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Purification of monoclonal antibodies by epitope and mimotope affinity chromatography[1]

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Abstract

Murine monoclonal antibodies raised against the carcinoma-associated MUC1 mucin have applications in the diagnosis and therapy of human cancer. Many of these antibodies define linear epitopes of three, four or five amino acids within an immunodominant region of the MUC1 protein core. Various synthetic peptides which incorporated this region were prepared and covalently linked to agarose beads for use as affinity matrices. An unrelated peptide was identified as a mimotope for one of the anti-MUC1 antibodies using phage display technologies and this was also evaluated as a potential ligand in an affinity matrix. Epitope affinity chromatographic purification of an anti-MUC1 antibody was performed using hybridoma tissue culture supernatants as sample. Following sample application and column washing, antibody was desorbed from the matrix by gradient elution with increasing concentrations of NaSCN. The procedure has proved efficient for the purification of anti-MUC1 antibodies and the concentration of NaSCN required for antibody desorption gives a measure of the relative binding affinity of the antibody for the peptide epitope matrix so that separation strategies may be optimised. © 1997 Elsevier Science B.V.

Keywords: Immunoaffinity ligands; Epitope affinity chromatography; Monoclonal antibody

1. Introduction

The MUC1 mucin is a high-molecular-mass glycoprotein which is normally expressed on the apical surfaces of specialised glandular epithelia. In malignancy, however, its expression is upregulated and the mucin is also secreted at higher levels into the serum of the cancer patient [1,2]. Consequently, there is considerable interest in the measurement of circulating MUC1 so that it can be used as a blood-borne marker of active disease. Many monoclonal antibodies have now been produced against MUC1 mucins and these have been incorporated into immunoassays for circulating MUC1 mucins. Around half of the antibodies produced react with determinants in a region of multiple repeated hydrophilic motifs within the protein core. It has been possible to identify the epitopes for these antibodies using synthetic peptides to interrogate the binding sites [3,4]. In this way, it was found that anti-MUC1 antibodies reactive with the MUC1 protein core define linear epitopes of three, four or five amino acids in the hydrophilic motif APDTRPAP. This

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suggested that if such peptides were immobilised on an affinity matrix, then the matrix could be employed to remove antibody selectively from complex feedstocks (hydridoma culture supernatants, ascitic fluids, fermentation broths etc.). Previous tests showed that fully immunoreactive and highly purified anti-MUC1 mucin antibodies could be isolated from hybridoma supernatants by adsorption and subsequent elution from a peptide epitope affinity matrix [5]. In the present investigation, we have selected one anti-MUC1 mucin antibody, C595, which defines the epitope sequence RPAP, to explore the performance of a panel of epitope matrices containing a variety of immobilised MUC1 epitope and mimotope sequences. We now report on these experiments which have been designed to evaluate the critical features of epitope affinity matrices for antibody purification.

2. Experimental

2.1. Monoclonal antibody

The murine anti-MUC1 mucin antibody C595 was prepared from exhausted media from static cultures of the C595 hybridoma. Cell-free supernatants were clarified by ultracentrifugation (40 000 *g*, 30 min) and ultrafiltration (0.45 μm) then stored at 5°C with sodium azide (0.05%, w/v) as a preservative.

2.2. Peptide epitopes

Synthetic peptides, shown in Table 1, were prepared on an Applied Biosystems 431A Peptide Synthesiser. The glycopeptide TAP-2 was prepared using tri-*O*-acetyl *N*-acetylgalactosamine-α-Fmoc threonine (D.I.R. Spencer, unpublished findings; Fmoc=9-fluorenylmethoxycarbonyl). Purity was assessed by mass spectroscopy and HPLC and found to be in excess of 96%.

2.3. Immunoaffinity matrices

Synthetic peptides were linked to beaded agarose (Sepharose 4B, Pharmacia Biotech, Uppsala, Sweden) via their N- or C-termini using CNBr-activated Sepharose or carbodiimide (0.1 *M* 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), Pierce & Warriner, Chester, UK) conjugation to epoxydiaminohexane (EAH) Sepharose, respectively. A standard coupling ratio of 1 μmol peptide per ml of gel was employed and conjugation procedures were as recommended by the manufacturer.

2.4. Affinity chromatography

Equal volumes (approximately 3 ml) of each affinity matrix were packed into chromatography columns (diameter 10 mm; Pharmacia Biotech) which were then fitted to an automated fast protein liquid chromatography (FPLC) system (Pharmacia Biotech). Chromatography was performed at room temperature (22–25°C) using reagents that were equilibrated at room temperature before use. All reagents contained 0.05% (w/v) sodium azide.

Hybridoma supernatant containing C595 antibody at 22 μg/ml (100 ml aliquots) was applied at 1 ml per min and the columns were washed with phosphate-buffered saline, pH 7.4 (PBS). Antibody was eluted from the column matrix by using a linear gradient of sodium thiocyanate (NaSCN) (Sigma, Poole, UK) in PBS from 0 (Buffer A) to 3 *M* NaSCN (Buffer B) over 20 ml. At the end of the gradient, Buffer B was held for 5 ml before switching to Buffer A for a further 15 ml. Data acquisition and analysis was performed using FPLCdirector Software (Pharmacia Biotech).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The immunoreactivity of isolated antibody fractions was determined using a standard ELISA procedure to determine antibody binding to MUC1 mucin dried to the wells of microtitre plates. After blocking non-specific binding sites by incubation with PBS containing 1% (w/v) bovine serum albumin (BSA), antibody preparations were titrated by serial dilution with 50 μl added per well. After incubation for 1 h, the plates were washed 4 times and horseradish peroxidase (HPO)-conjugated rabbit anti-mouse immunoglobulins (1/1000) (Dako, High Wycombe, UK) were added at 50 μl/well. Plates were incubated for 1 h, washed 4 times and ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] was added as the chromogenic substrate.

ABTS was prepared as a 0.033% (w/v) solution in 0.1 *M* citrate phosphate buffer (pH 4) with 33% (v/v) hydrogen peroxide added at 1 μl/ml.

Alternatively, the ELISA procedure was applied to measure antibody binding to aliquots of Sepharose beads (30 μl packed bead volume per tube) to which peptide epitopes had been linked. The ELISA procedure was essentially equivalent to that described for the microtitre plate assay although washing was performed by centrifugation (10 000 *g* for 5 s, 1 ml wash volume per tube). With both assays, development of colour was measured spectrophotometrically at 405 nm.

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

Affinity-purified C595 antibody samples were diluted 1:1 with SDS loading buffer [bromophenol blue (0.05%, w/v), sucrose (40%, w/v), EDTA (0.1 *M*, pH 8.0), and SDS (0.5%, w/v)] and pre-treated by boiling for 5 min prior to loading. SDS–PAGE was performed using a PhastSystem Separation and Control Unit (Pharmacia) in conjunction with PhastGel precast gels (homogenous acrylamide 12.5%, w/v). Protein on the gels was revealed by staining with Coomassie R 350 stain (0.1%, w/v, in methanol (30%, v/v), acetic acid (10%, v/v)) for 1 h.

3. Results

Table 1 summarises the synthetic peptides and glycopeptides employed as ligands coupled to beaded agarose matrices. The performance of these matrices was explored by assessing their capacity to adsorb the antibody C595 from tissue culture supernatants. This antibody recognises the peptide epitope RPAP in the MUC1 mucin protein core. All separations were performed under standard conditions of sample volume applied (100 ml), column size (3 ml), peptide ligand immobilisation (1 μmol/ml gel) and desorption with a linear gradient of NaSCN from 0 to 3 *M* over 20 ml. Fig. 1 illustrates the desorption of antibody from a series of three matrices incorporating antigenic MUC1-related peptides linked to CNBr-activated Sepharose via their N-termini. Desorption of antibody from the matrix containing the smaller peptide A-G was effected using a lower concentration of NaSCN (1.0 *M*) than that required for antibody elution from the longer peptides (TAP and TAP-2, 1.3 and 1.4 *M*, respectively). The order

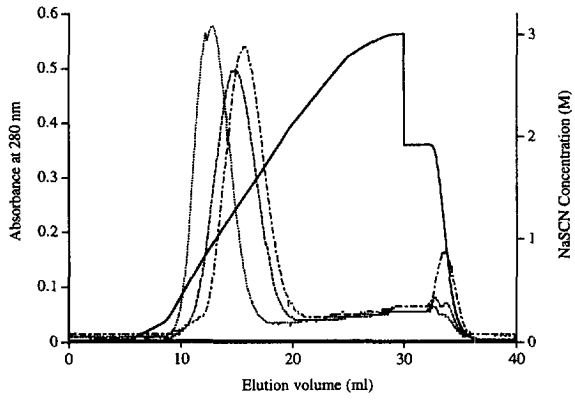
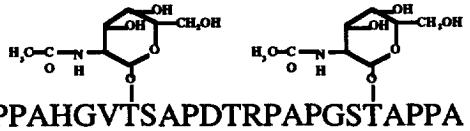


Fig. 1. Purification of C595 monoclonal antibody from hybridoma supernatant using Sepahrose–A-G (···), Sepharose–TAP (- - -) and Sepharose–TAP2 (– · –) conjugates. Desorption was achieved using a linear NaSCN gradient (——) from 0 to 3 *M* over 20 ml.

Table 1
Structures of peptides evaluated as ligands in epitope affinity chromatography

Peptide sequence	Description	Abbreviation
*APDTRPAPG	MUC1 epitope	A-G
KSKAGVC	MUC1 mimotope	KSK
TAPPAHGVTSAPDTRPAPGSTAPPA	MUC1 core sequence	TAP
 TAPPAHGVTSAPDTRPAPGSTAPPA	MUC1 glycopeptide	TAP2

*Refers to one-letter amino acid codes

of elution (from A-G, TAP followed by TAP-2) is equivalent to the order of increasing binding constants that the peptides display for this antibody in solution, as measured by their capacity to quench the fluorescence of the C595 antibody [6]. Small peaks were observed on elution profiles from all three matrices after approximately 35 ml (Fig. 1), these contained no antibody and were assumed to be an artefact caused by the change in buffer at that point. Elution of a column containing non-substituted Sepharose under the conditions of Fig. 1 confirmed this (data not shown). Each antibody preparation was evaluated for immunoreactivity by titration in ELISA and all preparations were equivalent showing high immunoreactivity (Fig. 2). The purity of antibody eluted from the columns was evaluated by SDS–PAGE on homogeneous 12.5% acrylamide gels with samples prepared under reducing and non-reducing conditions. All preparations were equivalent showing characteristic heavy and light chains of mouse immunoglobulin in reduced samples with no evidence of contaminating proteins (Fig. 3).

In previous studies, a mimotope for the C595 antibody was identified by reacting the antibody with a phage display library expressing random hexamers [7]. The mimotope sequence, KSKAGV, was unrelated to the native epitope, RPAP, defined by the C595 antibody. The mimotope peptide KSKAGV with a C-terminal cysteine was linked to Sepharose and compared with an affinity matrix of Sepharose–A-G prepared in an equivalent manner. As shown in Fig. 4, a peak of material (shown to be C595 antibody) was bound to the Sepharose–A-G matrix and eluted with 1 *M* NaSCN. No peak corresponding to elution of antibody from the mimotope column was observed. As illustrated in Table 2, ELISA tests on the materials which were not bound to the columns showed that there was no reduction in antibody activity in material which passed through

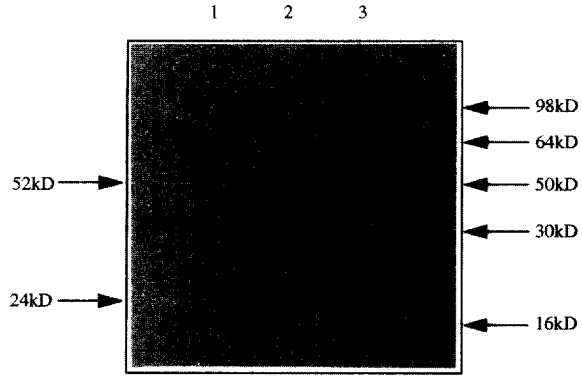


Fig. 3. SDS PAGE analysis of C595 antibody samples purified using Sepharose–A-G (lane 1), Sepharose–TAP (lane 2) and Sepharose–TAP2 (lane 3). Positions of standards are given down the right hand side and the approximate size of the bands corresponding to C595 antibody heavy and light chains are given on the left-hand side.

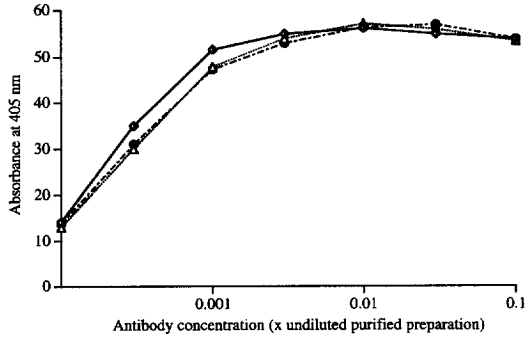


Fig. 2. Analysis of the immunoreactivity of antibodies purified using Sepharose–A-G (◊), Sepharose–TAP (△) and Sepharose–TAP2 (●) conjugates. Antibody preparations were titrated by serial dilution and binding to immobilised MUC1 antigen was measured by indirect ELISA.

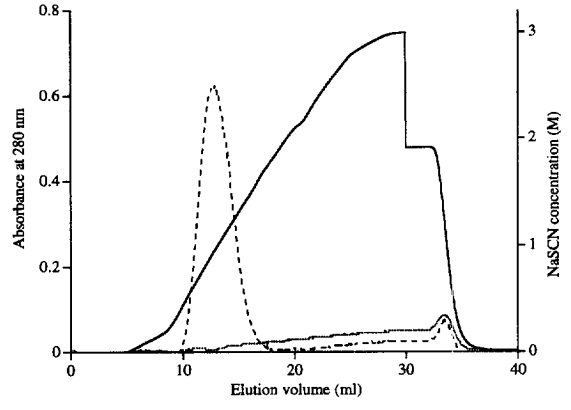


Fig. 4. Purification of C595 monoclonal antibody from hybridoma supernatant using Sepahrose–A-G (- - -) and Sepharose–KSK (. . .) conjugates. Desorption was achieved using a linear NaSCN gradient (——) from 0 to 3 *M* over 20 ml.

Table 2
Comparison of the antibody content of C595 hybridoma supernatant feedstock and column pass samples resulting from purification using various affinity matrices

Sample	Mean absorbance at 405 nm (AU)	Standard deviation
C595 supernatant	27.78	1.53
A-G column pass	13.86	0.42
KSK column pass	28.01	0.46
TAP column pass	3.95	0.18
TAP2 column pass	0.78	0.04
Positive control	40.53	0.35
Negative control	0.39	0.04

Analysis was performed using an indirect ELISA. Positive control was purified C595 antibody (10 μg/ml), negative control was an anti-*myc* peptide antibody (10 μg/ml). Standard deviation values were calculated from triplicate samples.

the mimotope column. Considerably reduced antibody-binding activity was demonstrable in the Sepharose–A-G passed fraction. The two affinity matrices containing the longer TAP peptide and TAP-2 glycopeptide were most efficient in removing antibody from the sample materials in accord with the previous experiments shown in Fig. 1.

Preliminary tests were performed in which the epitope peptide A-G and the mimotope peptide KSKAGVC were linked to Sepharose via C-termini rather than their N-termini. ELISA tests were performed on aliquots of the substituted matrices to determine the binding of C595 antibody to the various matrices (Table 3). Antibody binding was demonstrable with the A-G peptide in either orientation, with N-terminal coupling showing the higher binding capacity. C595 binding was also clearly evident using Sepharose linked to the mimotope peptide via its N-terminus, whereas the C-coupled mimotope peptide matrix failed to bind antibody. These tests demonstrate that the orientation of the epitope and mimotope sequence, as immobilised to an affinity matrix, is a significant regulator in modulating antibody recognition and binding to the substituted matrix.

4. Discussion

Epitope affinity chromatography has proved to be highly effective in the purification of anti-MUC1 antibodies from diverse biological feedstocks. The procedure has been applied to the purification of IgG and IgM antibodies from hybridoma supernatants and ascitic fluids [5]. Further, recombinant single-chain Fv fragments [8] and Fv heterodimers [9] as well as a humanised or reshaped anti-MUC1 mucin antibody [10], have all been isolated using this separative method. In addition, peptide affinity chromatography using T-cell receptor mimics has been used to purify autoantibodies from the serum of patients infected with human immunodeficiency virus (HIV) [11]. However, no systematic studies have yet been performed to explore critical features

Table 3
Analysis of antibody binding to peptides conjugated to Sepharose 4B in different orientations

Peptide Sepharose conjugate	Concentration (μmol peptide/ml gel)			
	10	1	0.1	0
N-terminally coupled A-G	1.42 (0.05)	0.80 (0.19)	0.63 (0.15)	0.15 (0.01)
C-terminally coupled A-G	1.08 (0.15)	0.99 (0.03)	0.23 (0.05)	0.22 (0.01)
N-terminally coupled KSK	0.70 (0.13)	0.62 (0.05)	0.17 (0.01)	0.16 (0.04)
C-terminally coupled KSK	0.26 (0.04)	0.24 (0.01)	0.26 (0.07)	0.22 (0.01)

Analysis was carried out using an enzyme-linked bead assay. Standard deviations calculated from triplicate samples are given in parentheses.

of the design and construction of the peptide ligand matrix which regulate its performance in immunoaffinity chromatography. The present investigation was initiated to resolve these unknowns.

The affinity of synthetic peptides for an antibody has been measured in solution using fluorescence quenching tests [6]. This study has shown that these measurements are related to the concentration of the chaotrope required to desorb the antibody from the peptide immobilised to an agarose-beaded matrix. Thus the affinity of an antibody–affinity matrix interaction may be regulated by the choice of peptide epitope and simple parameters such as length of peptide bearing an epitope sequence may be used to modulate matrix affinity for an antibody. The orientation of the peptide epitope to the matrix may influence the affinity of the resultant matrix for an antibody. Therefore, the mode of epitope immobilisation offers a second option for modulation of matrix affinity for an antibody.

The immobilised mimotope sequence KSKAGV failed to adsorb antibody from hybridoma supernatants. However, the fact that the mimotope substituted agarose-bound antibody in ELISA assays would indicate that such materials are able to retain antibody in tests which model batch separations, although clearly the procedure failed to translate into an efficient chromatographic separation method. Other researchers have demonstrated the use of sequences derived from peptide libraries as ligands in affinity chromatographic purification of ribonuclease A [12], and indeed the approach is now being marketed as a tool for large-scale purification [13]. The poor performance of the KSKAGV mimotope sequence as an affinity ligand may be due to slow reaction kinetics with C595 antibody which does not allow binding to take place during the short residency time of the antibody supernatant on the column. Immobilised library-derived peptide ligands have been shown to have affinity constants around an order of magnitude lower than those of peptides free in solution [12]. It is suggested that further research is required to increase the affinity of the mimotope for the C595 antibody in order to enhance its potential as a ligand for use in an immunoaffinity matrix for antibody purification.

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References

[1] S. Gendler, A.P. Spicer, E.-N. Lalani, T. Duhig, N. Peat, J. Burchell, L. Pemberton, M. Boshell, J. Taylor-Papadimitriou, Am. Rev. Respir. Dis. 144 (1991) S42.
[2] M.R. Price, S.J.B. Tendler, Breast 2 (1993) 3.
[3] J. Burchell, J. Taylor-Papadimitriou, M. Boshell, S. Gendler, T. Duhig, Int. J. Cancer 44 (1989) 691.
[4] M.R. Price, F. Hudecz, C. O'Sullivan, R.W. Baldwin, P.M. Edwards, S.J.B. Tendler, Mol. Immunol. 27 (1990) 795.
[5] M.R. Price, M. Sekowski, S.J.B. Tendler, J. Immunol. Methods 139 (1991) 83.
[6] D. Spencer, M.R. Price, S.J.B. Tendler, C.I. DeMatteis, T. Stadie, F.-G. Hanisch, Cancer Lett. 100 (1996) 11.
[7] P. Laing, P. Tighe, E. Kwiatkowski, J. Milligan, M.R. Price, H. Sewell, J. Clin. Pathol. Mol. Pathol. 48 (1995) M136.
[8] G. Denton, M.S. Sekowski, D.I.R. Spencer, O.D.M. Hughs, A. Murray, H. Denley, S.J.B. Tendler, M.R. Price, Br. J. Cancer (1997) in press.
[9] G.M. Davies, S. Bosze, F. Hudecz, M.R. Price, S.J.B. Tendler, Cancer Lett. 82 (1994) 179.
[10] M.E. Verhoeyen, J. Saunders, M.R. Price, J.D. Marugg, S. Briggs, E.L. Broderick, S. Eida, A.T.A. Mooren, R.A. Badley, Immunology 78 (1993) 364.
[11] D.F. Lake, S.F. Schluter, E. Wang, R.M. Bernstein, A.B. Edmundson, J.J. Marchalonis, Proc. Natl. Acad. Sci. USA 91 (1994) 10849.
[12] P.Y. Huang, R.G. Carbonell, Biotech. Bioeng. 47 (1995) 288.
[13] J. Maclennan, Biotechniques 13 (1995) 1180.