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PRODUCTION OF HUMAN ANTI-GLYCOPHORIN-A MONOCLONAL ANTIBODIES AND ITS PURIFICATION BY PSEUDOAFFINITY CHROMATOGRAPHY USING A CONVECTIVE INTERACTION MEDIA (CIM) MONOLITHIC COLUMN

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ABSTRACT
The group of red blood cell membrane glycoproteins known as glycophorins, in reference to their high carbohydrate content, have been used for years as a general model to probe the structure and function of membrane proteins and as specific markers of erythroid differentiation. The major glycoprotein of the human erythrocyte membrane, called glycophorin A (GPA), is a single polypeptide chain composed of 131 amino acid residues and approximately 125 sugar residues. GPA can act as receptor for malarial parasites, bacterial toxins and some viruses. This paper reports the production of anti-glycophorin A monoclonal antibodies (mAbs) and its purification using a convective interaction media (CIM) disk monolithic column. Hybridomas were generated by the fusion of mouse myeloma cell line (Sp2/0) and spleen cells from mouse immunized with Triton X-100 solubilized RBC membrane proteins. The hybridomas secreting antibodies specific for commercial glycophorin A were assayed by Enzyme Linked Immunosorbent Assay (ELISA) and the cloning of hybridoma cells was performed by limiting dilution method. The titers of the cell culture supernatant for the stable clones were found to be above 4096. The antibody produced by all the stable clones was found to be IgG1 with kappa (κ) light chain. Purification of IgG1 monoclonal antibodies from the supernatant of hybridoma cell culture was carried out by pseudobioaffinity chromatography using a CIM-Ethylene Diamine (EDA)-histidine disk. Higher purity of monoclonal antibody was obtained in the elution fractions of 25mM MOPS buffer, pH 6.5. The peak fractions eluted with 0.2M NaCl had a purification fold of about 8.27 and it showed good purity of IgG1 with higher specific activity based on SDS-PAGE analysis and ELISA. The results indicate that faster separation and efficient recovery of high purity anti-Glycophorin A mAbs could be achieved using CIM-EDA-histidine disk.

KEYWORDS: Monoclonal antibody, human glycophorin A, CIM disk, pseudoaffinity, purification.

INTRODUCTION
Of the large of proteins that comprise the red blood cell (RBC) membrane, the group of glycoproteins collectively designated the glycophorins, indicating their high carbohydrate content occupies a privileged position.[1] Glycophorin molecules are distributed uniformly over the red cell surface and appear to be associated with the intramembranous particles. Glycophorin A (GPA) is the major glycoprotein of the human erythrocyte membrane and accounts for 80% of Periodic-acid Schiff (PAS) positive material in SDS-PAGE of an erythrocyte membrane proteins extract.

GPA carries the MN blood group antigens. Glycophorin A serves as a receptor for viruses, bacteria and parasites.[2] It has been observed that the glycoproteins which are present on the RBC surface acts as the major receptors for the malarial parasite to invade. Malarial disease remains a major public health problem in many regions of the tropical world [2 billions of people, 40% of world population in 90 countries, are exposed].[3] There is an immediate requirement for developing vaccines that can provide protection against malaria. The ability of merozoites (blood stage) to invade erythrocytes rapidly indicates that it is a securely controlled process, which involves specific receptor ligand interactions between host and parasite. The *Plasmodium falciparum* uses sialic acid residues of glycophorin A as receptors to invade human erythrocytes[4-6], whereas *Plasmodium vivax* and *Plasmodium knowlesi* use the Duffy blood group antigen.[7-9] Parasite ligands that bind these receptors belong to a family of erythrocyte-binding proteins (EBP). The Plasmodium erythrocyte binding antigen (EBA-175) is a ligand for merozoite invasion into human erythrocytes that binds to GPA.[10]

The parasite proteins bind with erythrocyte receptors to mediate invasion. They are attractive candidates for malaria vaccine development since antibodies are directed against erythrocyte ligands, which may block erythrocyte invasion, reducing parasite multiplication and providing protection against malaria. Antibodies to Glycophorin A inhibit invasion of parasites.[4] Hence the development of monoclonal antibodies against glycophorins may inhibit the parasitic infection.

In this paper, we describe the production, characterization and purification of anti-glycophorin A monoclonal antibody. We also present here the successful separation of anti-glycophorin A IgG1 from cell culture supernatant by pseudobioaffinity chromatography, using monolithic convective interaction media (CIM)-EDA-Histidine disk. Convective interaction media (CIM®: BIA Separations, Ljubljana, Slovenia) stationary phases are designed for the separation and purification of macromolecules and nanoparticles. These are new polymeric macroporous material based on radical co-polymerization of glycidyl methacrylate and ethylene glycol dimethacrylate. CIM monolithic supports represent a novel generation of stationary phase used for liquid chromatography and bioconversion.[11-14] The CIM monolith supports are based on a highly cross-linked porous monolith polymer, with well defined bimodal pore-size distribution providing excellent separation power and offering exceptional chemical stability and flow characteristics. They are characterized by flow independent resolution separations and a flow independent binding capacity. They exhibit a high binding capacity for large molecules and low pressure drop at high flow rates. By this, the purification time can be significantly decreased, resulting in a pronounced reduction of the purification process cost. In this context, the monolithic CIM EDA-Histidine disk was used for fast pseudobiospecific separation and purification of monoclonal IgG1 from cell culture supernatant.

MATERIALS AND METHOD

Chemicals and Reagents

Human blood (A +) was obtained from John Scudder Memorial Blood Bank, Christian Medical College (CMC), Vellore, India. Disodium phosphate, monosodium phosphate, sodium chloride and potassium chloride were purchased from Sisco Research Laboratories (SRL), India. Triton X – 100 was purchased from Qualigens, India. Bovine serum albumin (BSA), glycophorin A, anti-mouse IgG-conjugated with horseradish peroxidase (HRP), anti-mouse IgG-conjugated with alkaline phosphatase, 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) tablets were purchased from Sigma, India. Tetra methyl benzidine (TMB)/H2O2 was purchased from Genei, India. CIM EDA-Histidine disks were obtained from BIA Separations, Slovenia.

Antigen Preparation

Erythrocyte 'ghosts' from human red blood cells were prepared by the method.[15] Blood was centrifuged at 1700xg for 10 minutes at 4°C. Plasma and Buffy coat were removed by aspiration. The pellet containing RBC was suspended in 3 volumes of isotonic phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10mM phosphate, pH 7.4) and washed three times with isotonic PBS at 1700xg for 10 minutes. Some erythrocytes were discarded in each wash step to ensure complete removal of the buffy coat. The packed erythrocytes were hemolyzed by suspending in hypotonic phosphate buffer (20mM phosphate buffer) (1:10 volume) and kept at 4°C for overnight stirring. Erythrocyte ghosts were harvested by centrifugation at 14000xg for 40 minutes. (Beckman Ultracentrifuge, USA). To remove hemoglobin, ghosts were washed several times, until it became clear (colorless) solution.

To the erythrocyte ghosts, 5mM phosphate buffer, pH 8.0, was added and centrifuged at 14000xg for 40 minutes (2 times). The supernatant was discarded and to the pellet, 50 ml of 36mM phosphate buffer, pH 7.5, containing 0.5% Triton X 100, was added and kept for overnight stirring at 4°C. Then the mixture was centrifuged at 10000xg for 40 minutes. The supernatant containing RBC membrane proteins was collected and concentrated using Amicon filters (10kDa cut-off, Millipore, USA). Protein estimation was done by bicinchonic acid (BCA) method.[16]

Immunization, fusion and screening

Three female BALB/c mice were immunized with total glycoproteins (100µg) emulsified in Freund's complete adjuvant in the ratio (1:1). Booster immunizations, spleenectomy and their fusion with Sp2/0 myeloma cells, culture of the resultant hybridomas and their cloning by limiting dilution were carried out according to the techniques described by.[17] The screening of hybridomas secreting anti-glycophorin A antibodies was done by indirect ELISA method using microplates coated with commercial glycophorin A.

Enzyme-linked immunosorbent assay (ELISA) - Antibody assay

Indirect ELISA method was used for screening culture supernatants for the presence of specific antibodies against glycophorin A. The wells of high binding capacity 96-well flat bottomed microtiter plates (Cellstar, Griener bio-one, Germany) were coated with glycophorin A (50ng/well) diluted in 100mM carbonate-bicarbonate buffer, pH 9.6 and incubated for 2 hours at 37ºC. The plate was then washed with wash buffer (phosphate buffered saline (PBS), pH 7.4, 0.1% Tween 20) thrice and blocked with 1% Bovine serum albumin (BSA) in PBS for one hour at 37ºC. After three washes cell culture supernatants were added and incubated for 1 h at 37ºC. After washing, the plates were incubated for 1 h with peroxidase-conjugated anti-mouse immunoglobulin diluted 1:3000 in 0.5% BSA in PBS-

Twen. Trimethylbenzidine (TMB)/H2O2 substrate solution (GeNei, India) was used as a substrate for the enzyme with 2M sulphuric acid as stopping solution. Optical density (O.D.) was read with a multiwell plate reader (FLUOstar Optima, BMG LABTECH, Germany) at 450nm.

Determination of Immunoglobulin Subclass

To determine the isotypes of the monoclonal antibodies produced by the hybridoma clones, a commercially available mouse mAb isotyping kit (Monoclonal Antibody Isotyping Kit Pierce, USA) was used according to the manufacturer's instructions.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The various protein preparations were analyzed for its purity by SDS-PAGE (12% and 8% gel) on a 1mm separating gel plate under non- reduced conditions using a Mini-protean system (Bio-Rad, US) following the method.[18] Human Immunoglobulin G (prepared from COHN fraction II and III) from Sigma, USA, was used as molecular weight marker. The gels were stained with silver nitrate.

Western Blotting

Approximately 10µg of the protein preparation was electrophoresed in 10% polyacrylamide gels. The proteins were electrophoretically transferred at 200mV for 1 h on to a nitrocellulose membrane (Millipore, USA) using a Trans blot apparatus (Bio-Rad, USA).Western blot analysis was carried out using the cell culture supernatant containing anti-glycophorin A monoclonal antibody. Membrane was incubated in 5% skim milk in wash buffer (phosphate buffer saline (PBS) + 0.1% Tween 20) and incubated with mAb for 2 h at 37˚C. Serum (1:500) from a non-immune mouse was used as a negative control. After extensive washing with PBS-Tween, alkaline phosphatase conjugated anti-mouse immunoglobulin was added, incubated at 37ºC for 1 hour. The bands were visualized by using 5-bromo-4 chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) as a substrate.

T-flask growth curve and antibody production

For static culture, cells were seeded at cell density of 2 x 10^5 viable cells per ml into a 25-cm^2 T-flask (in triplicates) containing 10 ml of medium.[19] Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) at 37ºC in a humidified incubator containing 5% CO_2. Cell culture samples were collected daily for over 12 days in order to determine the cell number, antibody production and glucose concentrations. Viable cell numbers were counted with (0.1 % (w/v)) trypan blue on a hemocytometer. Cell viability was estimated from the ratio of viable cell count to total cell count. Glucose concentrations were measured by enzymatic analysis using a colorimetric method for glucose (Span Diagnostics, India).

Cell Culture Supernatant Precipitation and Dialysis

Cells were grown in 150-cm^2 T-flask for 8 to 9 days and the culture medium with the cells was centrifuged (5000g, 5mins) (Eppendorf, Germany) to remove debris and the cell culture supernatant (CCS) was sterilized by filtering through 0.22µ filters. Then the CCS was precipitated with equal volume of 50% ammonium sulfate saturated solution according to the procedure.[20] The precipitated fraction was dialyzed (10kDa cutoff) in the respective buffer with which the chromatographic run was performed. All steps were carried out at 4ºC.

Purification of mAbs using pseudo affinity chromatography

The monolith CIM EDA-Histidine disk (BIA Separations, Slovenia) having a dimension of 12mm x 3mm I.D. (0.34mL) was used for fast separation of monoclonal antibodies from the CCS. All experiments were done atleast thrice and reproducible chromatographic profile was obtained.

The adsorption buffer used was 25mM MOPS (3-(N-morpholino) propanesulfonic acid) buffer, pH 6.5 (Sisco Research Laboratories (SRL), India). The elution was carried out using 0.2M, 0.4M, 0.6M, 0.8M and 1M sodium chloride (NaCl) (SRL), in the adsorption buffer. Column regeneration was done with 1M sodium hydroxide (SRL).

Cell culture supernatant, precipitated and dialyzed samples, containing anti-glycophorin A IgG1 mAbs were diluted (1:2 *v/v*) in the equilibration buffer and fed into the columns at a flow rate of 2ml/min. Chromatographic runs were carried out with the P-50 pump (GE, USA) at temperature (~22ºC). The outlet stream was connected to a fraction collector and the fractions (2mL) were monitored by measuring the absorbance at 280 nm using Beckman Coulter DU®730 spectrophotometer (Beckman, USA).

Protein quantification

The total protein concentration of the fractions collected in the chromatographic runs was quantified by[21], using Human immunoglobulin G (IgG) (Sigma, USA) as reference protein.

RESULTS AND DISCUSSION

RBC membrane proteins

Glycophorins are transmembrane glycoprotein and possess an intramembraneous hydrophobic portion that spans the membrane bilayer.[22] Glycophorin A (GPA) is the major glycoprotein of the human erythrocyte membrane and carries the antigenic determinants of the MN blood group. The SDS-PAGE analysis and silver nitrate staining of the Triton X-100 solubilized RBC membrane protein showed the presence of glycophorins, band3, actin and some other proteins which were found to be qualitatively similar to the method (15). Also, it showed the presence of 36 kDa protein which could be the protein of interest, Glycophorin A, in reference with the commercial glycophorin A (Figure 1). The

solubilized membrane protein also contained 90 kDa proteins which could be Band 3 and some higher molecular weight proteins which could be spectrin. Solubilization of membranes by a mild detergent is useful in order to estimate the association of membrane components with the membrane skeleton, which is defined as the insoluble material recovered after centrifugation of the detergent lysate.[23] Glycophorin B (GPB) does not bind to membrane skeleton, since 70% are recovered in the supernatant with as little as 0.1% detergent and it is quantitatively solubilized with 1% detergent. In contrast, Glycophorin C (GPC) and Glycophorin D (GPD) are tightly associated with the membrane skeleton, since only 20% of these components can be solubilized with up to 5% detergent. The behavior of GPA is intermediate indicating that it interacts loosely with the components of the membrane skeleton. The western blot analysis, of the solubilized RBC membrane protein, carried out using the cell culture supernatant containing anti-glycophorin A monoclonal antibody confirmed the presence of a 36 kDa protein which is Glycophorin A in reference with the commercial glycophorin A (Figure 2).

Hybridomas

Eight stable hybridomas producing mAbs against glycophorin A were produced by fusion of Sp2/0 myeloma cell line with spleen cells from female BALB/c mice immunized with the RBC membrane proteins. In order to characterize the antibody, it is necessary to clone the hybridoma several times by limiting dilution to assure the clone stability and therefore its monoclonality.[24] The titers of the cell culture supernatant for the above stable clones were found to be above 4096. The antibody produced by all the stable clones was found to be IgG1 with kappa (κ) light chain, using a commercially available isotyping kit. Only one clone G9G7 was selected for further experimental works.

T-flask growth curve

The T-flask growth curves were typical for murine hybridomas (Figure 3). There was no lag phase apparent for the cell line and the antibody production was found to be strongly growth associated. The cell count reached highest in the fourth day and the antibody production started increasing from the fourth day and reached maximum in the ninth day of culture (Figure 3). It was also observed that cells produced some additional antibody in the death phase.

Purification of mAbs using pseudo affinity chromatography

The addition of fetal bovine serum in the culture medium is an essential source for growth factors.[25-26] However, the addition of serum to a culture medium has some drawbacks especially when the maximal antibody purity and strict quality are essential. One of the most obvious drawbacks is that high amounts of albumin and other proteins are introduced into the system which challenges the downstream processess. Therefore, purification of the antibody from these protein mixtures is extremely difficult and always requires several additional steps. The most commonly used adsorption systems for the purification of monoclonal antibodies from cell culture supernatant or human serum are protein A and G, immobilized on different types of matrices such as porous particles, monoliths[27-28] or membrane.[29] These affinity ligands has high selectivity towards IgG but have some disadvantages which include high cost of biomolecules, difficulties in the immobilization of bioligands and the requirement of harsh elution conditions which may denature the protein. Owing to the disadvantages with protein A/G affinity ligands, pseudobioaffinity ligands were introduced and successfully used as suitable alternatives for affinity ligands. Pseudobiospecific ligands include single amino acids like histidine (His), immobilized metal ions and dyes. These ligands have been reviewed under the name “pseudobiospecific affinity ligands” by Vijayalakshmi.[30]

Pseudobioaffinity chromatography using l-histidine immobilized onto poly (ethylene-vinyl alcohol) hollow fiber membranes were developed to separate and purify protein in large amount as immunoglobulin (IgG) from human serum.[31-32] The main drawback in the membrane system is, increase in pressure drop due to fouling of membrane.[33] Therefore, it is necessary to develop a purification processes which are fast, effective, and suitable for large-scale production of monoclonal antibodies at a reasonable cost. In this line, CIM monoliths as stationary phases are characterized by large channel diameter, high surface accessibility and convective mass transfer due to which they exhibit flow independent binding capacity for macromolecules. High flow rates with low pressure drop enable rapid separations required for increased productivity.[34]

We used CIM-EDA-Histidine disk for the purification of IgG1 anti-glycophorin A monoclonal antibody. The figure 5 presents a chromatogram of the separation of monoclonal antibody on the CIM-EDA-Histidine. The run was carried out with 25mM MOPS buffer at pH 6.5 in a step gradient elution with increasing salt concentration (sodium chloride) in the binding buffer. The buffer was chosen based on the previous studies[35], where they observed the elution of purified human serum IgG at 0.2M NaCl, using histidine immobilized onto poly ethylene vinyl alcohol (PEVA) hollow-fiber membranes. Here in our experiment also, we observed that 0.2M NaCl was the maximum salt concentration required for the elution of IgG in the pure form which is being confirmed from SDS-PAGE analysis (Figure 5, Lane 2). It was also observed that majority of the contaminants in the cell culture supernatant comes in the flow through fraction (Figure 5, Lane 1).

SDS-PAGE analysis of 50% ammonium sulphate precipitated and dialyzed cell culture supernatant shows the relatively low concentration of mAb as compared to

other contaminant proteins which are present in higher concentrations (Figure 5, Lane 1). As it can be seen from figure 5, the major contaminating protein is bovine serum albumin.

Table 1 shows the antigen binding efficiency and purification fold of peak fractions obtained with CIM EDA-Histidine disk. From the results, it is clear that much higher purity of monoclonal antibody was obtained in the elution fractions of 25mM MOPS buffer, pH 6.5. The peak fractions eluted with 0.2M NaCl had a purification fold of about 8.27 and it showed good purity of IgG1 with higher specific activity based on SDS-PAGE analysis and ELISA.

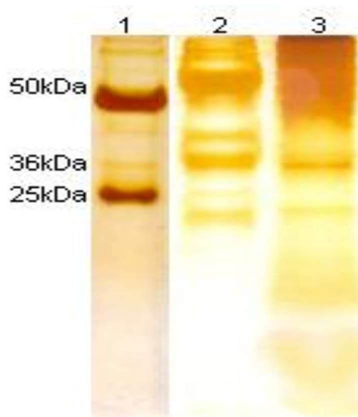

Figure 1: SDS-PAGE of Triton X-100 solubilised RBC membrane proteins.

Lane1- marker, Cohn Fraction IgG (reduced); lane 2- Commercial glycophorin A (Sigma); lane 3- Total glycoprotein. (12% acrylamide, silver nitrate staining)

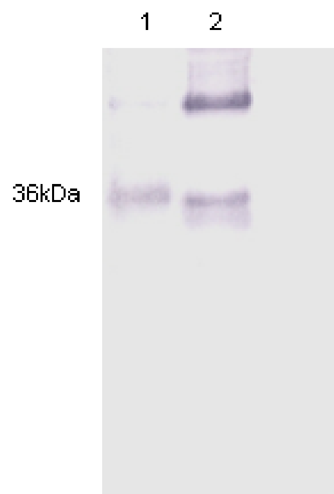

Figure 2: Western blot analysis of total glycoprotein.
Lane 1- Total glycoprotein; lane 2- Commercial anti-glycophorin A (Sigma).

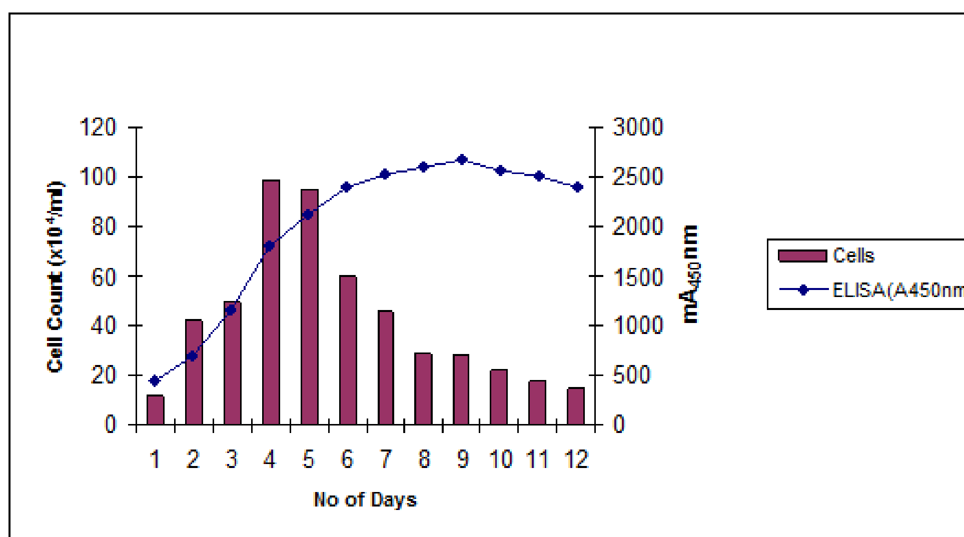

Figure 3: Growth and antibody production profile for cells grown in T-flasks.
Cells were inoculated at $2x10^4$ mL^{-1}

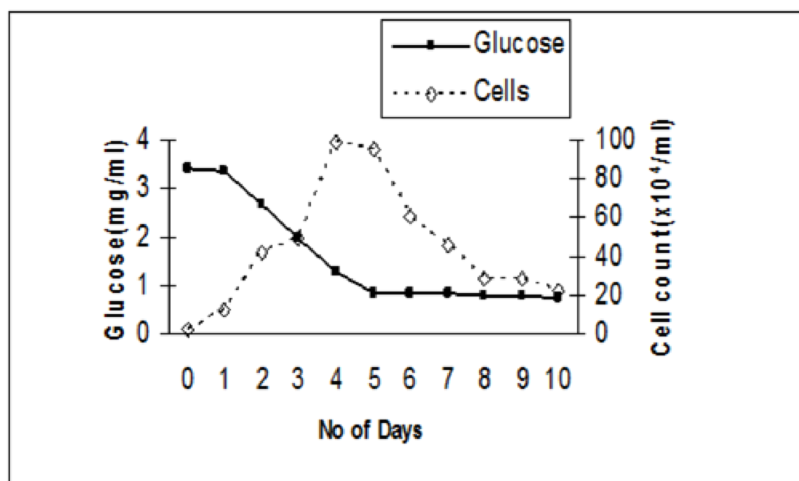

Figure 4: Cell growth vs Glucose concentration.

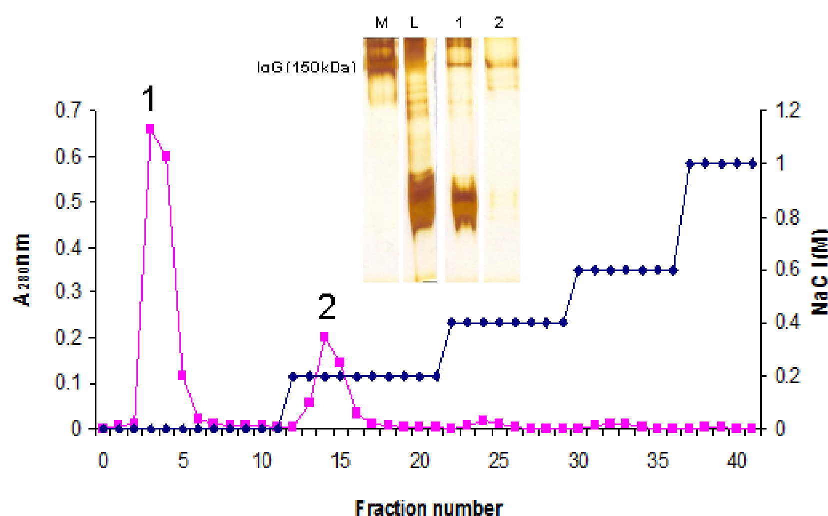

Figure 5: Chromatogram of monoclonal antibody (IgG_1) purification from cell culture sueprnatnat on CIM EDA-Histidine disk using adsorption buffer: 25mM MOPS buffer, pH 6.5. Desorption: discontinuous step gradient with increasing concentration of NaCl (0.2M, 0.4M, 0.6M, 0.8M and 1M) in the respective adsorption buffer. Column dimension: 12mm × 3mm I.D. (0.34mL), flow rate: 2mLmin^{-1}, fraction volume: 2mL, protein load - 500µl (~5.5mg). SDS-PAGE analysis under non reducing conditions of fractions from the chromatography: M – Marker (Cohn Fraction IgG); L – load; numbered lanes represent aliquots of the corresponding pooled fractions of the protein peaks obtained.

Table 1: Purification table – purification of monoclonal antibody (IgG_1) on CIM EDA-Histidine monolith disk.

Fraction	Total Protein (mg)	Total Activity (units)[a]	Specific activity (unit/mg)	Purification factor (fold)	% Recovery
Dialyzed cell culture supernatant	5.5	11450	2081.8	1	100
25mM MOPS, pH 6.5	FT: 3.8 0.2M: 1.355	16130 23330	4244.7 17217.7	2.04 8.27	93.73

[a] Arbitrary units for comparison purposes, where units are equal to scales of absorbance at 450nm.
FT – Flow through
0.2M – salt concentration

CONCLUSION

The purification results showed that CIM EDA-Histidine could be a potential alternative for the purification of IgG1, since anti-glycophorin A IgG1 monoclonal antibodies could be separated from the cell culture supernatant under mild conditions, at higher flow rates, and at room temperature. Faster separation of monoclonal antibody under non-denaturing buffer

conditions could be achieved with CIM-disks, which is more economical when compared to the conventional methods of purification.

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