Journal of Chromatography, 476 (1989) 187–194
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1584

ISOLATION OF A SPECIFIC MEMBRANE PROTEIN BY IMMUNOAFFINITY CHROMATOGRAPHY WITH BIOTINYLATED ANTIBODIES IMMOBILIZED ON AVIDIN-COATED GLASS BEADS

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SUMMARY

Avidin-coated, solid glass beads have been used as an immobilization support for attaching biotinylated antibodies. These beads have been packed into analytical, semi-preparative and preparative columns and used to isolate the B27 histocompatibility anigen (HLA) from human lymphocytes. The beads provided a suitable column material for all three chromatographic procedures and, depending on the size of the immunoaffinity column, B27 antigen could be isolated in nanogram to microgram quantities. Polyacrylamide gel electrophoresis demonstrated the presence of only a single band in the immunoaffinity peaks isolated by all three procedures. Enzyme-linked immunosorbent assay analysis of these immunoaffinity-isolated materials revealed that they were biologically active and could be used to determine the levels of anti-B27 antibodies in clinical studies.

INTRODUCTION

The isolation and recovery of membrane proteins can be a tedious and time-consuming job, but recent developments have greatly facilitated this task. Efficient isolation can be achieved by several different techniques, such as lectin affinity chromatography[1,2], immuno-precipitation[3–5] with monoclonal or polyclonal antibodies and immunoaffinity chromatography with antibodies, immobilized on either Protein A or streptavidin-coated glass beads, as the specific ligand[6–8]. The materials recovered by all of these techniques have been shown to be biologically active and, in the latter two techniques, the material was recovered in an immunologically pure form.

Analytical high-performance immunoaffinity separations, on avidin-coated glass beads as the immobilization support for biotinylated antibodies have been reported previously[7–9]. These reports demonstrated that the immobilized avidin–

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0021-9673/89/$03.50

biotinylated antibody complex coating of the glass bead was stable up to working pressures of 500 p.s.i.[9]. However, the solid glass bead–avidin complex can also be used as an antibody support for scale-up immunoaffinity isolations. In this paper, we describe the use of avidin-coated glass beads for both semi-preparative and preparative isolation of a lymphocyte membrane protein.

EXPERIMENTAL

Materials

Solid glass beads (diameter, 1 mm) were obtained from Kontes Scientific Glassware (Vineland, NJ, U.S.A.). Purified streptavidin was purchased as a lyophilized, pure product from Bethesda Research Labs. (Gaithersburg, MD, U.S.A.) and reconstituted in 50 m*M* carbonate buffer (pH 9.0). Mouse monoclonal antibodies (MAb), reactive with human leukocyte antigen (HLA) B27, were obtained as a purified immunoglobulin G (IgG) preparation from Chemicon International (El Segundo, CA, U.S.A.). The laboratory chemicals were obtained from Sigma (St. Louis, MO, U.S.A.); 3-aminopropyltriethoxysilane and 1,1′-carbonyldiimidazole from Pierce (Rockford, IL, U.S.A.); stainless-steel columns and column fittings from Alltech (Deerfield, IL, U.S.A.); and preparative glass columns were obtained from Kontes Scientific Glassware. HLA-B27 positive lymphocytes and human anti-HLA antibodies were obtained from normal volunteers.

Derivatization of the glass beads

The glass beads were prepared as previously described by Babashak and Phillips[9]. Briefly, the beads were washed by sedimentation in doubly distilled water to remove manufacturing impurities from the bead surface before peparing them for silanization and derivatization by placing 100 g of the washed beads into 500 ml of 1 *M* hydrochloric acid and gently sonicating for 25 min. This was followed by sedimentation in 1000-ml portions of 1 *M* hydrochloric acid and the process was repeated, using fresh acid solutions, until the acid supernatant became clear. The beads were then removed and air-dried, before refluxing them for 30 min in 500 ml of 1 *M* nitric acid, with constant agitation. The beads were recovered, air-dried, and resuspended in 500 ml of 10% 3-aminopropyltriethoxysilane in toluene. This suspension was gently refluxed for 16 h with constant agitation.

Following silanization, the beads were recovered and washed twice in 500 ml of 95% methanol before being transferred to fresh 95% methanol and refluxed for 20 min to remove the excess silanizing agent. The beads were allowed to settle, washed three times in doubly glass-distilled water, and air-dried prior to derivatization of the reactive side-groups.

The reactive carbonyldiimidazole (CDI) side-groups were attached to the bead surface by suspending the beads in 300 ml of dioxane and slowly adding 6 g of 1,1′-carbonyldiimidazole. The mixture was placed in a 500-ml capped conical flask and incubated for 6 h at room temperature in an oscillating shaker. The beads were then recovered and thoroughly washed in dioxane by sedimentation and decantation before being air-dried and immediately coated with streptavidin.

Bead coating procedure

A 100-g amount of the CDI-derivatized beads was suspended in 200 ml of double distilled water prior to the addition of 200 ml of 50 m*M* carbonate buffer (pH 9.0), containing 5 g of streptavidin. The mixture was placed into a 500-ml capped conical flask and incubated for 18 h at 4°C in an oscillating shaker. Following this incubation, the beads were allowed to settle and washed ten times in 0.01 *M* phosphate buffer by sedimentation and decantation. Attachment of the streptavidin to the beads was checked by incubating a 250-μl drop of the bead suspension, obtained from the last wash, with fluorescein-labelled biotin and examining 100 beads under a fluorescence microscope. Following satisfactory coating of the beads, they were sedimented, recovered and resuspended in 500 ml 0.01 *M* phosphate buffer.

Biotinylation of monoclonal antibodies

The hydrazine biotinylation technique[10] requires modification of the carbohydrate portion of the MAb, which was performed by suspending 100 mg of antibody in 10 ml of 0.1 *M* sodium acetate buffer (pH 5.0) and cooling to 4°C. A 10-ml volume of a 10 m*M* solution of cold sodium metaperiodate was added to the antibody before incubation for 20 min at 4°C in the dark. The reaction was stopped by adding 50 ml of 5% ethylene glycol and dialyzing the solution against 0.01 *M* phosphate buffer for 18 h at 4°C, with five changes of the dialysate. The antibody was then removed from the dialysis tubing and placed in a capped glass tube. To this was added 100 ml of phosphate buffer, containing 15 mg/ml of sodium cyanoborohydride and 25 mg/ml biotin hydrazine and the mixture was placed in a rotating mixer for 1 h at room temperature. The reaction was stopped by dialysis against 0.01 *M* phosphate buffer overnight, in a cold-room.

Column construction

The avidin-coated beads were packed into three different columns. Analytical 100 × 4.6 mm I.D. or semi-preparative 250 × 10 mm I.D. stainless-steel high-performance liquid chromatography (HPLC) columns were slurry-packed at 250 p.s.i., using a conventional pump-driven slurry packing apparatus. Preparative 15 × 1 cm I.D. glass columns were gravity-packed, using a bead–0.1 *M* phosphate buffer (pH 7.0) (1:3) slurry.

Isolation of lymphocyte membranes

Prior to disruption and solubilization of their membranes, human lymphocytes were isolated from whole blood by centrifugation at 400 *g* for 15 min in a Ficoll gradient[11]. The lymphocyte band was recovered, and $1 \cdot 10^8$ cells frozen and thawed until all cells were lysed and then sonicated in a Model 300 sonic dismembrator (Fisher Scientific, Columbia, MD, U.S.A.) for 2 min at 300 W. The sonicated pellet was resuspended in 5 ml of 0.01 *M* phosphate buffer, and the cellular debris sedimented by centrifugation at 10 000 *g* for 30 min. The membrane-containing supernatant was mixed with an equal volume of 1% sodium deoxycholate and incubated for 30 min at room temperature. Finally, the solubilized membrane sample was centrifuged for 1 h at 100 000 *g*, and the supernatant, containing 21–25 μg/ml protein was applied to the immunoaffinity columns.

Chromatography equipment

Analytical system. The avidin-packed analytical column was installed into a Beckman 340 isocratic HPLC system (Beckman, Palo Alto, CA, U.S.A.), equipped with a Model 112 pump, a Model 160 UV detector (set at 280 nm), and a Shimadzu C-R1B recording peak integrator (Shimadzu, Columbia, MD, U.S.A.). The elution profile was automatically controlled by a Model III OPG/S solvent selector/gradient controller (Autochrom, Milford, MA, U.S.A.). Samples were introduced into the system by injection through an Altex 210 injection port, equipped with a 100-μl sample loop.

Semi-preparative system. The semi-preparative column was installed into the same system as described for the analytical column, except that the Model 112 pump was replaced with a Model 110 pump, equipped with a semi-preparative pump head. Samples were injected into the system through an Altex 210 injection port, equipped with a 500-μl sample loop.

Preparative system. The preparative glass column was attached to a peristaltic pump (Pharmacia/LKB Biotechnology, Piscataway, NJ, U.S.A.) and the column effluent was monitored at 280 nm with a UV-2 ultra-violet monitor (Pharmacia/LKB). The peaks were recorded on the Shimadzu recording integrator. Gradient control was performed by the Autochrom Model III OPGS/S, used in both the analytical and semi-preparative studies. Samples of 2 ml were fed into the top of the column by gravity.

Immunoaffinity chromatography

Analytical system. The column was isocratically developed with 0.01 *M* phosphate buffer (pH 7.0) for 15 min at a flow-rate of 0.5 ml/min. Throughout the entire run, the column temperature was maintained at 4°C by a glass column jacket, attached to a recycling ice-bath. Following the initial 15-min run, during which the B27 antigen was bound to the immobilized antibody, an elution recovery phase was started. A chaotropic ion gradient was developed by adding 0 to 2.5 *M* sodium thiocyanate to the running buffer, over a further 15 min, and the upper limit of the gradient was maintained for a further 5 min before recycling the column by returning it to the initial running conditions. Fractions of the eluted material were collected in 500-μl Beckman microfuge tubes, in a modified ISCO Cygnet fraction collector (ISCO, Lincoln, NE, U.S.A.) and dialyzed overnight at 4°C against 0.01 *M* phosphate buffer.

Semi-preparative system. Chromatographic conditions similar to those described above for the analytical system were applied, with the exception that the flow-rate was slowed to 0.3 ml/min and the initial isocratic phase was extended to 60 min. The chaotropic ion gradient was started at 60 min, developed over a further 45 min, and then maintained at the upper limit of the gradient for 15–20 min before recycling the column. Fractions of the eluted material were collected in 1.5-ml Beckman microfuge tubes in the same fraction collector.

Preparative system. The preparative glass column was operated at a flow-rate of 1 ml/min. The initial phase was maintained for 120 min before gently developing the thiocyanate gradient over the following 90 min. The upper limit of the gradient was maintained for 30 min before recycling the column. Fractions of 1 ml of the eluted material were collected in an ISCO fraction collector.

Polyacrylamide gel electrophoresis (PAGE)

Analysis of the isolated materials were preformed by PAGE on a 10–30% linear gradient gel, containing 0.1% sodium dodecyl sulfate (SDS)[12]. Briefly, 25 μl of peak 2, isolated by all three procedures was reduced but not chemically modified by boiling for 5 min at 100°C in an equal volume of sample buffer (1% SDS and 5% β-mercaptoethanol dissolved in 0.01 *M* phosphate buffer, pH 7), and then allowed to cool to room temperature before use. A 10-μl sample of each specimen was placed into the sample wells in the analytical gel and run for 3 h at a constant voltage of 150 V. The gels were fixed in methanol–acetic acid (4:1) and then silver stained[13].

Enzyme-linked immunosorbent assay

The specificity of the immunoaffinity isolated material was tested by an enzyme-linked immunosorbent assay (ELISA)[14], using a battery of standard histocompatibility antisera and the anti-HLA-B27 MAb.

RESULTS AND DISCUSSION

Analysis of 80 batches of streptavidin-coated glass beads has shown that a 1-g batch of beads can be coated with between 0.72 and 1 mg of streptavidin. Once coated, the beads were able to bind between 100 and 124 μg of biotinylated antibody, which gave calculated bound antibody levels of 200 μg per analytical column; 550 μg per semi-preparative column, and 2.2 mg per preparative column. Stability analysis showed that the analytical columns could effectively be recycled between 20 and 30 times before loss of antibody could be detected. The semipreparative columns could be recycled 10–15 times and the peparative columns recycled 8–10 times. All columns remained stable for up to six months when stored in a refrigerator.

Antigen loading experiments showed that the analytical columns could isolate between 28 and 44 ng of B27 antigen, while the semi-preparative columns could isolate between 425 and 475 ng of the same antigen. The preparative columns could isolate

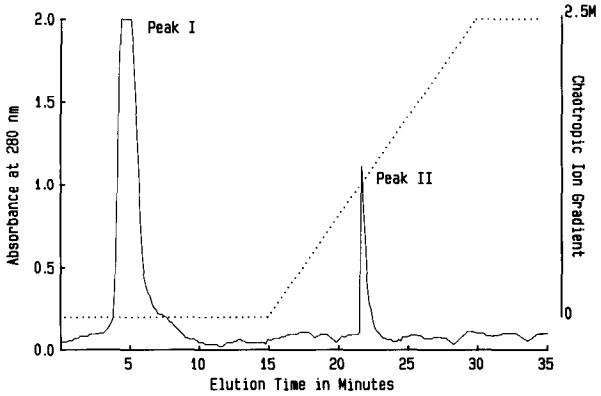

Fig. 1. Immunoaffinity isolation of HLA-B27 antigen from detergent-solubilized lymphocyte membranes. The separation was developed on a 100 × 4.6 mm I.D. analytical column. Flow-rate, 0.5 ml/min; detector, 280 nm, 0.005 a.u.f.s.; sample size, 100 μl. The dotted line represents the chaotropic ion gradient. Peak I represents the unbound material and peak II represents the immunoaffinity isolated material.

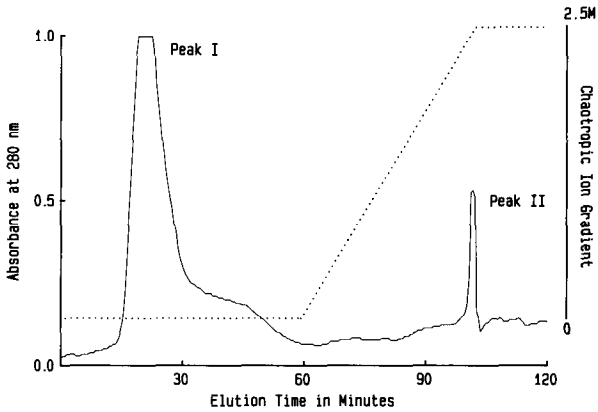

Fig. 2. Semi-preparative immunoaffinity isolation of B27 antigen on a 250 × 10 mm I.D. column. Flow-rate, 0.3 ml/min; detector, 280 nm, 0.08 a.u.f.s.; sample size, 500 μl. The chaotropic elution gradient is shown as a dotted line. Peaks I and II represent the same materials as described in Fig. 1.

between 3 and 8 μg of antigen depending on the protein content of the sample applied to the column and the expression of B27 antigen on the donor lymphocytes.

Fig. 1 demonstrates a typical chromatogram produced by performing an isolation of the B27 antigen on the analytical column. The non-B27 membrane material forms the first peak of the chromatogram, and the immunoaffinity isolated B27 antigen is eluted as the second peak, after 22.5 min.

Fig. 2 illustrates the chromatogram produced by the semi-preparative columns. Although the general profile is similar to that produced by the analytical column, the primary peak extends further into the chromatogram and the second peak is eluted further into the elution phase of the run (100 min). The delay in eluting the second peak is due to the decrease in flow-rate found to be essential when using the larger HPLC columns. Flow-rates of 0.5 ml/min and above caused a sharp decrease in the amount of bound antigen and a poor antigen recovery.

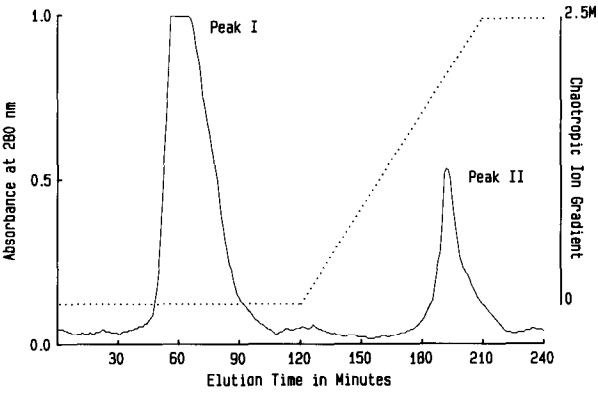

Fig. 3. Preparative immunoaffinity isolation of the same antigen on a 15 × 1 cm I.D. glass column. Flow-rate, 1 ml/min; detector, 280 nm, 0.08 a.u.f.s.; sample size, 2 ml. The elution gradient is indicated by a dotted line. Peaks I and II represent the same materials as described in Fig. 1.

TABLE I
ELISA ANALYSIS OF ISOLATED MATERIALS
All values expressed in absorbance units at 492 nm.

Antisera	*Peak II*		
	Analytical	*Semi-preparative*	*Preparative*
B27	1.389	1.101	0.988
B7-CREG[a]	0.258	0.291	0.300
Bw4[b]	0.206	0.242	0.260
HLA-B Loci (non-polymorphic)	0.816	0.861	0.772

[a] Common reactive group associated with HLA-B27.
[b] Common antigen associated with HLA-B27.

Preparative isolations were long and produced a larger, less well-defined second peak (Fig. 3). This peak was eluted after 190 min, although the immobilized B27 antigen–antibody complex appeared to dissociate earlier in the elution gradient. The antigenic material was also recovered in a large volume, which required concentration before SDS–PAGE and ELISA specificity testing could be performed.

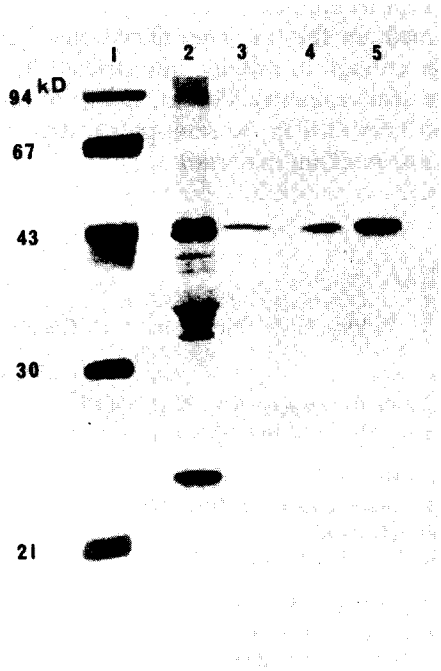

Fig. 4. Silver stained SDS–PAGE gel of the immunoaffinity-isolated material recovered in peak II from all three techniques. Lane 1, molecular weight standards; lane 2, membrane preparation; lane 3, peak II from the analytical column; lane 4, peak II from the semi-preparative column; and lane 5, peak II from the preparative column.

SDS–PAGE analysis of the second (immunoaffinity) peaks, isolated by the three techniques, showed the presence of a single peak at approximately 44 000 kilodalton (kD), which is similar to that described by others for the polymorphic chain of the HLA class I antigens[15,16]. This group includes the products of the HLA-B loci. No other protein bands could be detected in the immunoaffinity-isolated material, even though the gels were silver-stained for extra sensitivity (Fig. 4).

ELISA analysis of the immunoaffinity-isolated material showed strong reactivity with an antiserum directed against the common (non-polymorphic) area of the class I antigen polymorphic chain, associated with HLA-B loci products. This acted to confirm that the isolated material was derived from HLA-B class I antigens. The isolated materials also reacted strongly with the original anti-B27 MAb, which had been used as the immunoaffinity ligand. This reactivity was stronger than that observed with the anti-HLA-B loci antiserum and was strongest in the material isolated on the analytical column. No reactivity was seen with the antisera directed against other HLA-B antigens, even though the Bw4 and B7-CREG antisera have been reported to cross-react with a number of HLA-B antigens and especially B27[17]. The results of the ELISA analysis are shown in Table I.

Immunoaffinity isolation, of HLA, with avidin-immobilized anti-HLA MAb provides a rapid and simple technique for purifying not only these antigens but other membrane proteins[6]. In the HLA system, the molecular size and serological specificity of the immunoaffinity-isolated B27 antigen compared well with other reports on the isolation of class I HLA antigens by biochemical techniques[15,16].

The beads provide a suitable support medium for the immobilization of all classes of biotinylated antibodies and are large enough to provide unrestricted flow when used in semi-preparative and preparative-scale columns. The advantage of this bead system is that analytical procedures can be scaled up to semi-preparative or preparative procedures while still using the same packing material.

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