Efficient bispecific monoclonal antibody purification using gradient thiophilic affinity chromatography

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Abstract

Bispecific monoclonal antibodies (bsMAbs), due their unique design, have a wide range of potential applications in immunodiagnostics and immunotherapy. One of the major limitations for the use of bsMAbs produced by hybrid–hybridomas is the concomitant production of parental monospecific antibodies. The relative amount of bsMAb secreted may vary between different hybrid–hybridomas. Hence, the purification of the desired bispecific molecule from other forms is crucial. Current purification methods include anion-exchange, HPLC on different matrices, and dual affinity methods. Most of those methods include multiple steps and have limitations on the purity or yield of the desired species. We report here a simple single-step purification method, using inexpensive thiophilic chromatography. This new method can potentially be scaled up, for industrial proposes. Finally, based on the amino acid sequences and assembly of the two heavy chains we attempt to explain the possible mechanism by which thiophilic chromatography was able to resolve the bsMAbs from the monospecific species. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Bispecific monoclonal antibodies (bsMAbs) are unique immunoprobes incorporating two different paratopes in a single antibody molecule. They can be used in immunoassays [1–3], immunohistochemistry [4], immunotherapy [1,5,6] or immunoimaging [1,7]. Recently, we published two examples of bsMAbs used in cancer immunodiagnostics [2,3]. In both examples the bsMAb was designed to bind a tumor marker [CA125 or prostate specific antigen (PSA)] in one paratope and peroxidase in the other paratope. The assays developed using bsMAb as the tracer presented superior kinetics and sensitivity when compared to corresponding assays using mono-specific tracer. Although chemical cross-linkage [8] and genetic engineering methods [9] can be used to produce bispecific molecules, the hybrid–hybridoma method is the most common method [10]. In this approach, two pre-established hybridomas can be selected and fused to generate a cell which expresses both parental light and heavy chains. Assembly of these chains in a random fashion can potentially give rise up to 10 different types of antibody molecules as shown in Fig. 1. The hybrid–hybridoma could however produce fewer than the theoretical 10 types. Some degree of preferential association between homologous light and heavy chains may occur [11,12]. The qualitative and quantitative mechanics of the assembly process in the quadroma are largely

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Type of Heavy (H) and Light (L) chain combination	Diagrammatic representation
Monospecific (Parental H-L associations)	
Bispecific (Two homologous H-L and heterologous H-H associations)	
One non-homologous H-L association	
Two non-homologous H-L associations	

Fig. 1. Schematical representation of the 10 possible types of antibody molecules produced by a hybrid–hybridoma.

unknown. In our experience in generating over a dozen such hybrid–hybridoma cell lines, the relative amount of secreted bsMAb vary largely. Mono-specific contaminants can potentially compete with the bispecific antibodies causing decrease in its specific activity. Hence, purification of bsMAb is the most important obstacle to the widespread used of these immunoprobes. Current methods of bsMAb purification include anion-exchange, high-perform-ance liquid chromatography (HPLC) in different matrices, or double affinity methods [3,5,10–14]. All of these methods have some limitations in terms of purity, yield or bulk production.

In this paper we describe a simple, single-step purification of bsMAbs from the parental mono-specific MAbs using thiophilic adsorption chroma-tography with a simple gradient elution. We also use computerized protein homology modeling in an attempt to explain the possible mechanism by which

thiophilic interaction was able to resolve the mono-specific antibodies from the bispecific species.

2. Experimental

2.1. Hybrid–hybridomas

Two different bispecific monoclonal antibodies previously developed in our laboratory were used in our studies. They are: (a) P57.3R2 [3], obtained by the fusion of anti-PSA monoclonal antibody (MAb) hybridoma B80.1, secreting mouse immunoglobulin G1 (IgG1) (courtesy of Biomira, Edmonton, Canada), and YP4, anti-peroxidase hybridoma, sec-reting rat IgG2a (courtesy of Dr. C. Milstein, MRC Laboratory of Molecular Biology, Cambridge, UK); (b) P52.12R8 [2], obtained by the fusion of B27.1, mouse IgG1 secreting anti-CA125 hybridoma (cour-

tesy of Biomira) and YP4. Both hybrid–hybridomas were grown in a 1-l roller bottle apparatus using RPMI 1640 media supplemented with 2 mmol/l L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and 5% (v/v) of fetal bovine serum (Gibco, Gaithersburg, MD, USA). The cultures were maintained for 72 h. The supernatants were collected, pooled and precipitated with 50% saturated ammonium sulphate solution. The precipitated antibodies were resuspended in phosphate-buffered saline (PBS) and dialysed against PBS.

2.2. Thiophilic gel purification of bsMAbs

A 5 ml thiophilic gel (T-Gel) (Pierce, Rockford, IL, USA) column was prepared using the manufacturer's instructions. The packed column was equilibrated with binding buffer (0.5 mol/l potassium sulfate and 50 mmol/l sodium phosphate, pH 8.0). Solid potassium sulfate was added to the dialyzed crude antibody preparation until a concentration of 0.5 mol/l was reached. This sample was loaded the column (0.5 ml/min) and the unbound fractions collected. A UV detector with a 280-nm filter was used to measured the protein content eluted from the column. Once the absorbance at 280 nm (A_{280}) returned to the baseline the bound material was eluted from the gel. The elution protocol suggested by the manufacturer utilized 50 mmol/l sodium phosphate, pH 8.0 as elution buffer, (1 ml/min) to elute all bound proteins in one step. Fractions (2 ml) were collected until no more proteins were detected in the eluate. This suggested protocol was initially utilized to eluted the bsMAbs. In our new procedure, a decreasing potassium sulfate gradient was used to elute bound proteins in an attempt to increase resolution between monospecific and bispecific antibodies. This decreasing potassium sulfate gradient was made using 50 ml 0.5 mol/l potassium sulfate in 50 mmol/l sodium phosphate plus 50 ml of 50 mmol/l sodium phosphate. The elution fractions were collected as described above.

2.3. Immunoassays

2.3.1. (A) Anti-horse radish peroxidase (HRPO) activity

Goat anti-rat (Sigma, St. Louis, MO, USA) diluted 1:1000 in PBS was coated overnight at 4°C onto microtiter plates (Nunc, Naperville, IL, USA). The plates were blocked with 1% bovine serum albumin (BSA) (Sigma) in PBS solution for 1 h at 37°C. Following a wash step with 0.05% Tween 20 in PBS (PBST), aliquots from different fractions were incubated for 1 h at 37°C. The plates were again washed and incubated with HRPO (Sigma) 10 μg/ml in 1% BSA for 30 min at room temperature (RT). After a final wash, ABTS (2.2′-azino-di[3-ethyl-benzthiazoline sulfonate]) plus H_2O_2 (Kirkegaard and Perry Labs., Gaithersburg, MD, USA) substrate was added. After 15 min the absorbance was measured at 405 nm.

2.3.2. (B) Simultaneous measurement of monospecific anti-PSA and bsMAb anti-PSA activities

In experiments utilizing P57.3R2 (anti-PSA/anti-HRPO) material a very interesting assay was applied using two different enzymatic markers as described below. In an indirect immunoassay, if a biotinylated anti-mouse IgG1 in conjunction with streptavidin–alkaline phosphatase label was used as secondary antibody, both monospecific and bispecific anti-PSA antibodies would be detected. But if HRPO is also incubated in addition to the second antibody system, a peroxidase substrate can be used to detect only the presence of bispecific molecules.

Affinity purified PSA [3] 0.5 μg per well was coated overnight at 4°C onto microtiter plates (100 μl/well). The plates were blocked with 1% BSA for 1 h at 37°C. After a wash step, different T-gel fractions were incubated for 1 h under agitation. The plates were washed and a rat anti-mouse IgG1-biotin (ICN Biomedicals, Aurora, OH, USA) diluted 1:500 was added and incubated for 1 h to detect both monospecific and bispecific antibodies. The plates were washed and streptavidin–alkaline phosphatase (Sigma) 1:10 000 and 10 μg/ml of HRPO were added and incubated for 30 min. The plates were washed five times and the alkaline phosphatase substrate Sigma 104 (Sigma) was added to detect the elution profiles presence of monospecific and bispecific anti-PSA antibodies. The absorbance was measured at 405 nm using V_{max} (Molecular Devices, Sunnyvale, CA, USA) enzyme-linked immunosorbent assay (ELISA) reader. The plates were subsequently washed three times with PBST to remove the alkaline phosphatase substrate and re-

action products. Tetramethyl benzidine (TMB) (Kirkegaard and Perry Labs.), a peroxidase substrate, was than added to detect the elution profile of the bsMAbs. After 10 min of incubation, TMB reaction was stopped by adding 1 *M* phosphoric acid and the absorbance measured at 450 nm.

2.3.3. (C) Profile of anti-CA125 activities

Immunomax (Nunc) plates were coated with goat anti-mouse (Sigma) 1:1000 in PBS 100 μl/well overnight at 4°C, and blocked with 1% BSA. After a wash step T-gel fractions were incubated for 1 h at RT. The plates were washed again and rat anti-mouse IgG1-biotin diluted 1:500 was incubated for 1 h and steptavidin–alkaline phosphatase (1:10 000) was incubated for 30 min. Following a final wash step Sigma 104 alkaline phosphatase substrate was added and absorbance was measured at 405 nm after 15 min of reaction.

2.3.4. (D) bsMAb anti-CA125 activity

Immunomax plates were coated with B43.13 (courtesy of Biomira) anti-CA125 MAb at 1 μg/well overnight at 4°C. This second anti-CA125 was previously selected for its ability to capture CA125 on the solid-phase at a site that is non-overlapping with the B27.1 epitope [15]. The plates were washed three times with PBST and incubated for 3 h with approximately 1000 U/ml of CA125 antigen (Biomira) The plates were washed three times and 100 μl of each T-gel fraction incubated for 1 h at 37°C. The plates were again washed and incubated for 1 h with 10 μg/ml of HRPO in PBS. Following a final wash, the plates were incubated with ABTS plus H_2O_2 and the absorbance scored at 405 nm after 30 min.

2.3.5. Protein assay

The protein concentration was measured by BCA Protein Assay (Pierce). The assay was performed using the manufacturer's procedure. Purified B27.1 MAb was used as the protein standard. The absorbance was measured at 540 nm and the unknown values determined using the Softmax software. Fractions containing more than 1 mg/ml were diluted in PBS and retested.

2.3.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Pre-cast gradient SDS–PAGE gels (8–25%) were used in the Phast-gel apparatus (Pharmacia, Uppsala, Sweden). The gels were stained with Coomassie Blue R.

2.3.7. Immunoglobulin sequences analysis

Sequences of the constant region of a mouse Ig γ-1 chain (PIR accession No. G1MS) and a rat Ig γ-2a chain (PIR accession No. PS0019) were retrieved and aligned using the sequence analysis package SEQSEE [16]. Using these initial sequence alignments, it was possible to unambiguously identify the suspected disulfide pairs in both the homo- and heterodimers. The disulfide-rich linker region was manually adjusted to permit appropriate disulfide pairing between the two chains. The aligned sequences are present in Fig. 6.

3. Results

In this study two quadromas clones, namely anti-PSA/anti-HRPO (P57.3R2) and anti-CA125/anti-HRPO (P52.12R8), were used as representative examples of the ability of thiophilic interaction chromatography to resolve the desired bsMAbs. These quadromas were recently generated in our laboratory to develop sensitive ELISA assays for PSA and the ovarian cancer antigen CA125, respectively.

Approximately 2-l of each hybrid–hybridoma supernatant were precipitated with a 50% saturated ammonium sulfate solution. This initial step removed some of the contaminant proteins and also concentrated the supernatant approximately 20-fold from the original volume. This material was used in different experiments using the thiophilic chromatography strategy to purify the bsMAbs. The crude ammonium sulfate precipitated immunoglobulin fraction of P57.3 was initially purified using the standard T-Gel protocol. Thirty five ml were utilized in this initial experiment. The unbound material was reloaded in an attempt to increase antibody binding. After washing all the unbound proteins, the antibodies were eluted using the standard manufacturer's elution protocol of low ionic straight buffer as

described in Section 2.2. In this study only the total protein and the bsMAb activity was monitored. The purification profile is shown in the Fig. 2.

The peak of protein absorbance was seen in the fraction 3, but the bsMAb activity peaked between fractions 5 and 6. Another key observation was the fact that the unbound fraction did not contain any bsMAb activity. Even after the first load less than 1% the bsMAb activity remained in the unbound fraction. On other hand, there was a significant decrease in total protein concentration between the first and second unbound fraction. The reload of the unbound material only caused an increase in non-specific binding. The delay in the appearance of the bsMAb activity in relation to the total eluted immunoglobulin peak suggested that a further optimization of the purification method could improve resolution between the monospecific and bsMAb antibodies. Our hypothesis was based on the assump-

tion that a bsMAb containing a hybrid heterodimeric IgG1–IgG2a constant domain, may produce a different elution profile when compared with the homologous mouse $(IgG1)_2$ or rat $(IgG2a)_2$ dimeric combinations. A decreasing salt gradient elution was tested in order to increase the resolution of the various species.

3.1. Gradient elution

3.1.1. P57.3R2

In order to test this hypothesis and achieve better resolution of the different antibody species secreted by our two quadromas, we devised a linear gradient of decreasing K_2SO_4 starting at 0.5 mol/l down to zero. Thirty five ml of the ammonium sulfate material was loaded into the T-gel column. To reduce nonspecific binding the unbound fractions were not reloaded. The bound material was eluted using the
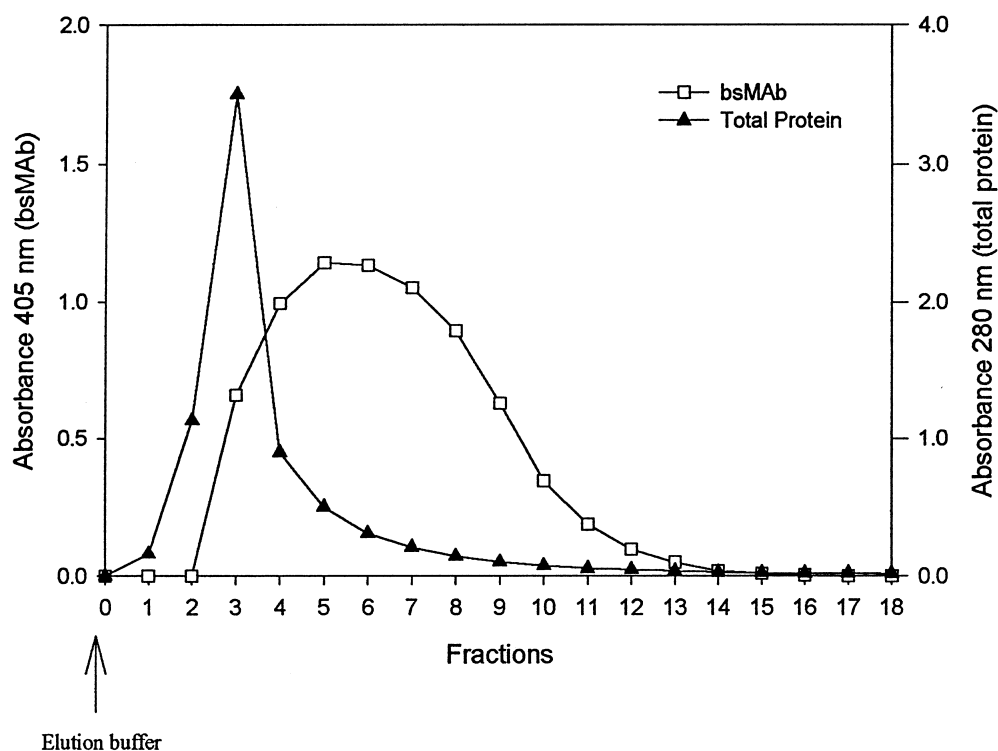

Fig. 2. Profile of P57.3R2 purification on T-gel column. The crude antibody sample adjusted to 0.5 mol/l K_2SO_4 was loaded at 1.5 ml/min on to the T-gel column. The bound antibody was eluted with 50 mmol/l sodium phosphate, pH 8.0, collecting 2-ml fractions. A UV detector with a 280-nm filter was used to measured the total protein content eluted from the column. PSA-coated plates were used to measured bsMAb activity (absorbance 405 nm). Undiluted fractions were used in bsMAb assay.
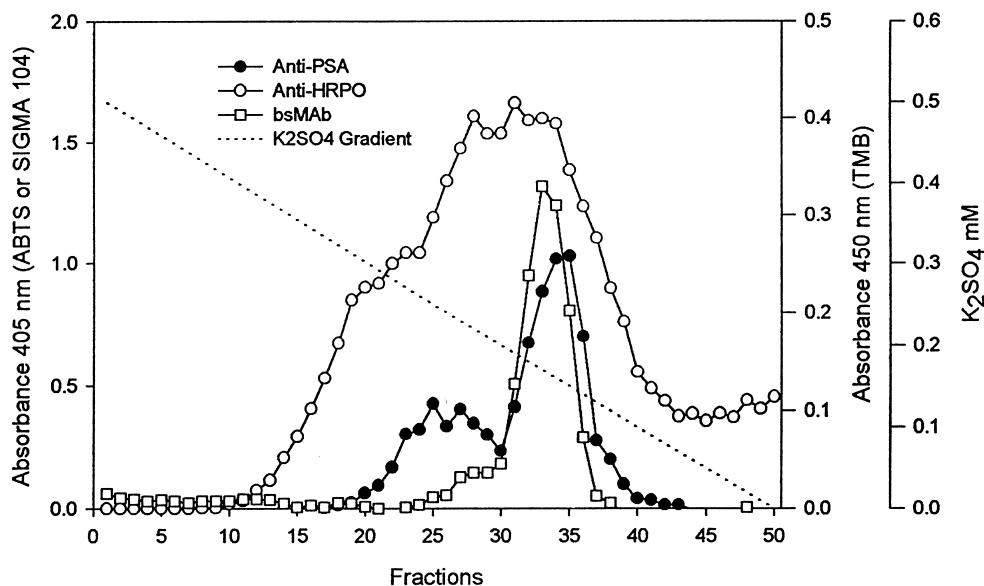
Fig. 3. Profile of P57.3R2 purification on T-gel column using a decreasing potassium sulfate gradient elution procedure. A gradient was set up with 50 ml of 0.5 mol/l K_2SO_4 in 50 mmol/l sodium phosphate, pH 8.0, and 50 ml of sodium phosphate only at the same molarity without K_2SO_4. Undiluted fractions were utilized in the anti-HRPO capture assay using goat anti-rat antibody-coated plates. ABTS substrate (absorbance 405 nm) was used to estimate the presence of anti-peroxidase antibodies. For the simultaneous measurement of anti-PSA and bsMAb the fractions were diluted 1:50 and anti-PSA was measured with PSA coated plates and detecting mouse antibodies with biotinylated rat anti-mouse and alkaline phosphatase–streptavidin. The bsMAb activity was detected by measuring HRPO activity using TMB substrate (absorbance 450 nm, right *y*-axis).

decreasing linear gradient as described in Section 2.2. The profiles of the various MAbs (anti-HRPO, anti-PSA and bsMAb) activities are shown in Fig. 3. Neat fractions were utilized in the anti-HRPO assay. For the simultaneous measurement of anti-PSA and bsMAb the samples were diluted 1:50 in 1% BSA in PBS.

The decreasing salt gradient elution clearly shows two peaks of anti-HRPO activity and two peaks of anti-PSA activity. The first peak of each activity seems to represent the monospecific species with no bsMAb activity detected. The bsMAb activity was confined to a single peak as shown in Fig. 3 above and well resolved from the monospecific species. It is particularly interesting to note that the first peak of anti-PSA and anti-HRPO activities have virtually no bsMAb activity. The bsMAb activity only corresponds and overlaps with the second anti-HRPO and second PSA MAb activities.

SDS–PAGE analysis of the various fraction showed a combination of one band corresponding to the heavy chain and two bands in the light chain

molecular mass region (data not shown). The two heavy chains could not be resolved as a doublet, unlike previously examples of bsMAbs [10].

3.1.2. Parental anti-PSA anti-HRPO

In order to produce a control elution profile, approximately 350 ml of YP4 supernatant (anti-HRPO) was spiked with 1 mg of purified B80.1 (anti-PSA). Solid K_2SO_4 was added to the antibody mixture until a concentration of 0.5 mol/l was reached. The loading and gradient elution was performed as described above. The elution profile is shown in Fig. 4.

The peak of elution of both parental antibodies were between fractions 16 to 34. These profile seems to correlate with the first peak (monospecific) obtained in the bsMAb purification (Fig. 3).

3.1.3. P52.12R8

The pooled supernatant from a second bsMAb P52.12 (anti-CA125/anti-HRPO) was used to further confirm and evaluate the general utility of our results
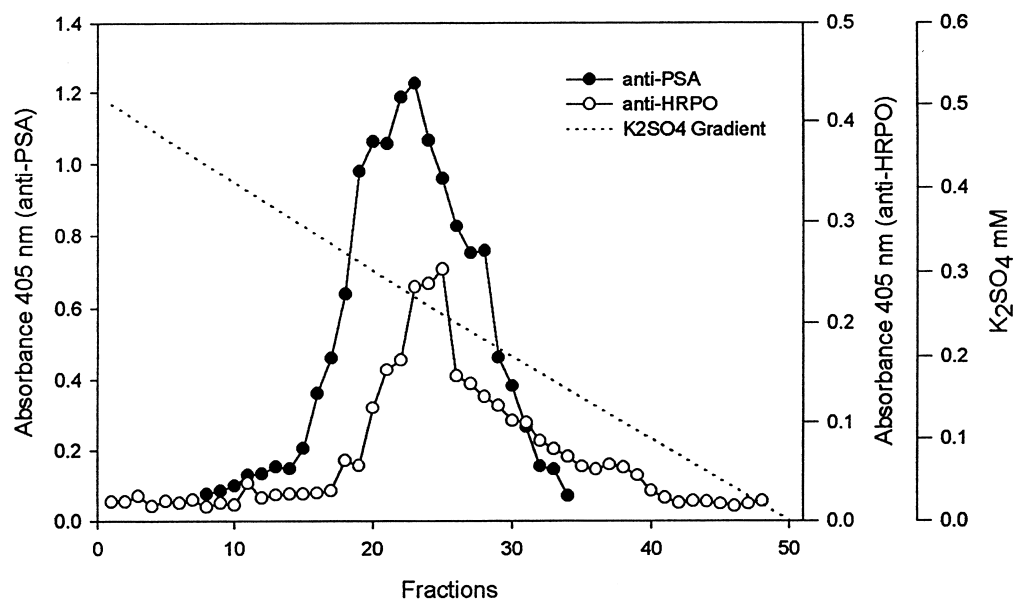
Fig. 4. Profile of parental anti-PSA and anti-HRPO purification using a decreasing potassium sulfate gradient elution procedure. Undiluted fractions were utilized in the anti-PSA assay (left *y*-axis) and anti-HRPO assay (left *y*-axis).

to other bsMAbs. The same conditions of gradient elution were applied and the profile is shown in Fig. 5.

Once again it is apparent that at least two peaks of each activity can be seen resolved from each other, although the results were not as clean as the previous P53.3R2 purification. The first peak of each activity had virtually no bsMAb activity and hence represents the bulk of the parental monospecific MAbs. The bsMAb activity appears much later (beginning fraction 43) and overlaps with the second peak each of the individual rat and mouse MAb profiles.

3.1.4. IgG alignments

Sequences of the constant region of a mouse Ig γ-1 chain (PIR accession No. G1MS) and a rat Ig γ-2a chain (PIR accession No. PS0019) were retrieved and aligned using the sequence analysis package SEQSEE. The overall homology between the constant domains of mouse IgG1 and the rat IgG2a was 78.5%. The disulfide-rich linker region was manually adjusted to permit appropriate disulfide pairing between the two chains (Fig. 6). The first and last inter-heavy chain disulfide bonds are indicated by arrows [17]. Mouse IgG1 contain three

disulfide bonds, and rat IgG2a has only two S–S bonds. The bispecific construct would probably contain two S–S bonds potentially leaving one free sulfidryl group.

4. Discussion

Thiophilic adsorption chromatography was first described in 1985 by Porath et al. [18]. Thiophilic adsorption is a highly selective type of salt-promoted protein–ligand interaction. It differs from simple hydrophobic interactions in some very important aspects. The latter is strongly promoted by high concentration of sodium chloride, whereas thiophilic adsorption is weakened. Albumin among the serum proteins is not at all adsorbed to a T-gel whereas it is the major protein interacting with a hydrophobic gel. The reverse is true for the immunoglobulins [19–21]. We have successfully used the T-gel in the purification of rat monoclonal antibodies [22].

The T-gel was capable of partially resolving (Fig. 2) monospecific forms from the bispecific even when the suggested manufacturer's elution protocol was used. The use of a decreasing K_2SO_4 gradient
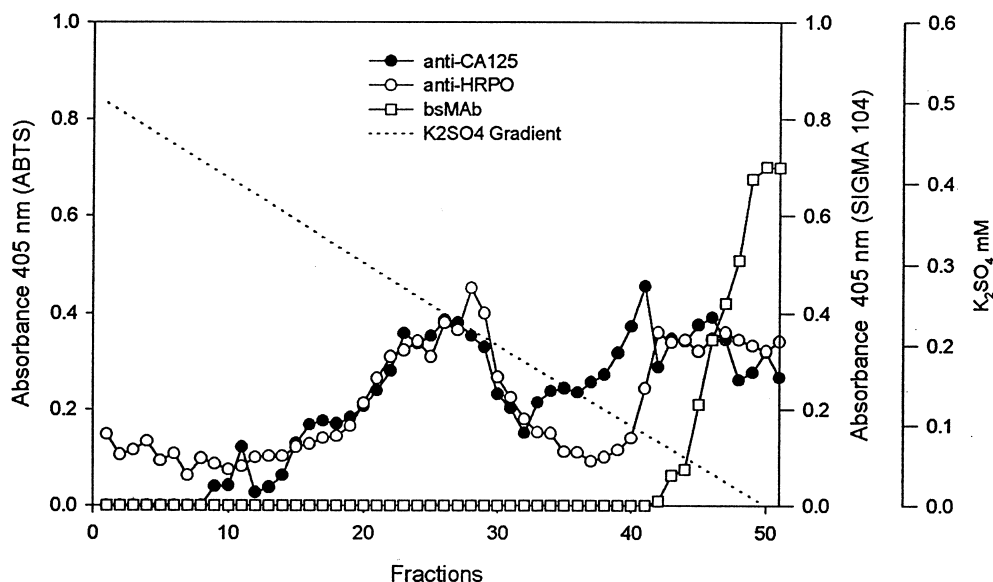
Fig. 5. Profile of P52.12R8 purification on T-gel column using a decreasing potassium sulfate gradient elution procedure. Undiluted fractions were utilized in all assays. Anti-HRPO and bsMAb activities were measured using ABTS substrate (left *y*-axis), and anti-CA125 activity was measured in a sandwich assay using solid-phase goat anti-mouse and solution phase biotinylated rat anti-mouse and alkaline phosphatase–streptavidin with Sigma 104 substrate (right *y*-axis).

elution further enhanced the resolution between the monospecific and bispecific forms of antibodies. Even though the mechanism of thiophilic adsorption remains uncertain, its utility is revealed by the selective and reversible immobilization of immunoglobulins from serum, ascites fluid and hybridoma cell-culture media [21,23]. The precise mechanism of this thiophilic interaction process is currently un-known, but interaction of the non-ionic sulfone–thioether ligand with appropriated acceptor sites on the protein surface appears to be quite specific, and is promoted in the presence of water structure-forming salts [19,24]. Thiophilic adsorption is a process that can be controlled experimentally by varying one of at least three variables, namely, pH, concentration and or type of water-structure-forming salt [20].
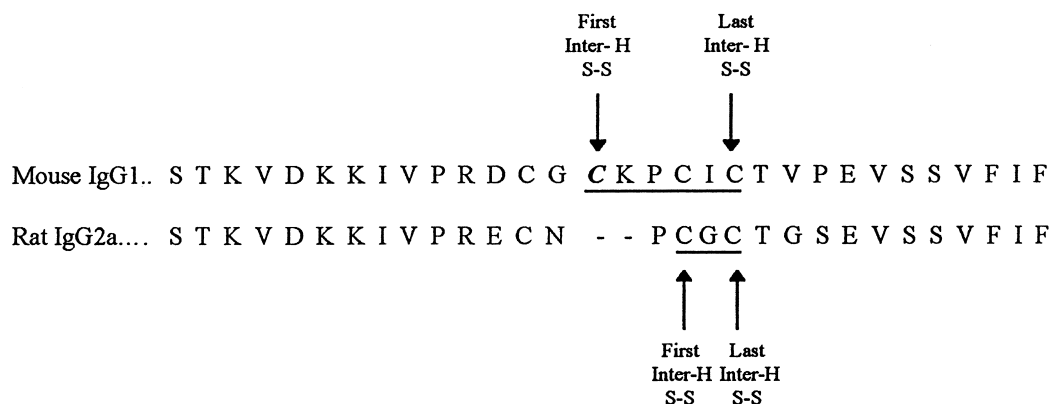

Fig. 6. Sequence alignment of mouse IgG1 (B80.3 and B27.1) with a rat IgG2a (YP4). The cysteine in bold/italic from the mouse IgG1 would not form a S–S bond. This free sulfidryl group may cause a stronger interaction with the thiophilic gel.

Because immunoglobulins are more thiophilic than most other serum proteins, buffer conditions have been developed for their selective adsorption during T-gel chromatography [19].

In a recent report [25], the affinity of the T-gel towards the different mouse IgG sub-classes was presented. The authors suggested that the hinge region of the immunoglobulin may be involved in the interaction with the thiophilic gel. Another experiment that further implicates the hinge region in the interaction with the T-gel was reported [26], wherein F(ab)$_2$ fragments were resolved from the Fc fragments in a step wise elution with different concentrations of the water structure-forming salt.

The formation of disulfide bonds requires cysteine residues aligned within a proper distance between the two heavy chains. One would expect proper alignment maximally in a dimer of two identical heavy chains with distinct extended domains. In the case of the bispecific heterodimeric heavy chain assembly, there is likely a misalignment between the two different species and subclasses of immunoglobulin resulting in fewer disulfides and potentially higher sulfidryl content. It is our hypothesis that this misalignment between to different immunoglobulin isotype or even between immunoglobulins from different species (rat and mouse) may cause differential affinity towards the thiophilic gel. Monospecific antibodies which have a prefect inter-chain sequence for maximal disulfide bond formation during post-translational assembly would behave differently than the bispecific antibody. This was demonstrated using our protein sequence alignment study (Fig. 6). The possible misalignment between the rat IgG2a and the mouse IgG1 would cause a higher affinity towards the T-gel than the monospecific species. One cysteine from the hinge region of the mouse IgG1 does not form a disulfide bond in the heterodimeric heavy chain assembly. Consequently, we expected and found higher affinity of the bsMAb species towards the functional groups of the T-gel, requiring a lower ionic strength buffer in order to be eluted. Further analysis will be require to identify to where, if any, the types of molecules with nonfunctional association (Fig. 1) would be resolved in this method. Theoretically, the extreme form of the non-functional associations (Fig. 1, middle of last row) should appear at the very end of the gradient run if the same

hypothesis is extended to all domains of immuno-globulins, particularly, if disulfides are not formed due to a similar misalignment. The difference in terms of resolution between the P53.3R2 and P52.12R8 may reflect different amount of nonfunctional association being secreted by the hybrid–hybridomas.

This new purification method could potentially solve some of the problems associated with the purification of bsMAbs secreted by hybrid–hybridomas. This method may not be useful for purification of bsMAb derived from two MAb of the same species and subclass. The resolution between mono-specific and bispecific forms seems to be satisfactory. Other protocols for purification bsMAbs contain usually multiple steps and are time consuming. The thiophilic purification described in this paper is simple, inexpensive, and can be potentially scaled up for industrial proposes.

Acknowledgements

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