Original Article

Purification of an IgA Monoclonal Antibody Specific for the Acr Protein of *Mycobacterium tuberculosis* by Immunoaffinity Chromatography

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Abstract

***Background*: A monoclonal antibody (mAb) of the IgA isotype, designated TBA61, is specific for the Acr protein of *Mycobacterium tuberculosis* (MTB). TBA61 has been used in studies exploring protection against tuberculosis (TB), and its efficacy has been proven using different challenge models. To purify the mouse IgA isotype, a combination of methods, such as globulin precipitation, ion exchange, and gel filtration, is usually required to achieve a satisfactory degree of purity.**

***Methods:* To minimise the number of chromatographic steps, we proposed to employ immunoaffinity chromatography using the Acr protein of MTB as a specific ligand for this mAb. For this purpose, the HspX gene was cloned and expressed in *Escherichia coli*, and recombinant Acr (rAcr) was coupled to a cyanogen bromide-activated Sepharose 4B matrix, which was used to purify TBA61 mAb from ascites produced in mice in a single step.**

***Results:* The recovery from the purification procedure was 1.46 mg per mL of ascites. Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot showed a high purity. The purified mAb retained its reactivity against the Acr protein based on enzyme-linked immunosorbent assay (ELISA) and western blot.**

***Conclusion:* The purification method used is rapid, simple, and specific and can be easily scaled up.**

Keywords: *Mycobacterium tuberculosis, IgA, monoclonal antibody, Acr protein, affinity, chromatography*

Introduction

Tuberculosis (TB) is second only to human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) as the greatest killer worldwide due to a single infectious agent (1). The control of TB has been hampered by the appearance of multidrug resistant strains, co-infection with HIV, and by the low diagnostic and therapeutic coverage in different regions. The only vaccine available is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG); however, its protective efficacy is extremely variable as it is effective against the severe forms of the disease in children but has limited effects on adult pulmonary TB and transmission (2).

Cell-mediated immune mechanisms have been traditionally considered as the sole immune mechanisms against TB. However, the overwhelming prevalence of TB around the world, the need for prolonged and complex therapy together with the emergence of multidrug-resistant and extensively drug resistant MTB strains (3), and the limited effect of the BCG vaccine (4) have encouraged investigators to examine novel approaches for the development of TB vaccines. With the new scientific tools that have become available over the past several decades, researchers have set out to re-evaluate the role of antibodies.

TBA61 mAb, an IgA subclass antibody targeted to the Acr protein of MTB (5), was recently shown to promote granuloma formation in mice infected intratracheally with MTB (6). In a different model of infection, the effect of TBA61 mAb was extended by the addition of IFN-γ (both administered intranasally) (7). In that study, treatment with IFN-γ three days prior to infection, at the time of infection, and at two and seven days after aerosol challenge with MTB resulted in the extension of the TBA61 effect in terms of bacterial load reduction and caused a decrease in granulomatous infiltration into the lungs of mice (7).

In another study, intranasal administration of TBA61 mAb and recombinant IFN-γ led to a more profound decrease in lung colony-forming unit (CFU) of MTB. IL-4 reconstitution reversed

the effect of IL-4, both in terms of CFU reduction and in terms of the beneficial effects of TBA61 mAb and IFN-γ (8). Furthermore, a combined immunotherapy consisting of intranasal recombinant IFN-γ, intranasal TBA61 mAb, and intravenous anti-IL-4 polyclonal antibody prevented disease relapse in mice infected with MTB and treated with isoniasid and rifampin for four weeks (9).

These results are particularly significant because they demonstrate that TBA61 can have a protective effect on various aspects of MTB infection using different models of infection and administration of the mAb.

To obtain a sufficient amount of highly purified TBA61 for experimental and pre-clinical evaluation, and taking into account the strong protective qualities of this mAb, the aim of this work was to explore a simple, fast, and specific method to purify TBA61 mAb by immunoaffinity chromatography in a single step.

Materials and Methods

Polymerase Chain Reaction (PCR) amplification, cloning, expression, and purification of rAcr

The nucleotide sequence corresponding to the HspX gene was PCR amplified from the MTB H37Rv genome using a forward primer containing an NdeI site (5'- CAT ATG ATG GCT ACC ACC CTG CCG GTT) and a reverse primer containing a BamH1 site (5'- GGA TCC GTT GGT GGA ACG GAT CTG GA). The PCR product was digested with Nde1 (Promega, Madison Wisconsin, USA) and BamH1 enzymes (Promega, Madison Wisconsin, USA), ligated to pET-15b (Novagen, San Diego, California, USA) (previously digested with the same enzymes), and transformed into the BL21 (DE3) *E. coli* strain (Novagen, San Diego, California, USA). To confirm the identity of the construct, purified recombinant plasmids were sequenced by Macrogen (Seoul, Korea).

Bacteria containing the recombinant pET-15b were grown in 1 L of Luria-Bertani (LB) broth supplemented with ampicillin (100 µg/mL). When the bacterial cells reached the mid-log phase of growth (OD_{600} measurements of 0.4–0.6), the expression of the rAcr protein was induced by the addition of isopropyl-β-D thiogalactoside (IPTG) to a final concentration of 0.4 mM, and the incubation was resumed at 37 ˚C for 5 hours. *E. coli* BL21 (DE3) carrying the empty pET-15b vector was used as a negative control.

Extraction of rAcr from the cytoplasmic fraction was performed as described in the QIAexpressionist Handbook (11). Briefly, the bacterial cell pellet was resuspended in 2–5 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) per g wet weight, and the cells were lysed by sonication. The insoluble material was removed by centrifugation, and rAcr was purified from the lysate supernatant by ion metal affinity chromatography (IMAC) on a HiTrap chelating Ni column (GE Healthcare, Piscataway, New Jersey, USA). After washing the column with 20 mM imidazole, rAcr was eluted with 250 mM imidazole. The fraction containing rAcr was dialysed against a phosphate buffered saline (PBS) buffer (pH 7.5) and was analysed by SDS-PAGE and western blot.

SDS-PAGE and western blot

We performed electrophoresis according to Laemmli (12) using a 12.5% resolving polyacrylamide gel under reducing conditions with Coomassie blue staining. Low molecular weight markers were obtained from GE Healthcare (Piscataway, New Jersey, USA).

The western blot procedure was performed as described by Towbin et al (13). Briefly, after SDS-PAGE and subsequent transfer onto nitrocellulose, purified rAcr (2 µg) was exposed to 5 µg/mL TBG65 (5), an IgG mAb specific for the Acr protein, and detected with a secondary antibody (anti-mouse IgG peroxidase conjugate [HRP]) (Promega, Madison Wisconsin, USA) diluted 1:3000.

Immunoaffinity column preparation

The rAcr (13.3 mg) was coupled to 2 g of CNBr-activated Sepharose 4B (GE Healthcare, USA) as recommended by the manufacturer (14). The coupling efficiency was determined by measuring the protein concentration using the BCA™ Protein Assay Kit (Pierce, Meridian Rd., Rockford, USA) according to the manufacturer's instructions.

Production of ascites fluid

Ascites fluid was obtained from BALB/c mice primed with 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane, Sigma, St. Louis, Missouri, USA) two weeks before intraperitoneal injection of 10^6 hybridoma cells. After 10–20 days, the ascitic fluid was collected, the cells were removed by centrifugation, and the supernatant was stored at –20 ˚C.

Immunopurification of TBA61 mAb

The supernatant from the ascites fluid containing TBA61 mAb was diluted 1:2 with PBS and filtered through 0.45 µm pore size filters

(Millipore, Billerica, Massachusetts, USA). A 10 mL aliquot of ascites was run through the column with a P-1 peristaltic pump (Pharmacia, Uppsala, Sweden) at a flow rate of 1 mL/min. After washing the column with 25 mL of PBS, TBA61 mAb bound to the column was eluted with 100 mM glycine-250 mM sodium chloride buffer (pH 2.7). The flow-through and elution fractions were collected, and their protein contents were measured using the BCA™ Protein Assay Kit (Pierce, Meridian Rd., Rockford, USA) according to the manufacturer's instructions. Three purification cycles were performed, and recovery was calculated in mg/mL of ascites.

Characterisation of the purified TBA61 mAb

Purified TBA61 mAb was evaluated by SDS-PAGE and western blot to evaluate its purity. SDS-PAGE was performed as described above, and western blot analysis was performed using the purified TBA61 mAb as an antigen and evaluated with either heavy chain-specific anti-mouse IgA, IgG, or IgM HRP conjugates (Sigma, St. Louis, Missouri, USA) diluted 1:3000.

Reactivity of the purified TBA61 mAb against the Acr protein of MTB

Reactivity against the Acr protein was determined by indirect ELISA and western blot. For the ELISA assay, polystyrene microwell plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 2 µg/mL Acr protein (GeneWay Biotech, San Diego, California, USA) in coating buffer (100 µL/well). After an overnight incubation at 4 ˚C, the coated wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 5% skim milk in PBS at 37 ˚C for 1 hour. Purified TBA61 mAb was then added (100 µL/well) at 5 µg/mL. The plates were incubated at 37 ˚C for 2 hours and washed three times with PBS-T prior to the addition of 100 µL of goat anti-mouse immunoglobulin (anti IgA: HRP) (Sigma, St. Louis, Missouri, USA). After 1 hour of incubation at 37 ˚C and four washes with PBS-T, freshly prepared 0.2% orthophenylenediamine (Sigma, St. Louis, Missouri, USA) containing 0.03% H_2O_2 in 0.1 M citrate buffer (pH 5.2) was added to each well (100 µL/well). The peroxidase reaction was stopped by the addition of 2.5 M H_2SO_4 (50 µL per well), and the optical density was measured at 492 nm.

The western blot procedure was performed as described above using the purified TBA61 mAb as the primary antibody and anti-mouse IgA HRP conjugate (Promega, Madison, Wisconsin, USA) diluted 1:3000 as the secondary antibody. In both ELISA and western blot assays, TBG65 (IgG mAb specific for Acr protein) was used as a positive control and 9A11D6 (IgG mAb specific for lipopolysaccharide of *Vibrio cholerae* O139) was used as a negative control.

Results

Expression and purification of rAcr

Amplification of the HspX gene using the specific primers resulted in a single 500 bp fragment (Figure 1) that was subsequently cloned into pET15-b.

Sequencing analysis revealed that the insert corresponds to a 500 bp open reading frame that encodes Acr, a polypeptide of 143 amino acids with an average molecular mass of 16 kDa. The homology and identity of the obtained sequences were examined using NCBI BLAST software. The results revealed that the sequences were completely identical to the HspX gene sequence (GenBank accession no. S79751). SDS-PAGE analysis of the rAcr from the IMAC elution fraction revealed a 19 kDa protein band (Figure 2a). This protein band includes a fusion protein consisting of the Acr protein fused to an N-terminal His tag (1 kDa) and 21 additional amino acids (2 kDa). Western blot analysis (Figure 2b) revealed the

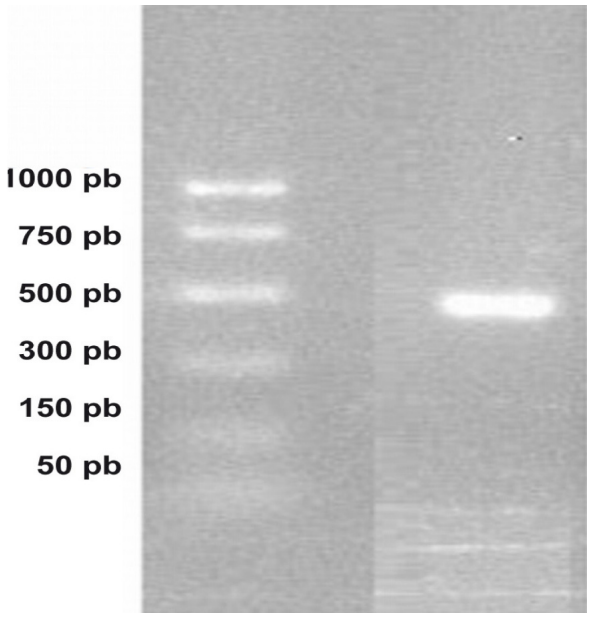

Figure 1: Amplification of the hspX gene using specific primers. Lane 1 shows 100 bp DNA ladder. Lane 2 indicates a band of 500 bp corresponding to the hspX gene.

presence of the purified rAcr protein. A total of 40 mg of rAcr was obtained.

Immunopurification of TBA61 mAb

Approximately 12.6 mg of rAcr was successfully bound to CNBr-activated Sepharose 4B, representing a coupling efficiency of 94.8%. The column bed volume was 5 mL. With this column, we purified TBA61 mAb in one single step by immunoaffinity chromatography. The average recovery obtained from the three immunopurification cycles was 1.46 mg per mL of ascites.

Characterisation of TBA61 mAb

SDS-PAGE analysis of the immunoaffinity purified TBA61 mAb showed bands corresponding to the heavy (55 kDa) and light (25 kDa) chains of the IgA (Figure 3a). Furthermore, the western blot (Figure 3b) analysis revealed the presence of IgA and the absence of other contaminant immunoglobulins such as IgG and IgM.

On the other hand, ELISA (Figure 4a) and western blot (Figure 4b) assays revealed the specificity of the purified TBA61 mAb for detecting the Acr protein.

Discussion

TBA61 mAb is specific for the Acr protein of MTB (5) and has been obtained from mouse ascites fluid, which contains a high concentration of the antibody. However, to purify IgA mAbs isotypes, a combination of methods is usually required (15–20) to achieve a suitable degree of purity. An alternative to this problem is to exploit the specificity of the antibody for its antigen using immunoaffinity chromatography (21–23).

For this purpose, we expressed and purified rAcr protein from *E. coli* BL21 (DE3). Although the purification of desired proteins from the insoluble fraction is difficult (24–26), in our study, this protein was expressed substantially in both the soluble and insoluble fractions. Therefore, we selected the soluble fraction to simplify the purification process. rAcr was easily purified in a single step by IMAC.

The purified rAcr was coupled to a CNBr-activated Sepharose 4B matrix to purify TBA61 mAb by immunoaffinity chromatography. Three immunopurification cycles were performed (Table 1), and the average recovery of the immunopurification was 1.46 mg per mL of

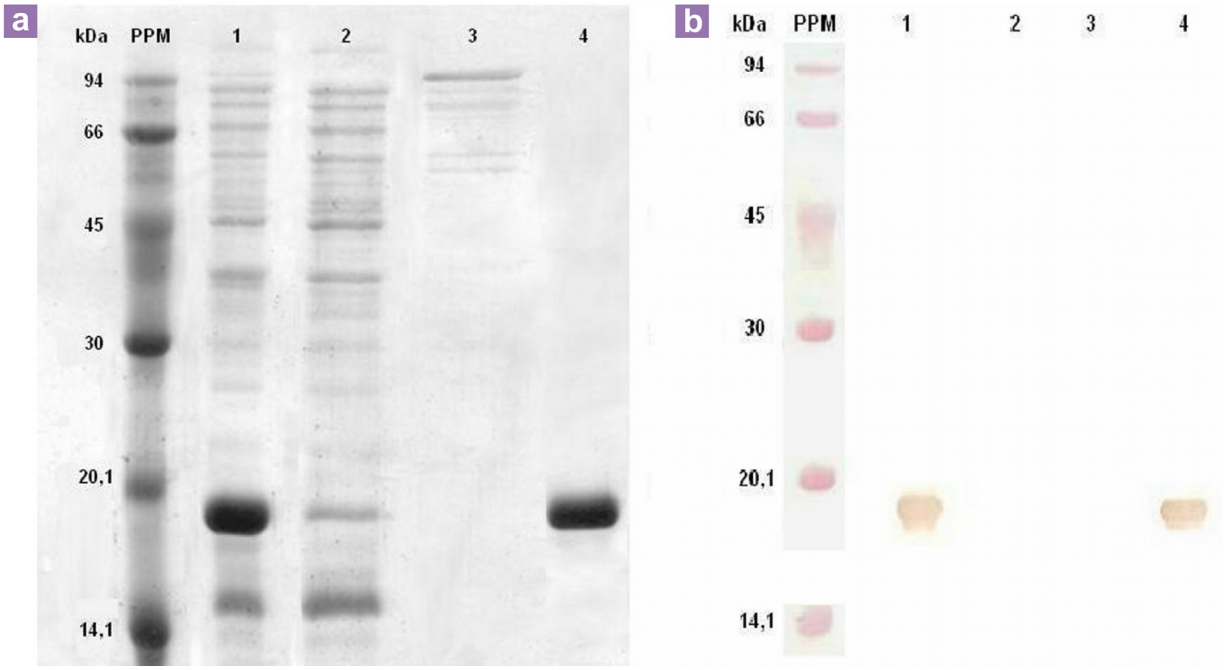

Figure 2: (a) Purification of rAcr protein: SDS-PAGE analysis of rAcr protein, purified by IMAC. Soluble fraction (Lane 1), flowthrough fraction (Lane 2), washing fraction (Lane 3), elution fraction (Lane 4). (b) Purification of rAcr protein: Western blot analysis of rAcr protein, purified by IMAC. Soluble fraction (Lane 1), flowthrough fraction (Lane 2), washing fraction (Lane 3), elution fraction (Lane 4).

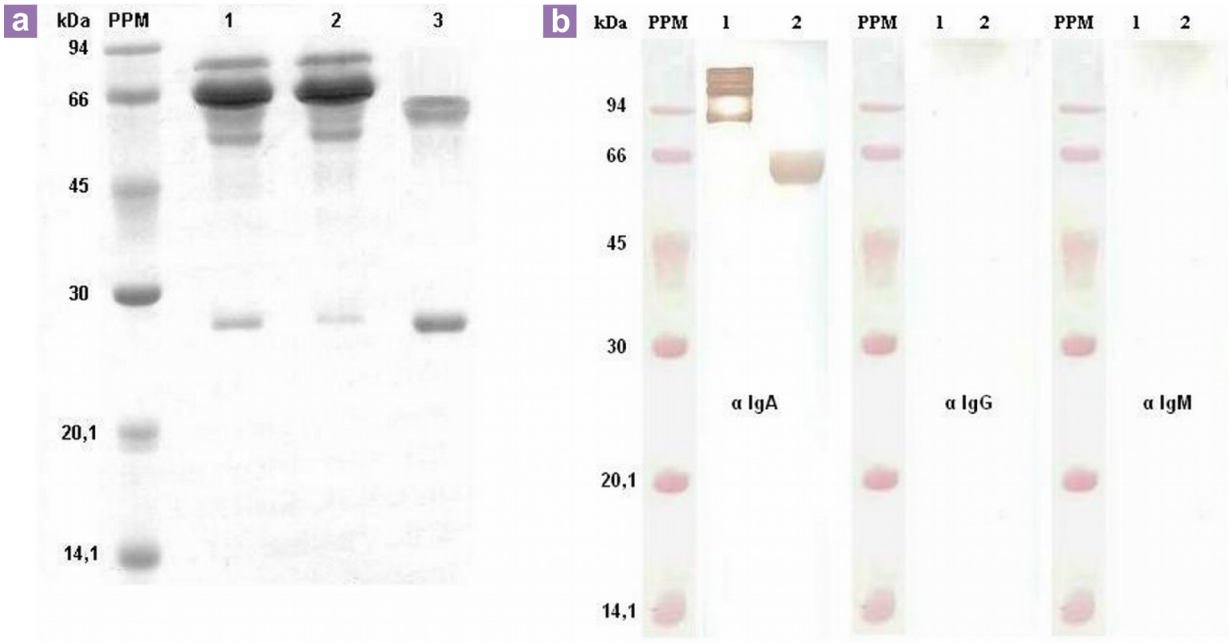

Figure 3: (a) SDS-PAGE analysis of the purified TBA61. Ascites (Lane 1), flowthrough fraction (Lane 2) and purified TBA61 (Lane 3). (b) Western blot analysis of the purified TBA61 employing different anti heavy chain specific mouse immunoglobulins (α IgA, α IgG, α IgM). Purified TBA61 under non reducing conditions (Lane 1), purified TBA61 under reducing conditions (Lane 2).

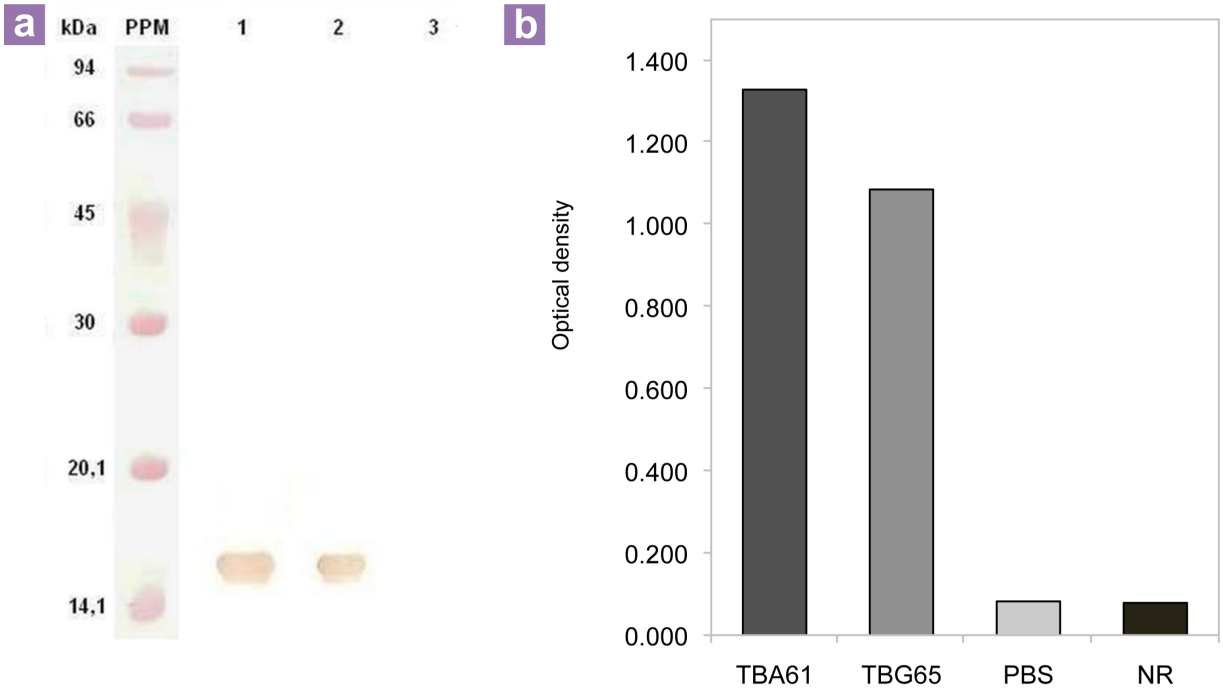

Figure 4: (a) Reactivity of the purified TBA61 against Acr by Western blot. Purified TBA61 (Lane 1), TBG65 MAb as positive control (Lane 2), 9A11D6 MAb as negative control (Lane 3). (b) Reactivity of the purified TBA61 against Acr by indirect ELISA. TBG65 mAb as positive control, NR: 9A11D6 MAb as negative control.

Table 1: Recovery of the immunopurification cycles

Cycles[a]	Elution fraction volumes	Protein content	Total	Recovery[b]
1	13.5 mL	1.04 mg/mL	14.04 mg	1.4 mg/mL ascites
2	14.0 mL	1.12 mg/mL	15.68 mg	1.5 mg/mL ascites
3	13.8 mL	1.1 mg/mL	14.18 mg	1.5 mg/mL ascites
Average	13.7 mL	1.08 mg/mL	14.64 mg	1.46 mg/mL ascites

[a] In each cycle were injected 10 mL of ascites into the column.
[b] Total divided by Elution fraction volumes of each cycle.

ascites. Some companies (27) have reported purification protocols for IgA mAbs using various standard chromatographic methodologies with typical recovery values ranging from 1–3 mg per mL of ascites. We previously developed a TBA61 purification protocol that involves ammonium sulphate precipitation followed by cation exchange and gel filtration (unpublished results); however, such an approach is complex, laborious, and time consuming.

The TBA61 mAb purified using the immunoaffinity chromatography method reported here retained its reactivity against Acr protein, as demonstrated by ELISA and western blot. Additionally, high purity levels were obtained, and undesired host immunoglobulins were removed, which is very important for future applications of this mAb. Co-purification of host immunoglobulins is a problem associated with non- immunospecific purification methods when using a native source such as ascites (28). Due to the similar physical characteristics of the contaminants and the target molecule (mAbs), the most suitable chromatographic technique must be carefully selected for purification. Because it is performed in a single step, immunoaffinity purification provides a significant time savings over less selective multistep procedures (14).

Conclusions

The purification of TBA61 mAb by immunoaffinity chromatography is clearly easy to perform (involving only one step), rapid (requiring only a few hours), and productive (recovery of 1.46 mg per mL of ascites). With this approach, we can obtain a sufficient amount of highly purified of TBA61 mAb for experimental and pre-clinical evaluation.

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Conflict of Interest

None.

Funds

None.

Authors' Contributions

Conception and design: FR, OO, FC, MES, AA
Analysis and interpretation of the data: FR, OO, FC, MES, AA
Drafting of the article and collection and assembly of data: FR
Critical revision of the article for the important intellectual content: OO, MES, AA
Final approval of the article: FR, MES, AA
Administrative, technical or logistic support: MES, AA

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