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Affinity chromatography on monolithic supports for simultaneous and high-throughput isolation of immunoglobulins from human serum

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Abbreviations

AFM	affinity monolith chromatography
CIM	convective interaction media
CIM r-protein G disc	monolithic analytical disc with immobilised recombinant protein G
CIMac r-protein L column	monolithic analytical column with immobilised recombinant protein L
HTP	high-throughput
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M

Keywords

Affinity chromatography, monoliths, immunoglobulins, high-throughput

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Abstract

Post translational modifications of immunoglobulins have been a topic of great interest and have been repeatedly reported as a major factor in disease pathology. Cost-effective, reproducible and high-throughput (HTP) isolation of immunoglobulins from human serum is vital for studying the changes in protein structure and the following understanding of disease development. Although there are many methods for the isolation of specific immunoglobulin classes, only a few of them are applicable for isolation of all subtypes and variants. Here, we present the development of a scheme for fast and simultaneous affinity purification of α (A), γ (G) and μ (M) immunoglobulins from human serum through affinity monolith chromatography. Affinity-based monolithic columns with immobilized protein A, G or L were used for antibody isolation. Monolithic stationary phases have a high surface accessibility of binding sites, large flow-through channels and can be operated at high flow rates, making them the ideal supports for HTP isolation of biopolymers. The presented method can be used for HTP screening of human serum in order to simultaneously isolate all three above mentioned immunoglobulins and determine their concentration and changes in their glycosylation pattern as potential prognostic and diagnostic disease biomarkers.

1. Introduction

Post translational modifications (PTM) have a crucial impact on the biological function and structure of proteins, altering their stability, activity, localization, and even protein-protein interactions. After glycosylation, covalently bound sugar moieties provide important recognition epitopes that influence regulatory or effector functions of the

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protein, while phosphorylation creates a direct link in a signalling network between upstream kinases and downstream transcription factors. The major secretory products of the adaptive immune system, immunoglobulins (Igs), are glycosylated, and both the Fc and the Fab fragments may contain glycans. Since immunoglobulin G (IgG) accounts for more than 70% of serum Igs, much more is known about this Ig isotype compared to the A, D, M or E classes. The role of IgG glycosylation has been extensively studied in cancer therapy and autoimmunity, linking disease severity to the changes in the composition and location of glycans [1,2,3]. Although most research has been focused on IgG, the link between glycosylation and pathology has been established in other Ig classes as well. Immunoglobulin A (IgA) glycosylation effects have been affirmed in the scope of allergies and IgA nephropathy [4,5] while sialylation influences the immunomodulatory effects of immunoglobulin M (IgM) on T cells [6]. While glycosylation as the most common PTM in antibodies has been a topic of many investigations, rarely studied modifications, such as methionine oxidation, deamidation and disulphide modifications, can all cause potentially immunogenic changes in immunoglobulin structure [7].

In order to study immunoglobulin structure and function, they first need to be isolated from biological samples. Many commercially available kits for targeted Ig isolation exist, using different specific proteins as purification ligands. However, they can be expensive and do not allow for simultaneous purification of different Ig classes. Chromatographic methods, on the other hand, provide a great platform for concurrent protein isolation, as was shown by Breen *et al.* who successfully isolated both IgG and IgM using protein A affinity chromatography followed by separation on a strong anion exchange columns [8]. High-throughput (HTP) fractionation of human plasma using the same approach allowed for the

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isolation of IgM from a large number of samples [9]. Traditional chromatographic particle-based supports, such as silica or agarose, can be replaced with polymethacrylate monolithic supports, which enable rapid and efficient analyses of biomolecules [10]. Monolith affinity chromatography (MAC) has been used for fast quantification of both IgG and IgM [11,12], as well as for the separation of IgG subclasses [13]. Pucic *et al.* developed a 96 well plate consisting of a monolithic stationary phase with immobilized protein G which enabled HTP isolation and analysis of human IgG of more than 2000 samples in only 12 hours [14].

The most common ligands for affinity chromatography are protein G and protein A, isolated from Gram-positive bacteria *Staphylococcus aureus* and Streptococcus sp, respectively [15,16]. Both of these ligands interact with immunoglobulins through the Fc fragment. Opposed to that, protein L, a membrane protein from *Peptostreptococcus magnus,* binds with high affinity to the Fab fragment [17]. Specifically, protein L interacts with kappa variable light chain regions of immunoglobulins, which allow for Fab fragment and single domain antibody isolation. It has to be noted that only around 35% of all human Igs contain kappa light chains, irrespective of their class.

In this work, an immunoaffinity purification method using convective interactive media (CIM) monolithic supports with immobilized protein A, G or L for IgA, IgG and IgM isolation from human serum is presented. Using a pipetting robot automation system, this method can be further optimized for HTP immunoaffinity applications.

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2. Materials and methods

2.1 Materials and chemicals

For connection to the high performance liquid chromatography (HPLC) system, a dedicated plastic housing (BIA Separations, Ajdovščina, Slovenia) was used. All chemicals were of analytical grade (Sigma-Aldrich, St. Louis, MO, USA). All buffers were prepared with ultra-pure water (Ωm≤18 S/cm) and filtered using a 0.22 m nitrocellulose filter (Millipore, Billerica, MA, USA). Blood serum was contributed from healthy individuals from the Clinical Medical Centre Rijeka. CIM r-protein G disc (0.34mL, pore size 1.3 µm) and CIMac r-protein L column (0.1mL, pore size 1.3 µm) were purchased from BIA Separations. These monoliths have a poly (glycidyl methacrylate-co-ethylene dimethacrylate) backbone with immobilized recombinant protein G or L produced in *E.coli*. Bradford colorimetric assay (Bio-Rad, Richmond, CA, USA) was used for protein quantification, with bovine serum albumin (BSA) as a standard.

2.2 Instrumentation

Affinity chromatography was performed at room temperature using an HPLC (Knauer, Berlin, Germany) system comprising of a quaternary pump, a solvent degasser, a conductivity/pH monitor, a UV-Vis detector with a 190 to 750 nm wavelength range and a 2 mL sample loop. Operation parameters were fixed and controlled through a computer using ClarityChrom Preparative software version 3.0.5.505.

Mass spectrometric measurements were performed on UltraflexExtreme MALDI TOF TOF

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instrument (Bruker, Bremen, Germany). The instrument was equipped with a nitrogen laser operating at a wavelength of 337 nm with a 2 kHz frequency in TOF/TOF mode.

2.3 Chromatographic procedure

Prior to sample application on a monolithic support, all samples were diluted 10 fold in equilibration buffer, centrifuged and filtered through a 0.22m filter. Protein G disc was employed using three mobile phases (Buffer A: 0.2 M Tris pH 7.2, for equilibration of the column; Buffer B: 0.5M NaCl/50mM Tris-HCl, pH 7.2, for washing of impurities before protein elution; Buffer C: 0.1 M glycine-HCl, pH 2, for protein elution). The first two mobile phases were identical when chromatography on protein L column was performed, while the preferred elution buffer was 0.5 M acetic acid, without pH adjustment. Elution was performed under isocratic conditions. The flow rate of the mobile phase was 1 mL/min and the column temperature was 25°C. The injection volume of the diluted human serum sample was 2 mL. The eluted proteins were monitored at an absorbance of 280 nm and were rapidly neutralized by addition of a concentrated buffer of 1 M Tris to avoid denaturation. Finally, the system was re-equilibrated with Buffer A.

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

The composition and purity of eluted protein fractions were tested by vertical SDS-PAGE (Mini Protean Tetracell system, Bio-Rad), using a 12% resolving gel and a 4% stacking gel under reducing conditions. Samples were boiled at 95 °C for 5 minutes. Proteins were separated in the gel for 1h at 110 V. Protein staining was performed with Coommassie

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Brilliant Blue R-250 (Sigma-Aldrich). Roti-Mark TRICOLOR marker (Carl Roth, Karlsruhe, Germany) was used as a gel standard.

2.5 In-gel digest and ZipTip purification

Protein bands of interest were excised from the gel and each band was de-stained using acetonitrile and 100mM ammonium bicarbonate (ABC). Proteins were reduced (with 20mM DTT, 56°C, 30 minutes) and alkylated (by use of 50mM iodoacetamide, at room temperature for 30 minutes in dark). After washing and further de-staining with acetonitrile and 100mM ABC, samples were dried in a vacuum concentrator. In-gel tryptic digestion was performed at 4°C for 40 minutes in 50mM ABC containing 400 ng/µL trypsin (sequencing grade, Promega, Madison, WI, USA). Fresh 50mM ABC was then added to the sample, and it was incubated over night at 37 °C. The resulting peptides were re-dissolved in 0.1% TFA and purified via Zip Tip (Millipore) according to the manufacturer's instructions.

2.6. MALDI TOF TOF mass spectrometry

Mass spectra were acquired in the reflectron positive ion mode in the 700-3500 m/z range. All samples were applied on an anchor chip MTP MALDI target plate (Bruker). α-cyano-4-hydroxycinnamic acid (α-CHCA) was used as matrix (Sigma-Aldrich). An external six-point calibration was performed using a peptide mix containing angiotensin I and II, substance P, bombesin, ACTH and somatostatin (Bruker). All obtained raw MALDI TOF TOF mass spectra were exported from BioTools software version 3.2 (Knauer) and subsequently processed by means of Mascot software (Matrix Science, Boston, MA, USA).

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2.7 High-throughput robotics

The Tecan Genesis WorkStation 200 (Tecan Group Ltd., Männedorf, Switzerland) was used for high-throughput pipetting, washing and sequential elution of immunoglobulins that are bound after application of human serum on 50µL affinity discs mounted into ELISA plates [14]. The program for the work station was elaborated in our laboratory, and its mode of function is presented at the film recording in Supplement.

3. Results and discussion

3.1 Optimization of immunoglobulin separation

Monolithic supports, whether bearing affinity, ion exchangers or other ligands, are slowly replacing the hitherto used particle-based supports for chromatographic separations. The relatively new polymethacrylate monoliths have several advantages over their silica or agarose beaded counterparts [18]. Most importantly, they are compact carriers containing open ended channels, unlike pores. This structure enables an uninterrupted flow of the mobile phase through the chromatographic unit, allowing for the interaction between the ligand and the analyte to take place only on the surface of the monolith, removing the diffusion process and leaving only the convective flow. This results in narrower peak width and up to several orders of magnitude faster interactions, compared to classical chromatographic columns filled with particles. Monolith affinity chromatography has already been used for a wide range of applications, from traditional immunoaffinity

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purification of IgG, to an enzymatic reaction (*e.g.* trypsinization) between an analyte in the mobile phase and an immobilized enzyme [19].

In this work, carbonyldiimidazole (CDI) -modified CIM columns were used for the immobilization of recombinant protein G (Reprokine, Rehovot, Israel) and recombinant protein L (Acro Biosystems, Beijing, China) following similar procedure, as described by Černigoj *et al*. for protein A immobilisation [20]. Measured dynamic binding capacity for protein G column was ≥ 9,0 and < 15 mg/mL, with a recovery of ≥ 90%. Calculated dynamic binding capacity from elution peak for protein L column was ≥ 9,0 and < 13 mg/mL, with a recovery of ≥ 80% (Figure 1).

At the start of our experiments, we compared the affinity and selectivity for serum Igs of protein A, G and L. Next to strong affinity towards IgG, protein A displays weak affinity towards IgA and IgM, while protein G exclusively binds with high affinity to the G class of immunoglobulins. On the other hand, protein L binds to the kappa light chains of all antibody classes. Under isocratic elution conditions, a similar elution protein profile for protein A and protein L could be obtained, with a noted absence of IgA and IgM in the protein G eluate lane (Figure 2).

Breen *et al.* optimized a fractionation scheme for fast-throughput isolation of IgM and enrichment of low-abundance proteins [9]. Here, we present a similar scheme for HTP isolation of IgA, IgM and IgG, using both protein G and protein L, in two distinct and successive chromatographic procedures. After the first AMC on protein G or L, the unbound proteins are collected and are later applied to the second monolithic column, protein L or G, respectively. This results in the isolation of not only all IgG subclasses (protein G), but of IgA

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and IgM molecules carrying kappa light chains as well (protein L) (Figure 3). In this way, a broader selection of immunoglobulin classes and subclasses can be purified simultaneously, compared to using only protein G or A for their isolation.

3.2 Identification of isolated immunoglobulins

To evaluate whether immunoglobulin A, G and M were successfully isolated and separated, MALDI TOF TOF mass spectrometry was used for their identification. Unbound material and the corresponding eluted fractions were loaded on an SDS PAGE gel and corresponding bands were excised: with an apparent molecular weight of about 55 kDa and 27 kDa for IgG, about 60 and 25 kDa for IgA and about 70 and 25 kDa for IgM (see Figures 2 and 3). “In gel” tryptic digestion was performed and peptides were introduced on a MALDI target plate. Mascot online search engine was used for sequence matching, allowing for 2 miscleavages and requiring a minimal accuracy of <20ppm. IgA and IgG heavy and light chains were positively identified, while in the case of IgM, only the heavy chain was recognized (see Supplement 2). This result is not surprising, considering the low amounts of IgM present in human serum, compared to the other two examined Ig classes, and also the low visibility of IgM light chain in SDS-PAGE (see Figures 2 and 3). Protein sequence coverage also correlates with the relative amounts of Ig classes preent, with the heavy chain of IgM having the lowest coverage of only 9% (see Supplement). Undoubtedly, if a larger amount of total protein in a fraction containing IgM was loaded on an acrylamide gel, the IgM light chain would be successfully identified as well. Taking into consideration the apparent molecular weight of both chains (about 70 and 25 kDa, respectively) and positive identification of the IgM heavy chain, these data are sufficient for a positive identification of this protein.

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3.3 High-throughput automation of immunoglobulin isolation

The continuous structure of a monolith results in high external porosity, leading to increased permeability, which lowers the back pressure to a minimum. Since high flow rates can easily be applied and analysis time can be very short, monoliths are perfect supports for HTP screening [18]. A semi-high-throughput isolation of human fibrinogen using monolithic supports with immobilized monoclonal anti-human fibrinogen antibodies was successfully performed [21]. Another group used monoliths for HTP isolation of transferrin from human plasma by use of anti-transferrin monoclonal antibodies immobilized on monoliths [22]. Pučić *et al.* developed a 96 well-plate consisting of a monolithic stationary phase with immobilized protein G which enabled HTP isolation and analysis of human IgG [14]. Similarly, we designed a HTP 96 well-plate with mounted monolithic units with immobilized protein L. The average binding capacity of the column for IgG is 9.4 mg/mL. Thus, the immunoglobulin purification scheme that we present in this article can be adapted for a HTP and simultaneous Ig isolation from 96 samples. A fully automated liquid handling system, namely the Tecan Genesis WorkStation 200, is needed to preform HTP experiments using the 96 well-plate. It is a robot for automating pipetting tasks, equipped with two arms: the liquid handler, an 8 channel pipetting arm and the robot manipulator, an arm that picks up and moves objects on the workstation. We programmed its software and adapted it to our experimental design for HTP Ig purification. A film record that shows the robot at work can be seen in the Supplement.

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4. Concluding remarks

Monolithic supports with immobilized recombinant protein A, protein G and protein L ligands can be used for simultaneous isolation of immunoglobulins A, G and M from human serum. In opposite to both protein A and protein G that bind to the Fc fragment, protein L binds with high affinity to the kappa variable light chain regions of immunoglobulins. This different interaction allows isolation of Fab fragments of antibodies, but also complete antibody molecules that do not bind to protein A and protein G. However, only around 35% of human IgGs contain kappa light chain. Isolation scheme that is presented can be applied for high-throughput isolation of immunoglobulins, and further analysis of their glycosylation changes as possible diagnostic and prognostic biomarkers. Miniaturized disks carrying these affinity ligands can be mounted into ELISA plates and applied for simultaneous isolation of antibodies by use of laboratory robotics.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure Legends

Figure 1. IgG dynamic binding capacities of CIM protein G (A) and protein L (B) columns.

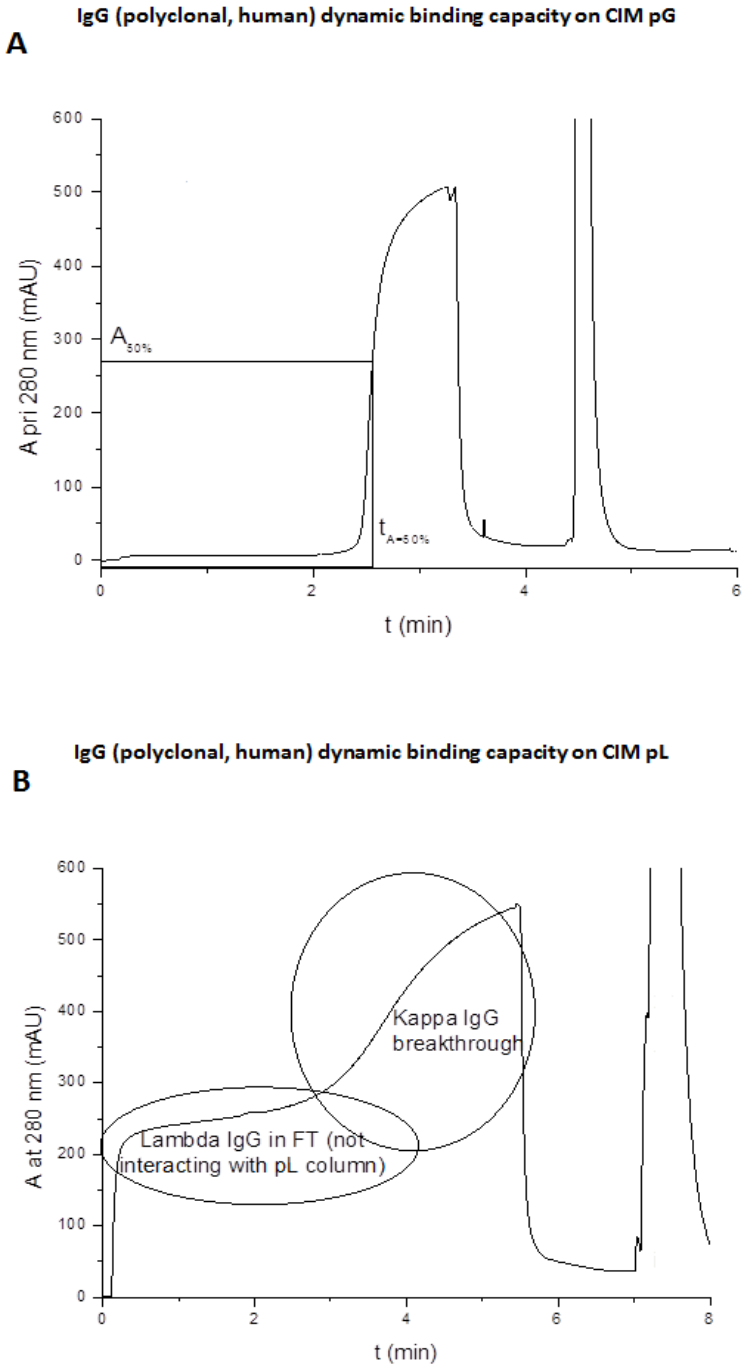

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Figure 2. A comparison of elution profiles after protein G, A or L affinity monolith chromatography. M - Roti-Mark TRICOLOR molecular mass standard. Lanes: (1) pG flow through; (2) pG eluate; (3) pA flow through; (4) pA eluate; (5) pL flow through; (6) pL eluate.

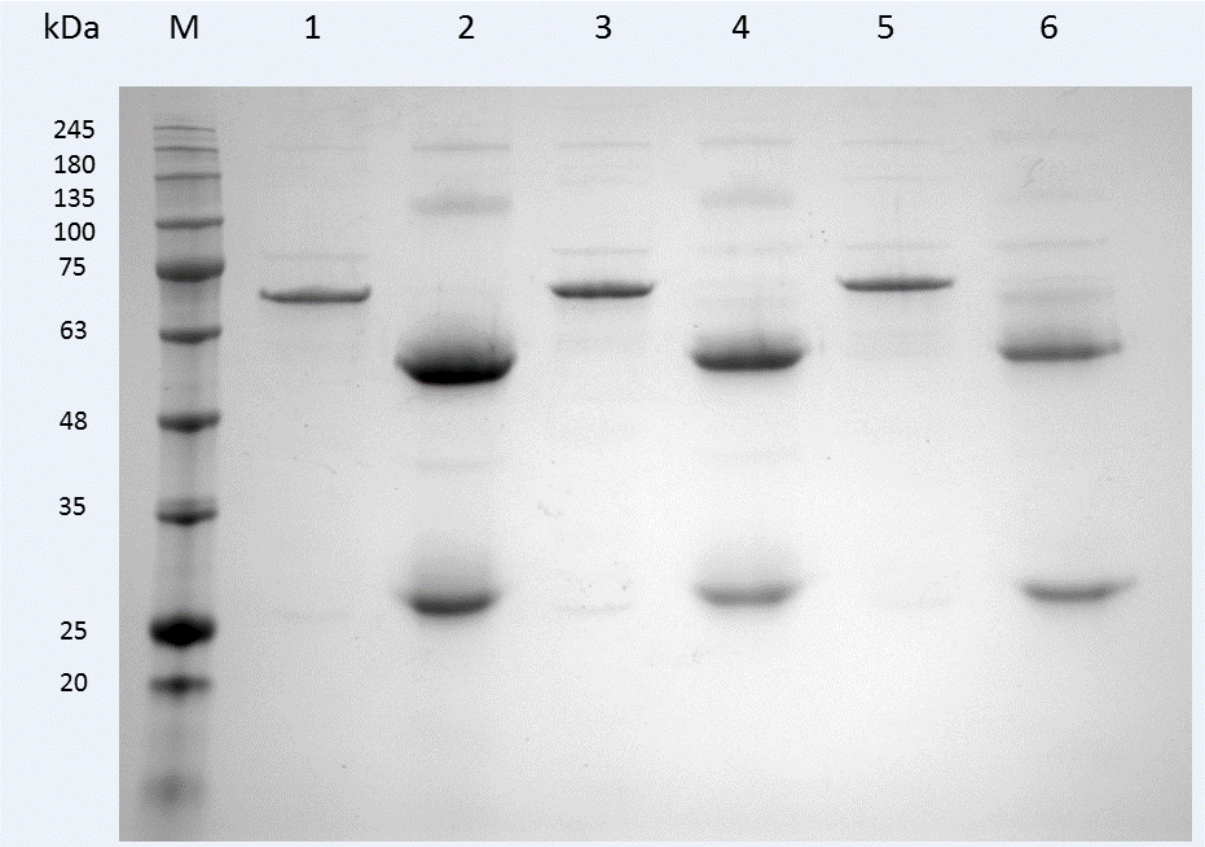

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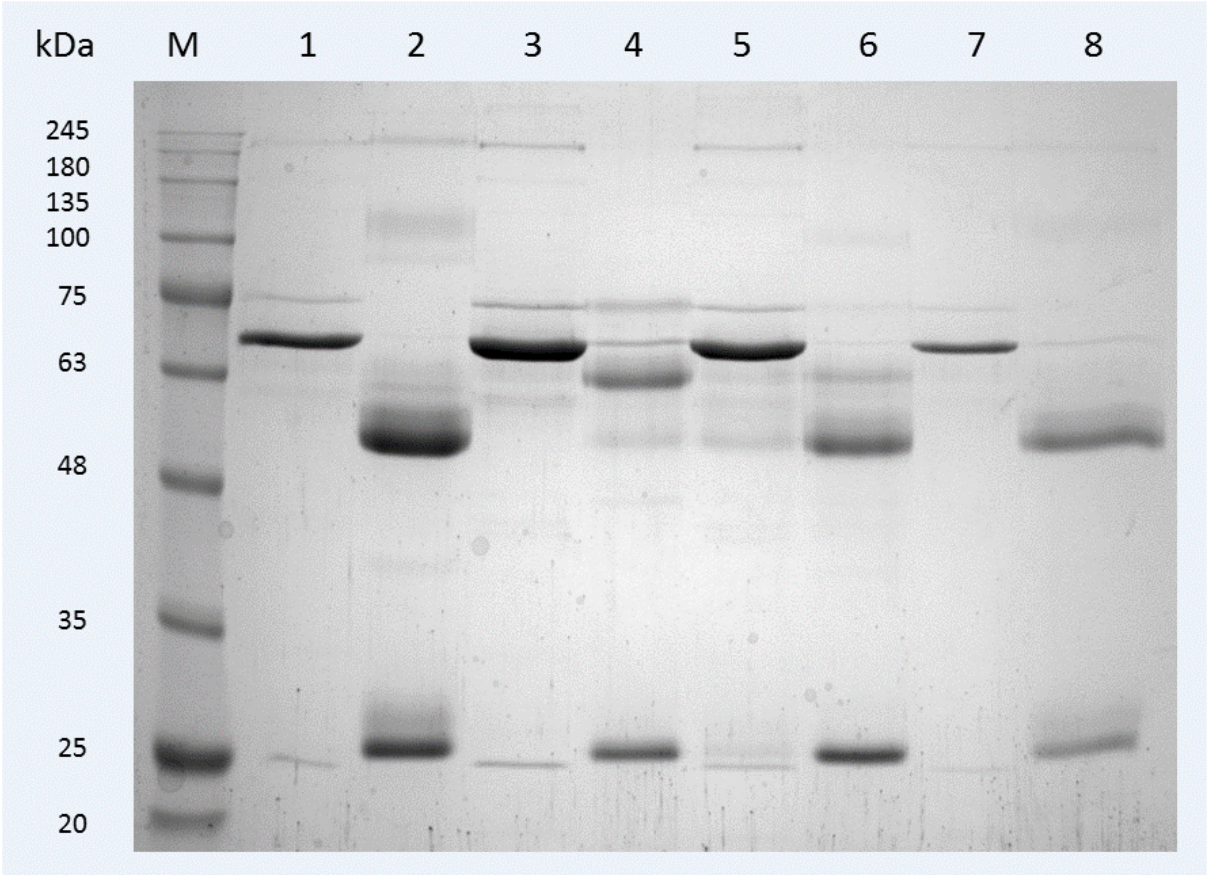
Figure 3. SDS PAGE of immunoglobulin A, G and M isolation using affinity monolith chromatography. Lanes marked with * refer to the second chromatographic step. M - Roti-Mark TRICOLOR molecular mass standard. Lanes: (1) pG flow through; (2) pG eluate; (3) pL flow through*; (4) pL eluate*; (5) pL flow through; (6) pL eluate; (7) pG flow through*; (8) pG eluate*.



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