Purification of an Acidic IgM with a Strong Anion Exchange Resin Tailored for Large Biomolecule Purification

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Antibody Purification

Bulletin 7127

Abstract

IgM antibodies are one of the fastest growing groups of diagnostic and therapeutic candidates. The large size of IgMs makes their purification very challenging. Bio-Rad has developed a new strong anion exchange resin (AEX), Nuvia HP-Q, to overcome the multiple issues faced when purifying large biomolecules. Purification of an acidic IgM was performed using Nuvia HP-Q Resin, which resulted in good binding and purification. Further polishing of the Nuvia HP-Q eluate pool on the mixed-mode CHT Ceramic Hydroxyapatite Media led to highly pure IgM, with minimal product- and process-related impurities. The workflow resulted in >99% IgM purity, as measured by size exclusion chromatography (SEC) and SDS-PAGE analysis. This AEX-based purification platform helps overcome the limitations of traditional ion exchange resins for IgM purification and provides a successful and scalable platform strategy. The purified IgM is suitable for diagnostic applications and potentially for pharmaceutical applications.

Introduction

IgM molecules are penta- and hexameric protein multimers with a size of 900–1,000 kD. Like other antibodies, they are involved in immune responses in mammals. Such recombinant antibodies are used in diagnostic and some therapeutic applications. IgM purification presents multiple challenges. IgM molecules do not have a strong Protein A binding site. Therefore, affinity purification with Protein A resins is not an option. Other affinity ligands, like Protein L and variants thereof, have not shown the same performance as Protein A resins with respect to dynamic binding capacity (DBC) and ligand stability over multiple cycles. Additionally, the large size of IgMs has direct impact on several process parameters in chromatographic purification. The dynamic binding capacity of IgM is significantly reduced in comparison to smaller proteins because of the inability of IgM to penetrate small pores and consequent insufficient utilization of the pore volume. The slow diffusion of these large molecules also impacts the chromatography steps that are dependent on mass transport.

To overcome these large biomolecule purification issues, Bio-Rad developed the Nuvia HP-Q Anion Exchange Resin (bulletin 7078). It is built on the UNOsphere epoxide base bead that provides fast mass transfer kinetics and low nonspecific binding. The particle size of Nuvia HP-Q is optimized to offer high DBC at fast flow rates without excessive backpressure. Its pore size is optimized for easy accessibility and adsorption of large biomolecules, like IgMs, and the internal spacer length and ligand density facilitate optimal binding of large biomolecules even at high flow rates. Traditional ion exchange resins have kinetic limitations due to nonoptimized pore sizes for large biomolecules, which affects purification productivity. The design and construction of Nuvia HP-Q Resin helps in overcoming this challenge.

In the present study, the purification of an acidic IgM is demonstrated with a two-step workflow using Nuvia HP-Q for capture and CHT Type II Ceramic Hydroxyapatite Media, 40 µm (CHT II 40) for polish purification. The capture step generated ~63% purity and ~83% recovery of the IgM. The polish step produced an IgM fraction with >99% purity and >77% recovery. In addition, excellent clearance of other impurities, such as host cell protein (HCP) and nucleic acids, was also achieved. These results establish Nuvia HP-Q as a great capture resin for large biomolecule purification.

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Materials and Methods

General

The acidic IgM (pI ~6.7) was expressed in HEK cells via transient expression. Foresight Prepacked Columns containing Nuvia HP-Q (catalog #12007020) and CHT Type II, 40 µm (#732-4736) were used and column chromatography was conducted on an NGC Discover 10 Pro Chromatography System (#7880011) (Bio-Rad Laboratories, Inc.). Protein fractions were analyzed by Criterion XT Gel (#3450129) under nonreducing conditions and Criterion TGX Stain-Free Precast Protein Gel (4–20% linear gradient; #5678093) under reducing conditions (Bio-Rad). Imaging was performed using the ChemiDoc MP Imaging System (#17001402; Bio-Rad). A Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific Inc.) was used to determine DNA concentration. HCP analysis was performed using a HEK 293 HCP ELISA Kit (Cygnus Technologies).

Optimization of Binding and Elution Conditions on Nuvia HP-Q Resin

Optimal binding and elution conditions were determined by on-column scouting of different pH conditions using linear NaCl gradients. For the initial screening, a pH range from 6.5 to 8.5 was tested. All chromatography runs were performed on an NGC Discover 10 Pro Chromatography System using 1 ml Foresight Columns.

Capture Purification of the Acidic IgM with Nuvia HP-Q Resin

The acidic IgM had a calculated pI of 6.7. Approximately 400 ml of the cell culture supernatant with ~2 mg of IgM antibody was adjusted to pH 7.5 with 800 ml of the equilibration buffer, buffer A (20 mM Na_2HPO_4/NaH_2PO_4, pH 7.5), before loading. The Foresight Nuvia HP-Q Column was equilibrated with 10 column volumes (CV) of equilibration buffer. The IgM sample was then applied to the column at 120 cm/hr. The column was washed with 20 CV of buffer A and elution of the bound IgM was done with a linear gradient to 200 mM NaCl.

Polish Purification of the Acidic IgM with CHT II 40 Mixed-Mode Media

CHT Type II Media, 40 µm (CHT II 40) was used for polishing purification. The pH of the pooled fractions A28 to A37 from the capture step was adjusted to 7.0 with 0.1 M H_3PO_4. The column was pre-equilibrated with 10 CV of 1 M NaCl, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.0 (buffer C). The pooled fractions from the capture step were applied to it at 120 cm/hr. The column was washed with 10 CV of buffer C. The purified IgM was eluted with a linear phosphate gradient to 400 mM NaPi with buffer D.

Size Exclusion High Performance Liquid Chromatography (SEC-HPLC)

Analysis was performed on a Dionex UltiMate 3000 System using a MAbPac SEC-1 Column (Thermo Fisher Scientific). A sample volume of 3 µl (Nuvia HP-Q eluate) or 10 µl (CHT II 40 eluate) was injected. Analytical separation was achieved in isocratic mode using 50 mM Na_3PO_4, 200 mM arginine, 300 mM NaCl, pH 6.8 as running buffer at 0.8 ml/min for 12 min.

Results and Discussion

Optimization of Binding and Elution Conditions

Multiple experiments were performed with a range of pH and salt conditions to determine the optimal binding and elution conditions of the IgM on Nuvia HP-Q Resin. Eluted protein from the initial trial runs was analyzed for purity and recovery. Using a linear NaCl gradient at pH 7.5 resulted in best purity and recovery values for this purification (Figure 1).

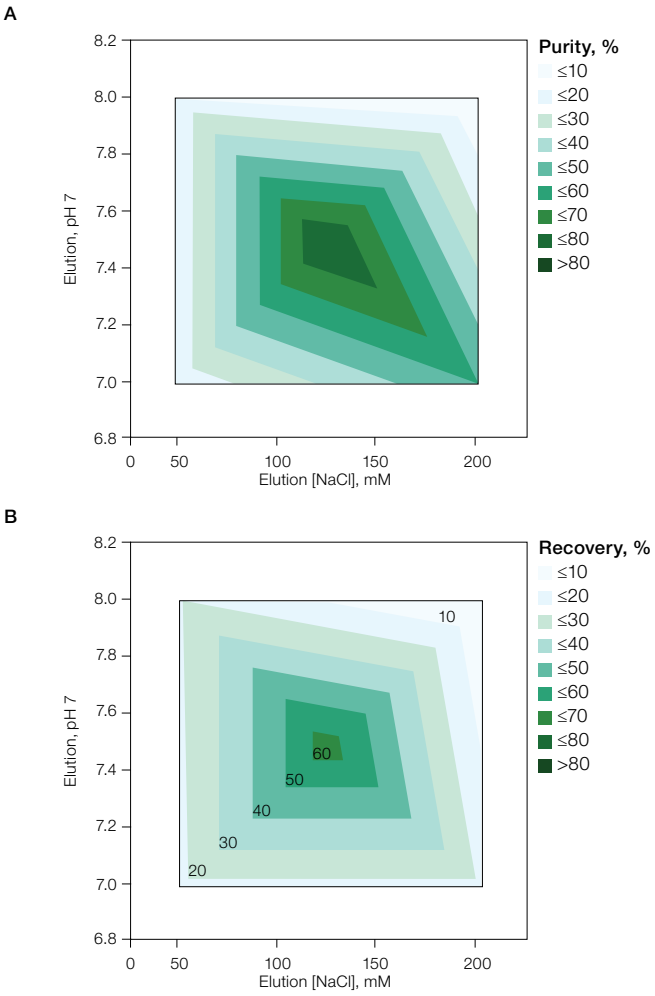
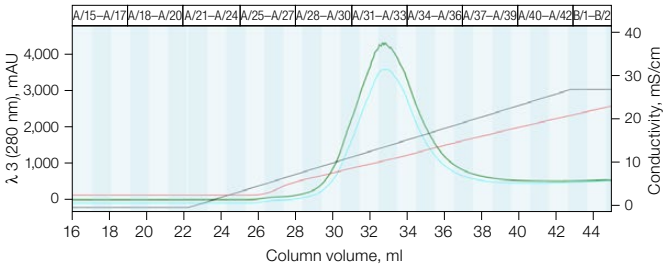


Fig. 1. Elution of the acidic IgM from Nuvia HP-Q Resin. A, effect of the buffer pH and NaCl concentration on the purity of the eluted IgM; **B**, effect of the buffer pH and NaCl concentration on the recovery of the eluted IgM.

Capture Purification of the Acidic IgM with Nuvia HP-Q Resin

Nuvia HP-Q is a high-capacity strong anion exchange resin with a pore size optimal for easy accessibility and adsorption of large biomolecules like IgMs. The capture purification was performed at pH 7.5. Since the calculated pI of the IgM was 6.7, it is expected to carry a negative charge at higher pH, making it ideal for capture with an AEX resin. The use of Nuvia HP-Q Resin eliminated the potential need for removal of any leached Protein A from downstream samples, which is a limitation of using a Protein A affinity resin for capture.

An IgM peak was seen with the 280 nm probe between fractions A28 and A37 (Figure 2). The peak is seen somewhere in the middle of the salt gradient, coinciding with the 125–150 mM optimal elution salt concentration. Small aliquots of the eluted samples were kept aside to analyze on gels for purity determination (data shown later). The remainder of the fractions was pooled to run on the CHT II 40 Column for subsequent polishing of the target protein.



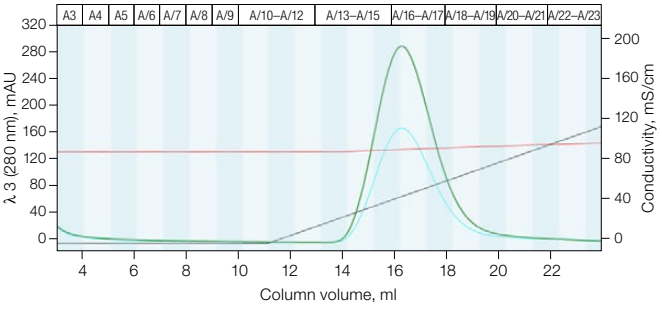
Column:	Foresight Nuvia HP-Q, 1 ml
Equilibration:	10 CV of 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.5 (buffer A)
Sample:	400 ml cell culture supernatant adjusted to pH 7.5 with 800 ml equilibration buffer
Flow rate:	120 cm/hr
Wash:	20 CV buffer A
Elution:	10 CV linear gradient from 0 to 20% buffer B
Buffer A:	20 mM Na_2HPO_4/NaH_2PO_4, pH 7.5
Buffer B:	1 M NaCl, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.5

Fig. 2. Capture purification of IgM on Nuvia HP-Q Resin. Chromatography run of IgM on Nuvia HP-Q with a 0–200 mM NaCl linear gradient. 260 nm (■); 280 nm (■); conductivity (■); %B (■).

Polish Purification of the Acidic IgM with CHT II 40 Media

CHT Ceramic Hydroxyapatite is a mixed-mode media widely used in monoclonal antibody polishing for removal of aggregates, endotoxins, HCPs, and DNA (Gagnon et al. 2006). Its applicability for IgM purification has been shown previously (Greenwood et al. 2017). CHT also has the advantages of operating well at neutral pH and generally requiring mild salt concentrations for elution (Gagnon et al. 2014).

The polish step utilized the capability of CHT II 40 to bind strongly to IgM relative to contaminants and IgG. CHT Type II has a very low affinity for albumin and is particularly well-suited for the purification of many species and classes of immunoglobulins. Biomolecules can bind to it by calcium affinity and/or cation exchange interactions (bulletin 6902). The carboxyl or phosphate groups on biomolecules bind to the calcium in CHT while amino groups or other positively charged moieties bind via cation exchange to the phosphate groups. Since hydroxyapatite binds IgM strongly at physiological pH and conductivity, mild nondenaturing conditions can be used for elution. We used a phosphate gradient for IgM elution. The IgM peak was seen with the 280 nm probe between fractions A13 and A20 (Figure 3). These fractions were collected for further analytical assays.



Column:	Foresight CHT II 40, 1 ml
Equilibration:	10 CV of 1 M NaCl, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.0
Sample:	Nuvia HP-Q elution pool, supplemented with NaCl, with pH adjusted to 7.0 using diluted H_3PO_4
Flow rate:	120 cm/hr
Wash:	10 CV buffer C
Elution:	20 CV linear gradient from 0 to 100% buffer D
Buffer C:	1 M NaCl, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.0
Buffer D:	1 M NaCl, 400 mM Na_2HPO_4/NaH_2PO_4, pH 7.0

Fig. 3. Polish purification of IgM on CHT II 40. Chromatography run of the previously eluted IgM on CHT II 40 with a 20–400 mM phosphate gradient in the presence of 1 M NaCl. 260 nm (■); 280 nm (■); conductivity (■); %B (■).

The resulting IgM after the polish purification was run on a nonreducing Criterion XT Gel (Figure 4A) and a reducing Criterion TGX Gel (Figure 4B) for purity analysis. The CHT II 40 eluate showed a single band of IgM in the nonreducing gel and two bands for the heavy and the light chains, respectively, on the reducing gel. Contaminant bands were not observed in these lanes.

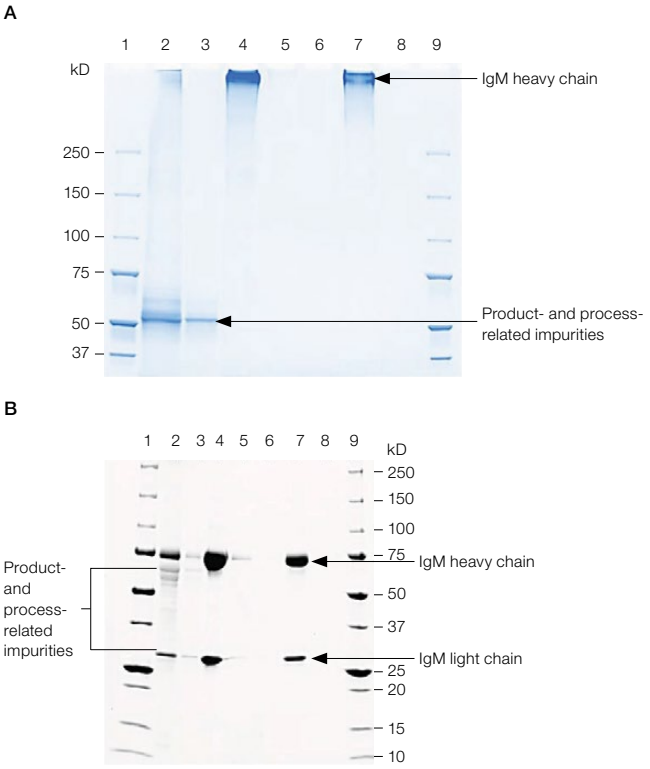


Fig. 4. Reduced SDS-PAGE analysis of IgM purified from the Nuvia HP-Q and CHT II 40 Columns. A, nonreducing Criterion XT Gel; **B**, reducing Criterion TGX Gel. **A** and **B**, lane 1, protein molecular weight (MW) marker; lane 2, culture supernatant; lane 3, Nuvia HP-Q flowthrough; lane 4, pooled Nuvia HP-Q eluate; lane 5, CHT II 40 flowthrough; lane 6, CHT II 40 wash; lane 7, pooled CHT II 40 eluate; lane 8, DNA peak from the CHT II 40 plot; lane 9, protein MW marker.

The purity was further confirmed by SEC analysis. Contaminant peaks were seen on the SEC plot with the Nuvia HP-Q eluate (Figure 5A) but only a single sharp peak for IgM was seen on the SEC plot with the CHT II 40 eluate (Figure 5B).

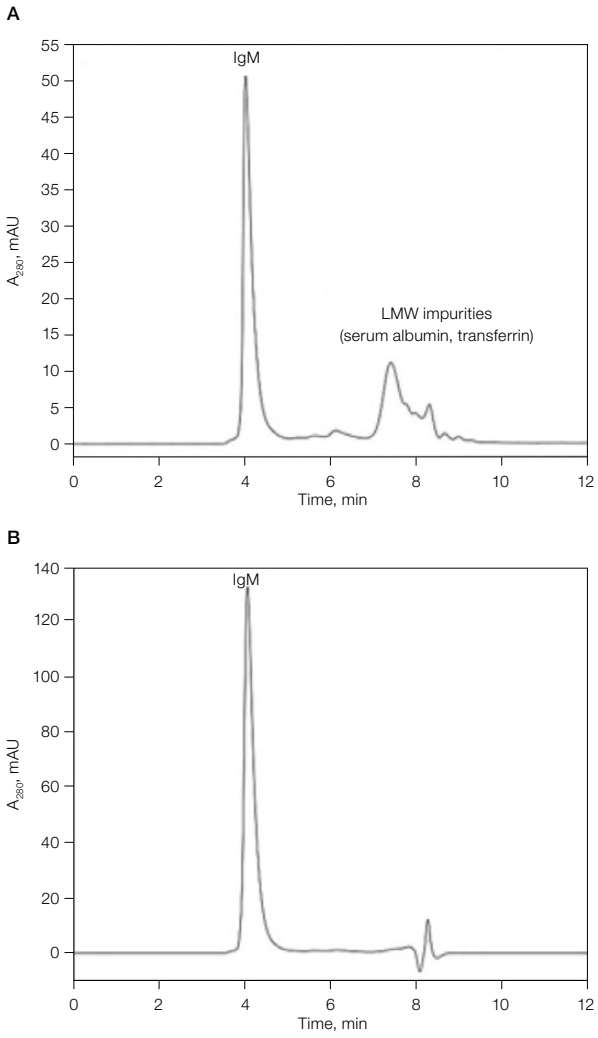


Fig. 5. SEC analysis of the Nuvia HP-Q and CHT II 40 eluates. Analysis was performed on a Dionex UltiMate 3000 System using a MAbPac SEC-1 Column. A sample volume of 3 µl Nuvia HP-Q eluate (**A**) or 10 µl CHT II 40 eluate (**B**) was injected. Analytical separation was achieved in isocratic mode using 50 mM Na_3PO_4, 200 mM arginine, 300 mM NaCl, pH 6.8 as running buffer at 0.8 ml/min for 12 min. LMW, low molecular weight.

Analysis of the contaminant levels in each of the samples showed a reduction in the DNA and host cell protein content after the Nuvia HP-Q purification. Polish purification with CHT II 40 resulted in minimizing these contaminants further as shown in Table 1.

Table 1. Final purification data.

Sample	Purity, %*	Recovery, %*	DNA, pg/µl**	HCP, ppm***
Culture supernatant	20	—	2,332	>200,000
Nuvia HP-Q EP	63	83	1,187	21,455
CHT II 40 EP	>99	77	Not detectable	109

EP, elution pool; HCP, host cell protein.
* Determined by SEC-HPLC.
** DNA content determined by Quant-iT PicoGreen dsDNA Assay Kit.
*** HCP content determined by HEK 293 HCP ELISA Kit.

Conclusions

This nonaffinity-based platform approach using Nuvia HP-Q Anion Exchange Resin produces a >99% pure IgM, which meets the purification requirements for diagnostic IgM manufacturing. This approach shows high potential for pharmaceutical purification as well. The process involves only two steps and requires a narrow range of optimized conditions, making it suitable for scalable purification. Using this state-of-the-art media with high capacity and selectivity for large biomolecules also helps overcome the affinity-based challenges of low binding and/or recovery during IgM purification.

Visit **bio-rad.com/AcidicIgM** for more information.

Acknowledgements

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References

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