ELSEVIER

Journal of Chromatography B, 766 (2001) 13–26

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Purification of anti-MUC1 antibodies by peptide mimotope affinity chromatography using peptides derived from a polyvalent phage display library

Richard G. Smith[a,*], Sotiris Missailidis[b], Michael R. Price[a,1]

[a]*University of Nottingham, Cancer Research Laboratories, School of Pharmaceutical Sciences, Nottingham NG7 2RD, UK*
[b]*The Department of Chemistry, The Open University, Walton Hall, Milton Keynes MK7 6AA, UK*

Received 8 June 2001; accepted 29 August 2001

Abstract

A polyvalent, lytic phage display system (T7Select415-1b) displaying a random peptide library has been investigated for its ability to discover novel mimotopes reactive with the therapeutic monoclonal antibody C595. Sequence analysis of enriched phage lead to the identification of a predominant sequence **RNREAPRGKICS**, and two other consensus sequences **RXXP** and **RXP**. The novel synthetic peptide **RNREAPRGKICS** was linked to beaded agarose and the performance as a mimotope affinity chromatography matrix evaluated. Antibody purified using the novel matrix was found to be of higher specific reactivity than antibody purified using the conventional epitope matrix (peptide **APDTRPAPG**). The **RNREAPRGKICS** peptide binding to C595 demonstrated a higher equilibrium association constant ($K_A = 0.75 \times 10^6$) than the epitope peptide ($K_A = 0.16 \times 10^6$). Circular dichroism showed that the novel peptide had a more highly ordered structure at 4°C and room temperature, than the epitope peptide.

Keywords: Affinity purification; Polyvalent phage display library; Anti-MUC1 antibodies; Peptides

1. Introduction

Advances in genetic engineering, cloning technology and the resulting increase in the number of protein based therapeutics [1–4], mean that the need to have techniques that enable us to isolate proteins of high activity, homogeneity and purity is ever increasing. Affinity purification is the method of choice [5]. The affinity purification of proteins employs the specific molecular interaction between an immobilised ligand and the protein of interest to effect an efficient separation of the protein of interest from the biological milieu. Peptide ligands offer many advantages over other ligands. They consist of only a few amino acids; and so in case of leakage into the product are less likely to cause problems with toxicity and immune response compared for instance to Dye ligands which are generally toxic and non-specific [6]. Peptide ligands are small and stable making them easily sanitizable compared to other more complex ligands (such as antibodies and protein A) and can be manufactured aseptically in large quantities. Peptide–protein interactions are generally moderate and this can result in mild elution

*Corresponding author. Fax: +44-1959-892506.
E-mail address: rgsmith@dstl.gov.uk (R.G. Smith).
[1]Deceased.

1570-0232/02/$ – see front matter
PII: S0378-4347(01)00422-4

conditions for the separation. Synthetic peptide ligands can also be made using (D) amino acid residues, compared to the naturally occurring (L) amino acids, reducing their susceptibility to proteolytic degradation in the biological milieu [6]. The key disadvantage of affinity purification of proteins is that (unlike for example protein A ligands) each ligand has to be individually discovered for a particular protein. Phage display offers a quick, non-costly way of exploring sequence space to look for these novel binding moieties. There are many examples of the use of peptide libraries (including phage display) to search for novel binding moieties [7–9], but few of these take advantage of polyvalent systems. In this study, the use of a polyvalent phage display system as a tool for finding novel binding moieties was investigated. The T7Select Phage Display System [10] utilizing the T7Select415-1b vector (Novagen, Inc., Madison, WI, USA), was used to display approximately 415 identical copies of each peptide on the capsid head. The peptides were encoded for by a degenerate oligonucleotide insert, consisting of a randomized library of 9-residue peptides flanked by cysteine residues (to impose a structural constraint on the C-terminus of the displayed peptides [11]. These cysteine residues were also flanked with serine residues, such that the N-terminus of the peptide was linked to the capsid head through a serine residue (see Fig. 1). The nine randomized residues were incorporated into the insert using a sequential synthesis strategy for each codon of NNK; where N is an equimolar mixture of the bases A, G, C and T; and K is an equimolar mixture of the bases G and T only. (Using this method the number of stop codons that could be coded for falls from three to one at each codon [12].

MUC1 mucin is aberrantly expressed on the surface of cancerous cells, and has been used as a successful tumour marker in bladder cancer [13,14]. Radiolabelled C595 has also been studied for the treatment of superficial bladder cancer [1,15]. Synthetic peptides corresponding to the immunodominant region of the protein core of MUC1 mucin have been used as effective affinity ligands for the purification of several anti-MUC1 monoclonal antibodies and antibody fragments [5,16,17]. Tethered synthetic peptides have also been used to examine the fine specificity of C595 binding to MUC1 related peptides, in order to identify peptides with higher affinity for the antibody [18]. The aim of the present study is to investigate the use of polyvalent phage display as a tool for identifying effective affinity ligands. The secondary structure of the peptides in solution was studied to identify reasons for the difference in chromatographic performance of the phage-derived peptide compared to the native epitope peptide.

2. Materials and methods

2.1. Materials

The T7Select415-1b vector was kindly donated by Dr. P. Tighe (Division of molecular and clinical immunology, The University of Nottingham, UK). The anti-estrone beta-D-glucuronide antibody was obtained from Unilever research (Sharnbrook, UK). The synthetic peptides **APDTRPAPG**, **APDTRPAPGC**, **APDTREAPG**, and **RNREAPRGKICS** were prepared on an Applied Biosystems 431A Peptide Synthesiser. Peptide purity was analyzed by mass spectroscopy using a Bio-Ion 20 plasma desorption mass spectrometer and reversed-phase high-performance liquid chromatography (HPLC) on a Brownlee C_{18} cartridge (30×2 mm) (Anachem, UK). Peptides were first equilibrated in buffer A (0.1% trifluoroacetic acid (HPLC grade, Fisher Scientific, UK) in dH_2O), and then eluted in a gradient elution from 0 to 50% buffer B (70% acetonitrile (HPLC grade, Fisher Scientific, UK) in buffer A). Peptides were supplied and analyzed by the Biopolymer Synthesis and Analysis Unit (Queens Medical Centre, Nottingham, UK). Phosphate buffered saline (PBS) tablets were from Oxoid (Basingstoke, UK). Bovine serum albumin (BSA) was obtained from Sigma (Poole, UK). Beaded agarose (CNBr activated) was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Rabbit anti-mouse IgG HRP conjugate was obtained from Dako Ltd. (Ely, UK). ExtrAvidin™–Horseradish Peroxidase conjugate was obtained from Sigma (Poole, UK). Shrimp alkaline phosphatase was obtained from USB Corporation (Cleveland, Ohio, USA). Exonuclease I was obtained from New England Biolabs Inc. (Hitchen, UK). Taq DNA polymerase was obtained from Advanced Biotechnologies (Epsom, Surrey, UK). All other chemicals were of a reagent grade or higher.

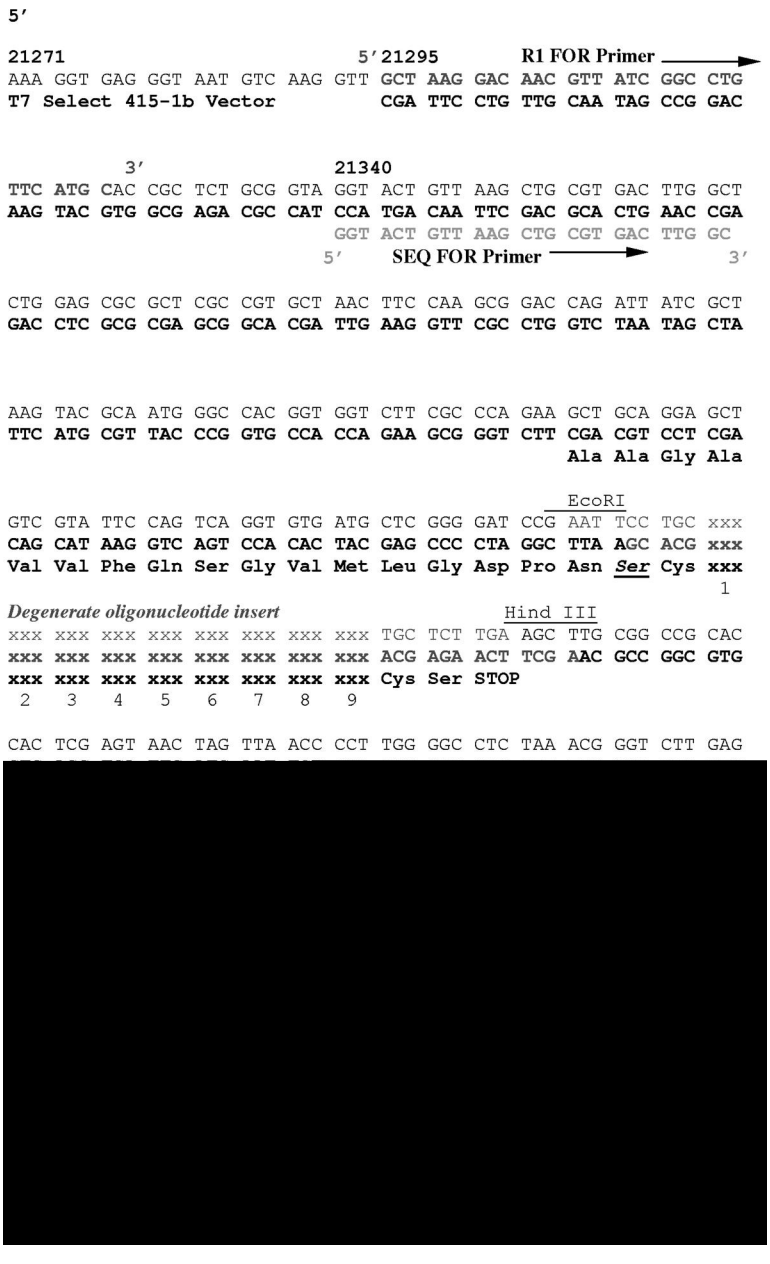

Fig. 1. T7Select415-1b cloning region, showing annealing regions of the two PCR primers R1FOR and R2REV, and the sequencing primer SEQFOR. Also shown is the insertion site of the degenerate oligonucleotide insert; which was inserted following an EcoRI/HindIII digest of the vector.

2.2. Monoclonal antibody production

The murine anti-MUC1 monoclonal antibody C595 (IgG3 subclass) was originally prepared by conventional hybridoma technology using spleen cells from a BALB/c mouse immunized against purified urinary MUC1 mucin [19]. Hybridoma supernatant was clarified by ultracentrifugation (40 000 *g*, 30 min) and ultrafiltration through a 0.2 μm filter (Sartorius, Gottingen, Germany). The

antibody was stored at 4°C with sodium azide (0.05% w/v) as a preservative.

2.3. Identification of binding sequences using phage display

2.3.1. Biopanning of phage library

The T7Select415-1b vector containing a degenerate oligonucleotide insert was used to screen antibody for binding peptides. The synthesized single-stranded oligonucleotide insert was converted into a clonable double-stranded DNA insert containing EcoRI and HindIII restriction sites by annealing a second short oligonucleotide and using Taq polymerase to fill in the second strand [20]. Restriction digestion of the resulting dsDNA allowed directional insertion into the cloning region of the T7Select415-1b vector. A total of 1.4×10^{8} independent clones were obtained from this initial ligation reaction. Propagation of the library gave a library consisting of 4.36×10^{10} pfu/ml, equivalent to 311 copies of each clone per millilitre. Measurements of phage titre were carried out using a plaque assay. *E. coli* (Strain BL21) were grown to mid-log phase, and aliquots inoculated with phage preparations serially diluted in LB media. These were then mixed with top agar and poured onto LB plates. The plates were then incubated at 37°C for 3 h. The number of phage plaques was then counted and the initial phage titre determined.

The phage library was screened against antibody passively adsorbed onto Maxisorb immunotubes (75×12 mm; NUNC). The library was first screened against an ''irrelevant'' antibody that would not be expected to bind specifically to MUC1 epitopes. Anti-Estrone beta-D-glucuronide antibody [21] was used for this purpose. The antibody, 2.5 ml @ 10 μg/ml in TBS buffer (50 m*M* Tris HCl pH 7.5, 150 m*M* NaCl) was passively adsorbed onto Maxisorp immunotubes by incubation at room temperature for 4 h. Unbound target protein was removed by washing the tubes three times with TBS buffer. The tubes were then incubated for 1 h at room temperature with 2.5 ml of blocking solution (1% w/v casein in TBS). The tube was then washed five times with deionised water and once with TBS buffer.

The phage library (2.5 ml phage lysate) was incubated in the immunotube (with anti Estrone beta-D-glucuronide antibody adsorbed) for 30 min at room temperature. The phage lysate in the tube was then gently agitated and transferred to an immunotube with C595 antibody adsorbed (10 μg/ml) and incubated at room temperature for 30 min. The phage lysate was then expelled from the immunotube and the tube was washed five times with TBS buffer containing 0.05% Tween20. A 1-min incubation was used for each wash. Excess buffer was removed by blotting on a paper towel. Bound phage were eluted using a 20 min incubation with sodium dodecyl sulphate (SDS, 1% (w/v), 2.5 ml), and transferred to a sterile glass tube. An aliquot of the eluted phage solution (250 μl) was then added to 50 ml of *E. coli* (BL21) grown to mid-log growth phase (OD_{600} ~0.5 AU) and incubated in a 250 ml Erlenmeyer flask with shaking at 37°C until lysis of the culture was observed. The phage lysate was then purified from the cell debris by centrifugation at $8000\times g$ for 10 min. The supernatant was transferred to a sterile glass tube. The phage titer of the eluted phage was determined using a plaque assay as described above. The amplified phage were used in the next round of biopanning. The phage titer of the eluted phage (prior to amplification) was monitored after each round of biopanning using the plaque assay.

2.3.2. PCR analysis of phage clones

Two primers (**R1FOR**$^{5'}$GCT AAG GAC AAC GTT ATC GGC CTG TTC ATG C$^{3'}$ and **R2REV**$^{5'}$CGT TGA TAC CGG AGG TTC ACC GAT AGA CGC C$^{3'}$) were designed to allow PCR amplification of the region surrounding the degenerate oligonucleotide insert. A third primer was designed to anneal inside this PCR amplification product (**SEQFOR**$^{5'}$GGT ACT GTT AAG CTG CGT GAC TTG GC$^{3'}$) to enable direct sequencing of the PCR product (see Fig. 1). A scrape from an individual plaque was dispersed in 100 μl of 10 m*M* EDTA, pH 8.0. The tube was briefly vortexed and heated at 65°C for 10 min. The mixture was cooled to room temperature and clarified by centrifugation at 14 000 g for 3 min. PCRs were performed in 50 μl aliquots using 0.1 μ*M* of each primer per reaction on an OmniGene Thermal Cycler Controller (Hybaid, Teddington, UK); and consisted first of a ''hot-

start'' by heating the tube for 5 min prior to the introduction of the Taq DNA polymerase (Advanced Biotechnologies, Epsom, UK); 35 cycles of 94°C for 50 s, 50°C for 60 s, 72°C for 60 s; and a final extension at 72°C for 6 min. Agarose gel electrophoresis was used to analyse the PCR products to ascertain that only a single PCR product of the expected size was present before sequencing.

2.3.3. Direct sequencing of PCR products

Exonuclease I (Sigma Biochemicals, Gillingham, Dorset, UK) (5U) and shrimp alkaline phosphatase (Sigma Biochemicals, Gillingham, Dorset, UK) (1U) were added to 5 μl of PCR product, heated at 37°C for 15 min and then at 80°C for 15 min. Sequencing reactions were conducted (ABI PRISM Big-Dye Terminator Cycle Sequencing reaction) in 10 μl aliquots using the SEQFOR primer (see Fig. 1). Using a program consisting of 96°C for 90 s; 25 cycles of 96°C for 30 s, 50°C for 15 s, 60°C for 240 s; and finally 28°C for 1 min. Prior to sequencing an ethanol precipitation step was conducted to remove excess dye terminator in the reaction mix [22].

2.4. Assays

2.4.1. Phage capture ELISA

C595 antibody was adsorbed onto the wells of a microtitre plate (96 well NUNC) by incubation at 37°C for 4 h. After blocking non-specific binding sites by incubation with phosphate buffered saline (PBS) pH 7.3 containing 0.1% casein, phage preparations were titrated by serial dilution with 50 μl added per well. After incubation for 2 h, the plates were washed four times with PBS (pH 7.3) containing 0.05% (w/v) Tween20 (PBS/Tween).

Antibody C595 was biotinylated using the succinimide ester of biotin as outlined in Ref. [22]. Biotinylated C595 antibody was added at 50 μl per well. After 2 h incubation, the plates were washed four times with PBS/Tween and HRP-conjugated ExtrAvidin™ (Sigma Biochemicals) was added at 50 μl per well. Plates were incubated for 1 h and then washed four times with PBS/Tween. A solution of 0.033% (w/v) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Merck) in 0.1 *M* citrate phosphate buffer pH 4.0, with 33% (v/v) hydrogen peroxide added at 0.3 μl/ml (ABTS substrate) was added to the wells at 50 μl per well. Colour development was measured using a Milenia Kinetic Analyser (Diagnostic Products Corporation, Llanberis, UK).

2.4.2. Immunoreactivity ELISA

2.4.2.1. Conjugation of peptides to BSA. BSA (10 mg) was dissolved in conjugation buffer (3.0 ml) in a clean glass vial. The mixture was rolled for 1 h. Peptide was also dissolved in conjugation buffer (~10.0 μmol/ml). Peptide solution (10.0 μmol) and glutaraldehyde (10 μl) were added to the BSA solution. The sealed vial was then agitated on a roller for 4 h at room temperature. The conjugate was then dialysed against sodium chloride (0.9% w/v) for 48 h at 4°C.

2.4.2.2. ELISA. A standard ELISA procedure was carried out to assess the reactivity of the purified antibody preparations, by binding to BSA–**APDTRPAPG** peptide conjugate dried to the wells of microtiter plates (96 well, NUNC). The BSA–conjugate was diluted in PBS buffer (pH 7.4), 50 μl was added to each well and left to dry down overnight. The plate was then washed four times with PBS/Tween and non-specific binding sites were blocked by incubating with PBS (pH 7.4) containing 0.1% casein for 1 h. Antibody preparations were added at 50 μl/well and incubated for 1 h. The plates were washed four times with PBS/Tween and HRP-conjugated rabbit anti-mouse immunoglobulin (DAKO, High Wycombe, UK) was diluted 1/1000 in PBS (pH 7.4) and added at 50 μl/well. Plates were incubated for 1 h, washed four times with PBS/Tween and ABTS substrate was added at 50 μl/well. Colour development was measured using a Milenia Kinetic Analyser (Diagnostic Products Corporation, Llanberis, UK).

2.4.3. Fluorescence quenching (FQ)

The antibody solution (2.5 ml in a 3 ml quartz cuvette, 1 cm path length) in PBS (pH 7.4) and previously filtered through a Minisart NML 0.2 μm pore membrane (Sartorius, Gottingen, Germany), was excited at 290 nm and the emitted light was measured at 345 nm using a Perkin-Elmer L-3000

luminescence spectrometer (Perkin-Elmer, Beconsfield, UK). The excitation slit width was set at 5 nm and the emission slit width was set at 10 nm. The test peptides were titrated into the antibody from a concentrated stock solution (~200 μM) until maximum fluorescence quenching of the antibody was observed. Dilution effects of titrating a peptide solution into the Ab solution were ascertained by titrating PBS solution into 2.5 ml of antibody solution as a control and were used for correction of the actual peptide titration data. The values of F_o (observed intensity of fluorescence in the absence of peptides) and F (observed intensity of fluorescence in the presence of varying amounts of peptides) were noted during all the titrations. In order to obtain equilibrium constants from the results for a single mode of binding, the following formula [23,24] was used:

$$A_{calc}(P0) = \left[\left[\frac{(E1-E2)\cdot\left[(1+K\cdot D+K\cdot P0-\sqrt{(1+K\cdot D+K\cdot P0)^2-4\cdot K\cdot K\cdot D\cdot P0}\right]}{2\cdot K} \right] + E2\cdot D \right] \cdot \frac{1}{D}$$

where $E1$ is the minimum value of F_o/F, $E2$ is the value of F_o/F corresponding to the fluorescence of the Ab on its own, K is the binding constant and D is the molar concentration of the Ab in solution. Using the observed F_o/F experimental data and the peptide molar concentrations added to the Ab solution, the $A_{calc}(P0)$ values were calculated by a method of computation involving an iterative procedure designed to satisfy the above equation.

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

Affinity-purified C595 antibody was electrophoresed on a PhastSystem separation and control unit (Amersham Pharmacia Biotech, Uppsala, Sweden) in conjunction with PhastGel precast gels (homogeneous acrylamide, 12.5% w/v) using SDS buffer strips (Amersham Pharmacia Biotech, Uppsala, Sweden). Silver staining was performed using the PhastGel silver-staining kit on the PhastSystem development unit.

2.5. Peptide affinity chromatography

2.5.1. Preparation of immunoaffinity matrices

Synthetic peptides were linked to beaded agarose (Sepharose 4B, Amersham Pharmacia Biotech, Uppsala, Sweden) via their N-termini using CNBr-activated matrix. Peptides were coupled at a ratio of 1 μmol peptide per millilitre gel using conjugation procedures as recommended by the manufacturer.

2.5.2. FPLC

Chromatography columns (10 mm internal diameter, Amersham Pharmacia Biotech, Uppsala, Sweden) were packed with 2 ml of each affinity matrix (column length = 2 cm). Chromatography was performed using an automated FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden). Data acquisition and analysis was performed using FPLCdirector Software (Amersham Pharmacia Biotech, Uppsala, Sweden). Columns were first equilibrated with 10 column volumes (20 ml) of PBS at a flow-rate of 1.0 ml/min. Loading of antibody solutions in PBS (100 ml) was conducted at room temperature at a flow-rate of 1.0 ml/min. Loading of clarified hybridoma supernatant (100 ml) was carried out at 4°C, with the supernatant circulating around the column for 48 h. The columns were then washed with 10 column volumes (20 ml) of PBSA at 1.0 ml/min, or until the trace from the UV monitor returned to zero. All elutions were conducted at room temperature. The gradient elution consisted of a linear gradient of NaSCN (Buffer B), in PBS (Buffer A) from 0 to 3 *M* NaSCN 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. Eluted fractions were desalted using PD10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.6. Circular dichroism (CD)

2.6.1. Structural analysis of peptides

The structural content of the four peptides, **RNREAPRGKICS**, **APDTREAPG**, **APDTRPAPG** and **APDTRPAPGC** was evaluated using circular dichroism (CD). The peptides were used at concentrations of 4 μM and CD spectra for the peptides

were recorded in the far UV region (190–250 nm) both at room temperature and at 4°C.

The monoclonal antibody C595 and peptides **RNREAPRGKICS**, **APDTREAPG**, **APDTRPAPG** and **APDTRPAPGC** were used at a concentration of 4 μ*M* and at a ratio of 1:1 of peptide to antibody binding site in 50 m*M* phosphate buffer for the room temperature measurements and for the temperature denaturation of the antibody. The thermal denaturation of the antibody was measured in the absence of the peptides as a control and in the presence of individual peptides at a 1:1 ratio to assess the possible stabilisation effect of the peptide binding to the antibody binding-pocket. For this experiment, pre-made mixture of peptide–antibody complex was used to measure the CD spectrum of the antibody at 25, 35, 45, 55, 65, 75 and 85°C. The temperature was peltier controlled and was increasing at a rate of 1°C/min. Although full spectra were obtained at the above mentioned temperatures, the $CD_{220\ nm}$ signal was recorded at every degree throughout the process. CD spectra were recorded under N_2 using a Jasco J720 Spectrometers (Jasco Inc., Tokyo, Japan). All measurements were taken using a 0.05 cm optical path length quartz cell. The band width was 2 nm and the slit width controlled automatically, the sensitivity was set at 20 mdegrees with a time constant of 4 (arbitrary units), a step resolution of 0.2 nm and scan speed of 10 nm/s. One scan was sufficient for accumulating spectra with high signal to low noise ratios.

2.6.2. Analysis of CD data versus temperature and pH

The CD signal from the C595 antibody in the presence and absence of antigenic peptides was recorded at 220 nm. The signal was analysed versus temperature to determine the melting temperature (T_m) of the antibody molecule and the possible ΔT_m (were $\Delta T_m = T_m$ of bound Ab $- T_m$ of free Ab) obtained as the difference between free and peptide-bound antibody stability. The Van't Hoff equation was used to fit the experimental results [23] and to determine the T_m of the antibody. The antibody was shown to have two melting transitions as previously determined [24], in which case two T_m values were determined (T_{m_1}, T_{m_2}). Similar, two-melting transitions were obtained from the peptide-bound antibody.

3. Results and discussion

3.1. Biopanning of phage library against immobilised C595 mAb, and analysis of phage clones

Biopanning (affinity selection) is a procedure for enrichment for molecules that bind to a given target protein. The target protein is non-specifically absorbed onto a solid support. Phage then bind to the target, whilst non-specifically binding phage are washed off. Bound phage are then recovered by an elution step, and then quantified and propagated for repeated rounds of selection and enrichment.

The titre of the eluted bacteriophage from each round of biopanning, as measured by plaque assay, increased until the fourth round of biopanning and leveled off in the fifth round (see Fig. 2). The phage capture ELISA of the eluted phage (results not shown) showed an increase in signal in the first three rounds, with little difference in the fourth round. Sequence analysis of individual, randomly chosen bacteriophage clones from each round of biopanning showed that a consensus sequence of **RNREAPRGKICS** appeared in the third round. Two other groups of consensus sequences were also

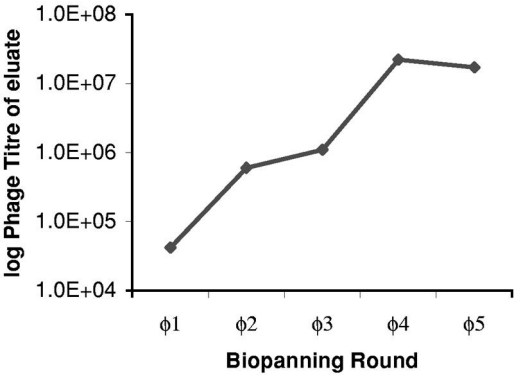

Fig. 2. Results of plaque assays on the eluted phage from each round of biopanning showing enrichment of phage library has occurred during five rounds of biopanning.

seen in all rounds; those containing an **RXXP** motif and those containing an **RXP** motif. The native epitope **RPAP** motif [25] was also present in round four (see Table 1). The emergence of the sequence **RNREAPRGKICS** was coincidental with the increased signal seen in the phage capture ELISA. The phage capture ELISA for randomly chosen clones taken from the fourth round of biopanning demonstrated enrichment, with some clones displaying pronounced activity in the assay (see Fig. 3); compared to a nearly level distribution of activity (majority of V_{max} <100 mOD/min) in the clones sampled from round one of biopanning (data not shown).

Analysis of the mucin core variable number of tandem repeat sequence (VNTR) **PDTRPAPGSTAPPAHGVTSA** [26] revealed that monoclonal antibody C595 binds to the simple linear peptide motif **RPAP** [19]. Replacement-net (RNET) analysis of this binding motif has shown that the 1st arginine and the 4th proline residues of the motif are crucial to the binding of monoclonal antibody C595. The 2nd and 3rd residues can be replaced with any of the 20 naturally occurring amino acids, and still bind to the antibody [27]. The phage derived sequence **RNREAPRGKICS** contains such an **RXXP** motif (**REAP**). In order to assess the importance of the other residues in the phage-derived peptide

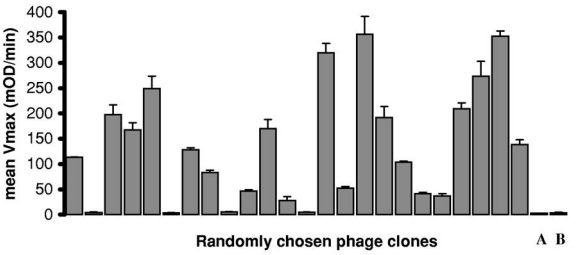

Fig. 3. Results of phage capture ELISA of randomly chosen phage clones from the fourth round of biopanning. A is phage lacking degenerate oligonucleotide insert (see Fig. 1) and B is the background with no phage present.

RNREAPRGKICS, three other peptides were synthesized: (i) The mucin core peptide sequence **APDTRPAPG**, (ii) the mucin core peptide sequence with proline substituted by glutamic acid **APDTREAPG** and (iii) the mucin core peptide with a terminal cysteine residue as a control for dimerisation **APDTRPAPGC**.

3.2. Peptide affinity chromatography

The synthetic peptides **APDTRPAPG**, **APDTREAPG**, **APDTRPAPGC** and **RNREAPRGKICS** were conjugated to Sepharose 4B at a concentration of 1 μmol/ml gel, to produce immunoaffinity matrices. The performances of the matrices in the

Table 1
Comparison of sequence analysis results from individual phage clones from each biopanning round and binding ability of phage clones from round 4 in the phage capture ELISA (✓ represents a +ve result, where the mean V_{max} observed in the capture ELISA >40 mOD/min; hyphens (–) represent residues where codons could not be determined from the sequencing data (see Discussion)

Biopanning round				
1	2	3	4	ELISA
CE**RGP**GKSRSCS	CS**RVAP**NRK	**RNREAPRGKICS**	**RNREAPRGKICS**	✓
CGNRVSKAPK–S	CVKRTASGSGCS	**RNREAPRGKICS**	**RNREAPRGKICS**	✓
GRKVKCS	CSMRASGGPKCS	**RNREAPRGKICS**	**RNREAPRGKICS**	✓
R–AA–MEKP–S	CTVPVRPQQKCS	**RNREAPRGKICS**	**RNREAPRGKICS**	✓
RRAAVRMEKPCS	CPATTHLG	**RNREAPRGKICS**	**–NREAPRGKICS**	✓
	CHLAGT	**RNREAPRGKICS**		
	CEE	**RNREAPRGKICS**	C**RPAP**SAKVACS	✓
	C	**RNREAPRGKIC–**	FE**RIAP**KGGNCS	✓
			CDSE**RTAP**KCS	✓
		RRPPMTTASCS		
		FE**RIAP**KGGNCS	RQAG**RKP**VNNCS	✓
			CS**RGP**AGRTVCS	✓
		CS**RGP**AGRTVCS	CSD**RMP**CEPSCS	✓
		C**RAP**AGSKKMCS	**RRP**SR	✓

concentration of monoclonal antibody C595 from PBS were compared under standard conditions of sample loading (0.3 ml/min), column size (2.0 ml swollen gel volume), ligand density (1 μmol/ml gel) and elution (3 *M* NaSCN gradient).

Representative chromatograms illustrating the elution of monoclonal antibody C595 from the **APDTRPAPG** and **RNREAPRGKICS** matrices are shown in Fig. 4(a). Chromatograms demonstrating the elution of monoclonal antibody C595 from the affinity matrices containing peptides **APDTREAPG** and **APDTRPAPGC** are omitted for clarity. The elution profiles from the **APDTREAPG** and **APDTRPAPGC** matrices were identical in shape and size to the elution profile of the **APDTRPAPG** matrix (Fig. 4(a)). The maximum of the peaks in the elution of monoclonal antibody C595 from the **APDTREAPG** and **APDTRPAPG** matrices were directly in line on the gradient elution. The maximum of the peak from the elution of monoclonal antibody C595 from the **APDTRPAPGC** matrix was further up the gradient than any of the other peak maxima, this has been previously attributed to dimerisation of the peptide leading to an increased

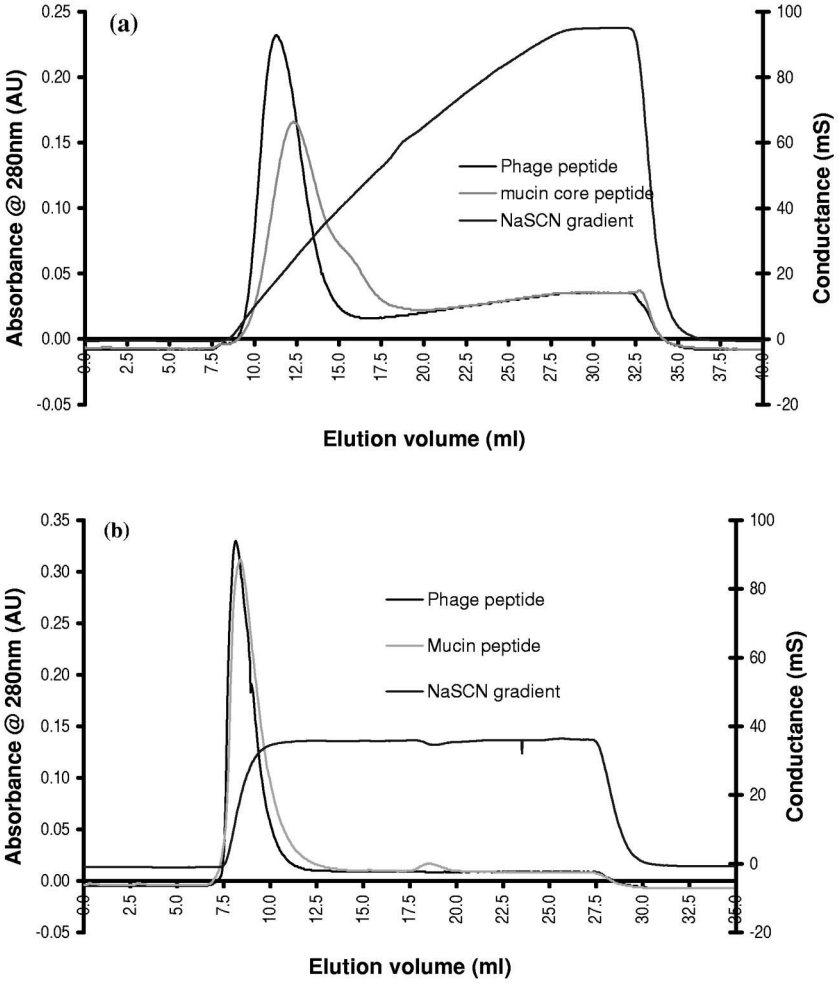

Fig. 4. (a) Gradient elution of the phage and mucin core peptide–Sepharose affinity matrices following column loading with 100 ml C595 antibody at 15 μg/ml in PBS. (b) Step elution of the phage and mucin core peptide–Sepharose affinity matrices following column loading with 100 ml C595 hybridoma supernatant.

affinity (A. Murray, University of Nottingham – personal communication). The chromatogram (Fig. 4(a)) for the gradient elution of antibody from the **RNREAPRGKICS** affinity matrix has a sharp symmetrical peak. The maximum of this peak occurs lower down in the gradient elution than antibody eluted from any of the other matrices.

Monoclonal antibody C595 was then purified from 100 ml of clarified hybridoma supernatant (200 ml sample divided into two), using the **APDTRPAPG** and **RNREAPRGKICS** affinity matrices. A step elution was carried out, using a step to 1 *M* NaSCN to elute antibody from the respective columns. Chromatograms for the elution are shown in Fig. 4(b). There is very little difference between the two chromatograms of the step elution profile.

3.3. Analysis of affinity purified antibody preparations

ELISA was used to analyse the immunoreactivity of the purified antibody samples. Mass of protein in each sample was determined spectrophotometrically at 280 nm by applying the Beer–Lambert Law and assuming a molar extinction coefficient $\varepsilon = 214\,500$ M^{-1}/cm for IgG [18]. Concentration of antibody from the clarified hybridoma supernatant (feedstock) was demonstrated for both the phage derived synthetic peptide (**RNREAPRGKICS**) and mucin core synthetic peptide (**APDTRPAPG**) affinity matrices. The activity per unit volume, of the antibody recovered from the **RNREAPRGKICS** peptide matrix was approximately 10 times the activity per unit volume of antibody in the feedstock. The activity per unit volume of the antibody recovered from the **APDTRPAPG** matrix was approximately six times the activity per unit volume of the antibody in the feedstock (see Fig. 5). The activity per unit volume of the antibody in the column pass samples of both the synthetic phage and synthetic mucin core peptide affinity matrices was approximately 30% of the activity per unit volume of the antibody present in the feedstock. SDS–PAGE analysis of the purified antibody preparations showed they were equivalent, showing heavy and light chains of the C595 antibody with no evidence of contamination. Spectrophotometric determination of protein levels in the purified antibody samples revealed that the antibody from the

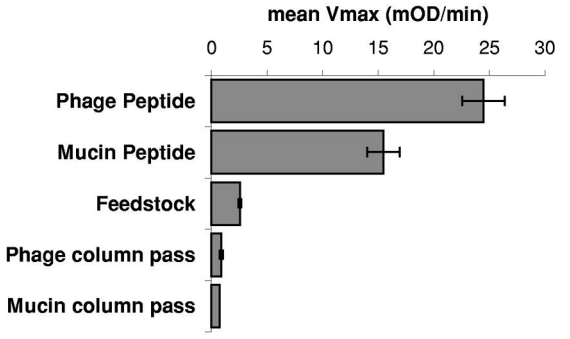

Fig. 5. Results of ELISA to evaluate the immunoreactivity of the eluted and then de-salted antibody from the phage and mucin core peptide–Sepharose affinity matrices. Comparison with hybridoma feedstock and column pass.

APDTRPAPG affinity matrix contained a total of 1.01 mg of protein, whereas the sample purified from the **RNREAPRGKICS** matrix contained a total 0.78 mg of protein.

3.4. Fluorescence quenching

The interaction of the synthetic peptides **APDTRPAPG**, **APDTRPAPGC**, **APDTREAPG** and **RNREAPRGKICS** with C595 antibody were measured and expressed as an equilibrium association constant using the antibody fluorescence quenching technique. By plotting the changes in the fluorescence of the antibody at its emission maxima versus the antigen concentration at each titration step, experimental binding curves were obtained (see Fig. 6). The experimental fluorimetric data were fitted to a mathematical equation describing the binding [23] (see Materials and methods), thus allowing the calculation of the association constants K_A for the above reactions (summarised in Table 2). The four peptides under study exhibited significant affinity towards the C595 antibody, as expected from the results of the chromatographic and ELISA studies. The phage display peptide (**RNREAPRGKICS**) was shown to have the highest affinity for the antibody ($K_A = 0.75 \times 10^6$ M^{-1}) followed by the substituted peptide (**APDTREAPG**) with an association constant ($K_A = 0.4 \times 10^6$ M^{-1}), the natural antigenic peptide with the cysteine residue at the C-terminus (**APDTRPAPGC**) ($K_A =$

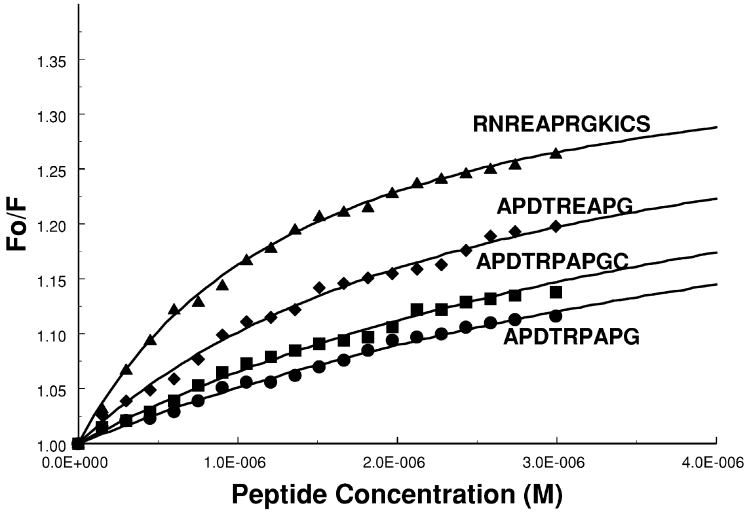

Fig. 6. Binding of the APDTRPAPG, APDTRPAPGC, APDTREAPG and RNREAPRGKICS peptides to C595 antibody. Changes in the natural fluorescence of the antibody were plotted versus peptide concentration to calculate the equilibrium association constants for formation of the antibody–antigen complexes. An increase of the equilibrium association constant was observed with increasing structural content of the peptides.

0.21×10^{6} M^{-1}) and the natural antigenic peptide (**APDTRPAPG**) ($K_A=0.16\times10^{6}$ M^{-1}).

3.5. Circular dichroism analysis of peptide conformation

Synthetic peptides **APDTRPAPG**, **APDTRPAPGC**, **APDTREAPG** and **RNREAPRGKICS** were analysed by CD for evaluation of their structural content. The examination of these peptides by CD at room temperature (Fig. 7) indicated that the peptides were not highly ordered, but there was a distinct trend of a preferred left-handed extended polyproline II helix (PII conformation) [28]. All peptides under study presented positive bands at 217–225 nm and negative bands at 195–200 nm. The presence and intensity of the positive band at 217–225 nm and the negative band at 195–200 nm confirms the population of a PII conformation [29,30]. Furthermore, the intensity of the positive band is directly proportional to the stability of the PII helix. It has previously been demonstrated for the synthetic peptide that represents the natural antigen of the C595 mAb, i.e. the **APDTRPAPG** peptide, that it exists in a PII helical conformation, which is stabilised in cryogenic studies and with increased glycosylation [24]. However, the two novel peptides **APDTREAPG** and **APDTRPAPGC**, as well as the phage display derived peptide **RNREAPRGKICS** have not previously been studied. The later peptide presents the strongest signal at room temperature, indicating its increased structural content and its ability to adopt a PII conformation more readily than the other peptides in the absence of its receptor molecule. Furthermore, the peptides have been evaluated for change in their structural content between room temperature (20°C) and 4°C. All three peptides have demonstrated an increase in their PII helical content with

Table 2
Results of fluorescence quenching experiments; the values shown in the table are calculated from the experimental data (in triplicate) by a method of computation involving an iterative procedure designed to satisfy the equation given in Section 2.4.3; the precision of these values = $\pm0.01\times10^{6}$ M^{-1}

Peptide	Equilibrium association constant K_A (M^{-1})
APDTRPAPG	0.16×10^{6}
APDTRPAPGC	0.21×10^{6}
APDTREAPG	0.40×10^{6}
RNREAPRGKICS	0.75×10^{6}

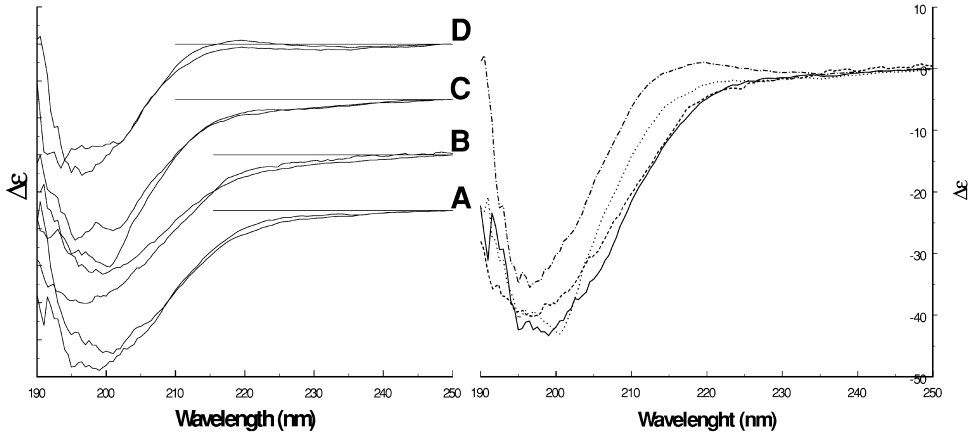

Fig. 7. (i) Structure/temperature relationship at 20 and 4°C examined using CD on: the MUC1 core peptide **APDTRPAPG(A)**, the MUC1 core related peptides **APDTRPAPGC(B)**, and **APDTREAPG(C)**, and the phage derived peptide **RNREAPRGKICS(D)**. Lines for $\Delta\varepsilon=0$ have been inserted at each case to assist assessment of the peptide signal. (ii) Comparison of the four peptides at 4°C.

the decrease in the temperature. This observation for the **APDTRPAPG** peptide has already led to an increased purification of the C595 MAb on affinity purification matrices at 4°C as opposed to room temperature. However, the change in the phage display derived peptide was far more pronounced. Its already increased structural content at room temperature was far more easily stabilised and the peptide presented a well structured form at 4°C (Fig. 7).

3.6. Circular dichroism and temperature denaturation analysis

The C595 antibody (IgG3 class) has previously been studied by CD and its denaturation with temperature and pH variation has been reported [24]. In this experiment, we re-evaluated the melting profile of the C595 monoclonal antibody, and evaluated the possible stabilising effect of the binding of synthetic peptides containing the natural epitope or phage display derived sequences to the antibody's binding pocket.

When the antibody was subjected to thermal denaturation the clear melting profile previously reported [24] emerged, with melting transitions at 61 and 68°C. Addition of peptides **APDTRPAPG**, **APDTRPAPGC**, **APDTREAPG** and **RNREAPRGKICS** did not have any stabilising effect on the antibody melting profile. This is not unexpected as these short peptides are occupying necessarily one only binding site. The binding of a single peptide per binding pocket would not seem to have any effect on the disulphide bonds stabilising the antibody's molecular structure.

4. Discussion

The fully conserved nature of all the residues in the predominant consensus sequence **RNREAPRGKICS** was striking in comparison to the variable nature of the residues flanking the **RXXP** motif and the newly discovered **RXP** motif. The absence of an N-terminal cysteine residue in the **RNREAPRGKICS** peptide is the result of a mutation of one of the nucleotides in the Cys codon. All of the **RNREAPRGKICS** peptides were coded for by the same nucleotide sequence. The Cys–Arg mutation was the result of a T–C transition (data not shown). Sequences displaying a mutation in the N-terminal cysteine residue were observed in rounds one, three and four. No mutations were observed in the second round of biopanning. The majority (7/10)

of these mutations were transitions (T–C or T–G); with the remainder C–G transversions.

It is not possible to say whether these mutations resulted in the phage displaying them acquiring a growth advantage. If a growth advantage had been acquired by a particular phage clone, one would expect to see an increase in the number of clones displaying this particular peptide sequence in the fourth round – this was not the case. The number of clones sampled displaying this peptide decreased in the fourth round. The coincidental emergence of the phage capture ELISA peak in the third round, and the frequency of occurrence of the **RNREAPRGKICS** sequence in the third round of biopanning compared to the frequency in the fourth round are indicative of affinity selection rather than selection for growth. The leveling off of phage titre in the fourth round suggests that affinity enrichment has reached a maximum in the third round of biopanning.

The fact that the nucleotide sequences were identical for all of the **RNREAPRGKICS** clone does not prove that this particular clone had a growth advantage. Measurements of initial phage titre indicated that 311 copies of each clone would be displayed per millilitre of lysate. A 2.5 ml volume of phage lysate was used in the biopanning process, which equates to 777 copies of each phage clone. It is conceivable that a single clone that had acquired a point mutation could be propagated in the amplification stages of the propagation process and be selected for enhanced affinity.

The CD data for the four peptides comes in agreement with the fluorescence quenching data as well as with the chromatography and the ELISA data. It this sense, a correlation between structural content of the peptides and their binding affinity to the C595 monoclonal antibody is profound. The peptides showed a structural content in the order **APDTRPAPG** < **APDTRPAPGC** < **APDTREAPG** <**RNREAPRGKICS**. In the other studies, the **RNREAPRGKICS** peptide had a distinct behaviour and increased activity. The peptide-matrix presented a narrow peak in the affinity chromatography studies, eluting lowest of the three peptides in the sodium thiocyanate gradient. Purified antibody from the affinity matrix presented an increased activity in the ELISA test. The peptide demonstrated the highest association constant in the fluorescence quenching experiments ($K_A = 0.75 \times 10^6$ M^{-1}, as opposed to $K_A = 0.4 \times 10^6$ M^{-1} for the **APDTREAPG** peptide, $K_A = 0.21 \times 10^6$ M^{-1} for the **APDTRPAPGC** peptide and $K_A = 0.16 \times 10^6$ M^{-1} for the natural antigenic peptide **APDTRPAPG**).

The results of the gradient elution of antibody from the **RNREAPRGKICS** peptide affinity matrix are in contrast to several authors who have suggested that tolerance to thiocyanate elution is proportional to the strength of the antigen–antibody interaction. Examples of the disruption of antigen–antibody interactions using thiocyanate elution in ELISA based techniques to determine relative affinities of interaction have previously been published [31–33]. This relationship has also previously been used as an empirical measure of the affinity of the interaction between an immobilised antigen or ligand and the antibody by eluting a loaded chromatography column with thiocyanate [17,18,6]. Other chaotropic solvents have been employed to fractionate polyclonal antibodies [34]. Here too, the authors note that ‘‘*. . . tighter binding pAbs would require increasingly more denaturing solvents to elute them*.’’ However Ref. [6] comment on the relatively low concentration of NaSCN required to elute antibody from an affinity column, even though good separations were achieved.

What the results of these experiments have shown, is that the relationship between equilibrium affinity constant (K_A) and the susceptibility of the antigen–antibody complex to dissociation by the chaotropic thiocyanate ion is not valid for the **RNREAPRGKICS** peptide.

In conclusion, the results of the fluorescence quenching and CD experiments, together with the performance of the **RNREAPRGKICS** peptide as an effective affinity matrix, and in association with previously published data demonstrate that the ability of an antigenic peptide to readily adopt a favorable conformation in the absence of its receptor molecule reduces the energy barrier required to be overcome by an unstructured peptide to adopt such conformation upon binding to this receptor, thus accounting for higher affinities and increased chromatographic performance compared to the other peptides studied. Further studies of the kinetics of the interaction between the phage derived peptide and the antibody,

using SPR or resonant mirror technology, could establish the values of the rate constants (k_{on} and k_{off}) and give greater insight into the peptide–antibody interaction.

Here too we demonstrate that in this case, a phage display library can be used to directly obtain an improved peptide mimotope molecule which functions as an effective affinity ligand without the need for further manipulation.

Acknowledgements

This research was funded from a Biotechnology and Biological Sciences Research Council (BBSRC) studentship to R.G.S.

Dr. P. Tighe (Division of molecular and clinical immunology, The University of Nottingham, UK) for providing the T7Select415-1b phage display vector and *E. coli* bacteria.

Mr. Ian Davies for his assistance in the biopanning experiments.

References

[1] O.D.M. Hughes, M.C. Bishop, A.C. Perkins, M.L. Wastie, G. Denton, M.R. Price, M. Frier, H. Denley, R. Rutherford, P.A. Schbiger, J. Clin. Oncol. 18 (2) (2000) 363.
[2] P.J. Hudson, Curr. Opin. Immunol. 11 (5) (1999) 548.
[3] S.J. DeNardo, L.A. Kroger, G.L. DeNardo, Curr. Opin. Immunol. 11 (1999) 563.
[4] H. de Haard, P. Henderikx, H.R. Hoogenboom, Adv. Drug Deliv. Rev. 31 (1998) 5.
[5] M.R. Price, M. Sekowski, S.J.B. Tendler, J. Immunol. Methods 139 (1991) 83.
[6] A. Murray, R.G. Smith, K. Brady, S. Williams, R.A. Badley, M.R. Price, Anal. Biochem., 296 (2001) 9.
[7] P. Bailon, G.K. Ehrlich, W.J. Fung, W. Berthold (Eds.), Methods in Molecular Biology – Affinity Chromatography Methods and Protocols, Humana Press Inc, New Jersey, 2000, p. 209.
[8] G.A. Baumbach, D.J. Hammond, BioPharm., May issue (1992) 24.
[9] D.J. Hammond, Chromatographia 46 (1998) 475.
[10] A. Rosenberg, K. Griffin, F.W. Studier, M. McCormick, J. Berg, R. Novy, R. Mierendorf, Innovations 6 (1996) 1.
[11] S.J. McConnell, M.L. Kendall, T.M. Reilly, R.H. Hoess, Gene 151 (1994) 115.
[12] B.K. Kay, J. Winter, J. McCafferty (Eds.), Phage Display of Peptides and Proteins. A Laboratory Manual, Academic Press, London, 1996, p. 21.
[13] M.S. Simms, O.D.M. Hughes, A. Murray, M.R. Price, M.C. Bishop, Br. J. Urol. 84 (1999) 350.
[14] O.D.M. Hughes, A.C. Perkins, M. Frier, M.L. Wastie, G. Denton, M.R. Price, H. Denly, M.C. Bishop, BJU Int. 87 (2001) 39.
[15] A. Murray, M.S. Simms, D.P. Scholfield, R.M. Vincent, G. Denton, M.C. Bishop, M. R Price, A.C. Perkins, J. Nucl. Med. 42 (2001) 726.
[16] G. Denton, M. Sekowski, D.I.R. Spencer, A. Murray, O.D.M. Hughes, M.R. Price, Br. J. Cancer 76 (5) (1997) 614.
[17] A. Murray, M. Sekowski, D. Spencer, G. Denton, M.R. Price, J. Chromatogr. A 782 (1997) 49.
[18] A. Murray, D.I.R. Spencer, S. Missailidis, G. Denton, M.R. Price, J. Peptide Res. 52 (1998) 375.
[19] M.R. Price, J.A. Pugh, F. Hudecz, W. Griffiths, E. Jacobs, I.M. Symonds, A.J. Clarke, W.C. Chan, R.W. Baldwin, Br. J. Cancer 61 (1990) 681.
[20] A.B. Sparks, J.E. Rider, N.G. Hoffman, D.M. Fowlkes, L.A. Quilliam, B.K. Kay, Proc. Natl. Acad. Sci. USA 93 (1996) 1540.
[21] R.A. Badley, M.J. Berry, P. Porter, T. Wattam, US Patent US005989926A (1999) 1.
[22] J.E. Sambrook, F. Fritsch, T. Maniatis (Eds.), Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Press, New York, 1989.
[23] S. Missailidis, W.V. Cannon, A. Drake, X.Y. Wang, M. Buck, Mol. Microbiol. 24 (1997) 653.
[24] D.I.R. Spencer, S. Missailidis, G. Denton, A. Murray, C.I. De Matteis, M.S. Searle, S.J. B Tendler, M.R. Price, Biospectroscopy 5 (2) (1999) 79.
[25] M.R. Price, F. Hudecz, C. O'Sullivan, R.W. Baldwin, P.M. Edwards, S.J.B. Tendler, Mol. Immunol. 27 (1990) 795.
[26] S. Gendler, J. Taylor-Papadimitriou, T. Duhig, J. Rothbard, J. Burchell, J. Biol. Chem. 263 (1988) 12820.
[27] S. Briggs, M.R. Price, S.J.B. Tendler, Eur. J. Cancer 29A (2) (1993) 230.
[28] A.P. Campbell, B.D. Sykes, Ann. Rev. Biophys. Biomol. Struct. 22 (1993) 99.
[29] L. Dalcol, M. Pons, M.D. Ludevid, E. Giralt, J. Org. Chem. 61 (1996) 6775.
[30] G. Siligardi, A.F. Drake, Biopolymers (Peptide Sci.) 37 (1995) 281.
[31] G.R.M. Pullen, G. Fitzgerald, C.S. Hosking, J. Immunol. Methods 86 (1986) 83.
[32] M.U. Ferreira, A.M. Katzin, J. Immunol. Methods 187 (1995) 297.
[33] R.A.C. Macdonald, S. Hosking, C.L. Jones, J. Immunol. Methods 106 (1988) 191.
[34] L.O. Narhi, D.J. Caughey, T.P. Horan, Y. Kita, D. Chang, T. Arakawa, Anal. Biochem. 253 (1997) 246.