NaOH on a HWRGWV adsorbent produced by direct on-resin synthesis caused a massive ligand leaching resulting in a loss of IgG binding capacity. A preliminary study is then presented to demonstrate the validity of the modification protocol. A chromophore amino acid (tryptophan) was coupled on a batch of modified resin with the new synthesis protocol, which showed a negligible amino acid leakage when washed in alkaline conditions. Finally, the ligand HWRGWV was synthesized on the modified resin to produce an alkaline-stable affinity adsorbent. Consecutive cycles of IgG purification were performed, each followed by an alkaline CIP step. After 200 cycles using regeneration with aqueous 0.1 M NaOH solution, the IgG yield decreased from 91% to 85%. When the regeneration was performed with 0.5 M NaOH and 1 M NaOH, the IgG yield decreased respectively to 80% over 100 cycles and to 76% over 50 cycles. However, under all these conditions, the IgG purity remained approximately constant in the range of 93–96%.

2. Experimental

2.1. Materials

HWRGWV resin having a peptide density of 0.15 mequiv./g was purchased from Creosalus (Louisville, KY, USA). Fmoc coupling chemistry was used to synthesize the peptide directly on Toyopearl AF-Amino-650M resin (particle size 65 μm). The resins Toyopearl AF-Amino-650M and Toyopearl HW-65F were purchased from Tosoh Bioscience (King of Prussia, PA, USA). Toyopearl HW-65F is a base resin having hydroxyl groups and is derivatized to produce Toyopearl AF-Amino 650M that has amine functional groups, as well as available hydroxyl groups. The reagents for peptide synthesis, Fmoc-protected amino acids (Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Phe-OH, Fmoc-Trp(Boc)-OH, Fmoc-Trp-OH, Fmoc-Val-OH) and the coupling agents (2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU)) were purchased from ChemPep Inc. (Wellington, FL, USA). Boc-Gly-OH, Boc-diaminoethane and carbonyl diimidazole were from Fisher Scientific (Pittsburgh, PA, USA). Diisopropylethylamine, ethanedithiol, thioanisole, anisole, piperidine (99%), trifluoroacetic acid and acetic anhydride were from Sigma Aldrich (Saint Louis, MO, USA). The solvents employed for peptide synthesis and analysis, dimethylformamide (extra dry), dichloromethane (extra dry), acetonitrile (HPLC grade) and trifluoroacetic acid (HPLC grade) were from Fisher Scientific (Pittsburgh, PA, USA). Human polyclonal immunoglobulin G (IgG) in lyophilized form was purchased from Equitech-Bio, Inc. (Kernville, TX, USA). Sodium chloride, sodium hydroxide, sodium acetate, hydrochloric acid and glacial acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). Phosphate buffer saline (PBS) of pH 7.4, ethanol, methanol and isopropanol were from Sigma Aldrich (Saint Louis, MO, USA). All the solvents were of analytical grade. Cell culture medium (Eagle Minimum Essential medium, EMEM) was from Quality Biological (Gaithersburg, MD, USA). Fetal calf serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (Logan, UT, USA) and Becton Dickinson (Sparks, MD, USA), respectively. The complete mammalian cell culture medium (cMEM) was prepared by combining EMEM with 10% FCS and 5% TBP. NuPAGE® Novex gels (4–12% Bis–Tris), NuPAGE® MOPS and MES running buffers, NuPAGE® LDS sample buffer, NuPAGE® reducing agent, SeeBlue plus2® pre-stained molecular weight marker, SimpleBlue™ SafeStain were all from Invitrogen (Carlsbad, CA, USA). A HiTrap™ Protein G column was purchased from GE Healthcare (Piscataway, NJ, USA). A Waters 626 LC system integrated with 2487 UV detectors (Milford, MA, USA) was used for all chromatography runs. This Waters 626 inert quaternary solvent pump can deliver a flow rate between 0.01 and 20.00 mL/min. Microbore stainless steel columns (30 mm long × 2.1 mm I.D.) were from Altech-Applied Science (Somerset, PA, USA). All experiments were carried out at room temperature.

2.2. Methods

2.2.1. On-column alkaline CIP of HWRGWV-Toyopearl resin prepared via direct on-resin synthesis

Thirty five milligrams of HWRGWV-Toyopearl resin were dry-packed in 30 mm × 2.1 mm I.D. Microbore columns (0.1 mL). The resin was swollen with 20% (v/v) methanol and then washed with PBS, pH 7.4. The column was equilibrated using PBS buffer containing 1 M NaCl. A 100 μL feed solution of IgG, prepared by spiking 10 mg of IgG into 1 mL of complete cell culture medium (cMEM), was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 4 mL of equilibration buffer at flow rate of 0.2 mL/min (346 cm/h). Elution was then performed with 4 mL of 0.2 M acetate buffer pH 4 at a flow rate of 0.4 mL/min (692 cm/h). The column was then regenerated with 0.1 M sodium hydroxide at the flow rate of 0.1 mL/min (174 cm/h) for 10 min. The effluent was monitored by UV absorbance at 280 nm. Different cycles of IgG purification followed by alkaline regeneration were performed. Fractions were collected and concentrated five times