ZZ polyester beads: An efficient and simple method for purifying IgG from mouse hybridoma supernatants

John G. Lewis a,⁎, Bernd H.A. Rehm b

a Steroid and Immunobiology Laboratory, Canterbury Health Laboratories, P.O. Box 151, Christchurch, New Zealand
b Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

Abstract

ZZ polyester beads are polyhydroxyalkanoate granules which display, at high density, the ZZ domain of protein A as a fusion protein covalently linked to the polyester core. These granules are produced directly, in one step, by recombinant E. coli. We have used these granules produced in a one step process to purify mouse IgG1, IgG2a and IgG2b from mouse hybridoma supernatants. Purified immunoglobulins were analysed by enzyme-linked immunosorbent assay, agarose gel electrophoresis and SDS-PAGE. The results showed that the recovery of IgG is 70% or greater with a significant degree of purity. ZZ polyester beads hence offer a rapid and novel method to purify IgG from mouse hybridoma culture supernatants. As a negative control we used culture supernatant from an IgM secreting hybridoma and showed that it did not bind to ZZ polyester beads.

Keywords:
ZZ polyester beads
Protein A
Mouse monoclonal antibodies
ZZ domain

1. Introduction

There is often the requirement to purify IgG from mouse hybridoma supernatants and this can be conveniently carried out using protein A, usually coupled to an inert matrix. Protein A purification of IgG occurs through the Fc region of IgG and the binding of different IgG classes, subclasses and species to protein A is well documented (Harlow and Lane, 1988). The Fc binding is mediated through the bivalent Z domain of protein A (Chen et al., 2006). Recently the ZZ domain from Staphylococcus aureus had been fused with the N terminus of the polyhydroxyalkanoate (PHA) synthase from Cupriavidus necator and expressed in E. coli which mediated, in one step, the production of granules which are available commercially as ZZ polyester beads (Brockelbank et al., 2006). Meanwhile the recombinant production of various polyester granules displaying various binding domains was demonstrated and their suitability for affinity purification was shown (Rehm, 2007; Grage and Rehm, 2008; Jahns et al., 2008; Peters and Rehm, 2008). Here we used ZZ polyester beads to directly isolate 3 different IgG subclasses from mouse hybridoma supernatants. The IgG subclasses were tested by enzyme-linked immunosorbent assay (ELISA) and showed reasonable recovery and purity as assessed by agarose gel electrophoresis and SDS-PAGE. Hence these novel ZZ polyester beads offer a simple and feasible option to purify IgG from mouse hybridoma culture supernatants.

2. Materials and methods

2.1. ZZ polyester beads

ZZ polyester beads were kindly donated by Polybatics Limited, Palmerston North, New Zealand as a 5 mL suspension containing the beads at a concentration of 30 mg/mL with a binding capacity of at least 3 mg human IgG/mL suspension. This equates to a capacity of at least 1 mg of IgG/10 mg of beads. The ligand density is 15 mg/mL in suspension and the beads range in size from 100–200 nm. The operating range is between pH 1.9 and pH 9.0.
2.2. IgG isolation

Hybridoma culture supernatants (20 mL) were diluted 1/4 with Tris-saline buffer (10 mM Tris, 37.5 mM NaCl pH 7.4), mixed and incubated with the ZZ polyester bead suspension for 60 min at room temperature following centrifugation, 3000 rpm for 10 min. An aliquot of the supernatant was retained and pelleted washed with 25 mL of Tris-saline buffer and twice with 10 mM Tris buffer containing 0.5 M NaCl, pH 7.4 and finally twice with the original Tris-saline buffer. Bound material was eluted from the beads using 5 mL of 50 mM glycine buffer pH 2.7 and the supernatant passed through a 0.2 μm filter and the pH adjusted (7–8) using solid Tris. The absorbance (280 nm) of each fraction was measured and purity assessed by agarose electrophoresis and SDS-PAGE. Additionally enzyme-linked immunosorbent assay (ELISA) was performed on dilutions of the intact supernatants, unbound fractions and the low pH eluates from the ZZ polyester beads.

2.3. Mouse hybridomas and supernatants

Culture supernatants from four mouse monoclonal hybridomas were used. Hybridomas were raised according to institutional guidelines with approval from the local animal ethics committee. They were derived from clone 11F11, a human sex hormone-binding globulin (SHBG) monoclonal antibody isotype, IgG2a κ (Lewis et al., 1999), clone C5, a pregnanediol-3-glucuronide monoclonal antibody, isotype IgG2b κ (Lewis et al., 1990), clone 8B11, a dehydroepiandrosterone sulphate (DHEAS) monoclonal antibody, isotype IgG1 κ (Lewis et al., 1996) and clone 8C6, a tetrahydrocortisone-16-glucuronide (THE-16-G) monoclonal antibody, isotype IgM κ as a negative control. All cells were cultured in RPMI 1640 containing 10% fetal bovine serum and the supernatants were harvested after the cells had grown to exhaustion. They were filtered (0.4 μm) and stored at −20 °C until required.

2.4. Enzyme-linked immunosorbent assay

Supernatants were tested by ELISA using standard curves and antibody in the intact supernatant, the unbound fraction and the low pH eluate. Equivalent sample dilutions, i.e. corrected for volume, were compared across the fractions thus allowing a qualitative measure of antibody recovery in each fraction. Quantitative recovery was determined using low pH eluate dilution curves and comparing these with the theoretical dilution relative to the intact supernatant to cause a 50% reduction in the absorbance change at 450 nm over the range of the assay. The two-site ELISA for SHBG has been described previously (Lewis et al., 1999). Briefly 96 well plates were coated overnight with a rabbit polyclonal antibody to SHBG in phosphate buffered saline (PBS) and following “blocking” with assay buffer, PBS containing 0.1% gelatin and 0.1% Tween 20, dilutions of standard added (100 μL/well in assay buffer) for 60 min at room temperature. The plates were then washed and bound SHBG detected by SHBG monoclonal antibody (diluted culture supernatant from clone 11F11, or fractions diluted in assay buffer) for 30 min at room temperature. Following further washing bound monoclonal antibody was detected with antimouse IgG-peroxidase (Chemicon, Australia) (50 μL/well and diluted 1/1000 in assay buffer) and following further washing tetramethylbenzidine (TMB) substrate added (100 μL/well). Colour development was stopped with 1 M HCl (100 μL/well) and the absorbance read at 450 nm. The ELISA’s for pregnanediol-3-glucuronide and DHEAS have also been described previously (Lewis et al., 1990, 1996) and both are competitive assays. They employ either pregnanediol-3-glucuronide-thyroglobulin or DHEA-3-CMO-thyroglobulin conjugates bound to the wells of the microtitre plate. Following “blocking” soluble standards, in assay buffer, were added (50 μL/well) followed by the monoclonal antibody, or fractions diluted in assay buffer (50 μL/well), for 30–60 min incubation at room temperature. For pregnanediol-3-glucuronide diluted supernatant or fractions were from clone C5 and for DHEAS diluted supernatant or fractions or fractions were from clone 8B11. The plates were then washed and antimouse IgG-peroxidase added, 100 μL/well diluted 1/1000 in assay buffer, for 30 min at room temperature. The plates were finally washed and TMB substrate added, colour development stopped with 1 M HCl (100 μL/well) and immediately followed by diluted monoclonal antibody supernatant or fractions from clone 8C6 (50 μL/well) for 30 min at room temperature. The plates were then washed and antimouse IgG-peroxidase added for 30 min at room temperature. The plates were finally washed with further processing as described.

2.5. Electrophoresis

Protein electrophoresis was carried out on 1% agarose gels prepared in 0.1 M Tris-barbitone buffer, pH 8.6. Proteins were visualized by silver staining (Harlow and Lane, 1988). SDS-PAGE was carried out in 10% polyacrylamide gels following denaturation of samples in 2% SDS containing 3% mercaptoethanol (Laemmli, 1970). Proteins were visualized by Simply Blue™ SafeStain, Invitrogen.

3. Results

3.1. ELISA standard curves

The ELISA standard curves for the four mouse monoclonal antibody supernatants tested are shown in Fig. 1. For each antibody comparisons are shown for the intact antibody, the unbound fraction or supernatant following the initial centrifugation and the supernatant retrieved following the pH 2.7 treatment. The dilution of each fraction was adjusted to account for the volume differences of each fraction. Hence the different fractions were comparable for each antibody and therefore represented a measure of recovery. Quantitative recovery for the SHBG monoclonal antibody isotype IgG2a κ was 100%, for the DHEAS monoclonal antibody isotype IgG1 κ recovery was 70% and for the pregnanediol-3-glucuronide monoclonal antibody isotype IgG2b κ recovery was 70% and the yield (OD 280 nm) was between 1.0 and 2.5 mg IgG/20 mL of culture supernatant. The THE-16-glucuronide monoclonal antibody isotype IgM κ was not retained by the ZZ polyester
beads and 90% appeared in the unbound fraction, the remainder was in the first wash and no detectable antibody appeared in the low pH eluate.

3.2. Electrophoresis

Silver staining of protein bands following agarose gel electrophoresis is shown in Fig. 2. Comparison is shown with the intact supernatant in each case and indicates that ZZ polyester beads purified the monoclonal antibody from the DHEAS (clone 8B11) and SHBG (clone 11F11) culture supernatants to homogeneity, lanes 2 and 8 respectively. There was diffuse silver staining in the gamma globulin region of the low pH eluate from pregnanediol-3-glucuronide (clone C5) supernatant, lane 4, and no visible staining in the low pH eluate from the THE-16-glucuronide (clone 8C6) culture supernatant, isotype IgM κ, lane 6. Protein staining following SDS-PAGE of reduced samples is shown in Fig. 3. Shown are molecular weight markers (lane 1), a typical hybridoma supernatant containing 10% fetal bovine serum in RPMI 1640 (lane 2) and...
ZZ polyester bead purified monoclonal antibody from the DHEAS (clone 8B11, lane 3), pregnanediol-3-glucuronide (clone C5, lane 4) and SHBG (clone 11F11, lane 5) culture supernatants. For comparison lane 6 shows SHBG monoclonal antibody 11F11 purified from 200 mL of culture supernatant using protein A (Affi-Gel, Biorad), on an earlier occasion. The positions of heavy chains with a molecular mass of 50 kDa (H) and light chain chains with a molecular mass of 25 kDa (L) are shown. Lanes 3 to 6 show variable protein loadings.

4. Discussion

We have shown that there was reasonable recovery of monoclonal antibody, as determined by ELISA, and acceptable purity of mouse monoclonal antibodies of isotypes IgG1, IgG2a and IgG2b isolated from culture supernatant using ZZ polypester beads. Agarose electrophoresis and silver staining showed diffuse rather than homogenous staining for the IgG2a antibody in the gamma globulin region, but on SDSPAGE this antibody showed acceptable purity (Fig. 3, lane 4). Supernatant from an IgM secreting mouse hybridoma was used as a negative control and there was no detectable antibody in the low pH eluate as analysed by either ELISA or agarose gel electrophoresis, indeed there was total recovery of antibody in the unbound and first wash fractions. We chose to analyse ZZ polyester purified IgGs by both agarose gel electrophoresis and the more conventional SDS-PAGE as it confirmed the ZZ purified material as a gamma globulin as well as characterizing the molecular weight and purity. Agarose gel electrophoresis and SDS-PAGE of the culture supernatants, which contain 10% fetal bovine serum, contrast with the material purified by ZZ polyester beads and show the predominance of bovine serum albumin in the alpha globulin region with a relative molecular weight of 68,000 Da. Although the agarose and SDS-PAGE gels showed variable loadings, due in part to inherently different IgG expressions in the various

20 mL hybridoma supernatants used, there is nevertheless acceptable purity using ZZ polyester beads. Furthermore calculated recoveries are an index of extraction efficiency which are also acceptable and ranged between 70 and 100%.

The use of ZZ polyester beads for purifying mouse IgG monoclonal antibodies from culture supernatant is novel and we have demonstrated that this method is feasible. ZZ polypester beads can be subjected to repeated purification cycles, at least five, with consistent purification performance and the beads can be sanitized. They are heat stable but at higher temperatures (60 °C) there was a 40% loss in human IgG binding capacity by ZZ polyester beads but no data on the purification of mouse monoclonal antibodies was reported (Brockelbank et al., 2006). On the basis of our results we consider these ZZ polyester beads a novel and useful method to purify IgGs from mouse hybridoma culture supernatants which could also have commercial application.

Acknowledgments

The authors are grateful for production and purification of ZZ polyester by Dr. R. Palanisamy as well as for the formulation and analysis of these beads by J.A. Atwood. This study was funded by the Massey University and the Foundation of Research, Science and Technology of New Zealand.

References


