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Immunoaffinity chromatography in biorecovery: an application of recombinant DNA technology to generic adsorption processes

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ABSTRACT

The constant region of human kappa light chain (C_k) was linked to *Escherichia coli* β-galactosidase, using standard molecular cloning techniques. The binding of C_k–β-galactosidase fusions to a number of different murine monoclonal antibodies, specific for C_k, was improved by the insertion of spacers between C_k and β-galactosidase: a cleavable linker was then introduced. Over-expressed C_k–β-galactosidase fusion protein was purified using monoclonal antibodies immobilised on Sepharose 4B. Elution conditions were found that maintained β-galactosidase activity so purified enzyme could be released on breaking the cleavable linker. A number of practical problems associated with maintaining stable fusion proteins and immunoaffinity column performance were identified.

INTRODUCTION

Affinity chromatography relies on the formation of specific, reversible interactions between immobilised ligands and their complementary bioproducts [1–3]. Although affinity chromatography has considerable potential for the purification of a wide range of protein products, a different affinity system needs to be developed for each bioproduct. This problem can be eliminated by the application of recombinant DNA technology: by engineering a common characteristic to different bioproducts, purification can be achieved using a common matrix [4–6]. Immunoaffinity chromatography is a process to which this generic concept can be applied [7–12]. An antigenic determinant (epitope) may be employed as a molecular "hook" by which fusion proteins can be immunoaffinity-purified using antibody specific for the epitope. A cleavable

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linker can be introduced between the hook and the bioproduct allowing, on treatment, release of product.

In this paper we describe the use of the constant region of human kappa light chain (C_k) as the molecular hook and exploitation of murine monoclonal antibodies (Mabs), directed against C_k, to purify fusion proteins. Using recombinant DNA techniques, we have constructed a tripartite fusion protein comprised of C_k, a designed cleavable linker (Cl.lnk) and β-galactosidase. This fusion was expressed in a stable form in *Escherichia coli*. A panel of Mabs specific for C_k have been screened to identify candidates suitable for the development of an immunoaffinity matrix. We describe the development of an immunoaffinity purification of the fusion protein and a number of process problems.

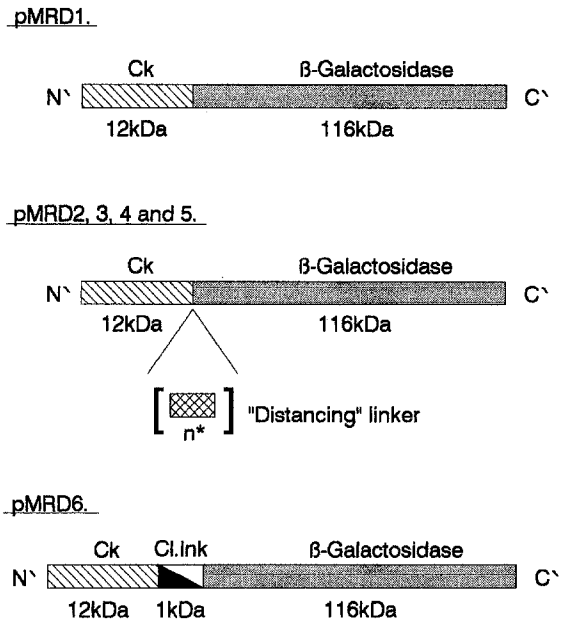

Fig. 1. Diagrammatic representation of fusion proteins. Each plasmid carried fusion protein with C_k at the N-terminus (N') and β-galactosidase at the C-terminus (C'). pMRD2-5 carry multiples of a 12-base pair insert distancing linker. pMRD6 carries a nine-amino acid cleavable linker (Cl.lnk) introducing two hydroxylamine-labile asparagine–glycine sites between the C_k and β-galactosidase domains.

EXPERIMENTAL

Plasmid manipulations

The *E. coli Δlac* host strain M182 was used throughout this work [13]. All plasmids were derivatives of pBR322 [14] and were constructed using standard recombinant DNA methodology [15]. The fusion proteins constructed are illustrated in Fig. 1. The C_k domain had previously been cloned downstream of the constitutive *E. coli gal*P2 promoter (M. Downham, unpublished experiments). The *gal*–C_k fragment was then cloned into pAA204 [16] to give pMRD1, which expresses C_k–β-galactosidase fusion protein from the *gal*P2 promoter (Fig. 1). Multiples of a 12-base pair oligodeoxynucleotide were then ligated between C_k and *lac*Z in pMRD1. The resulting plasmids (pMRD2, -3, -4 and -5), expressed C_k distanced from β-galactosidase domains. A 27-base pair oligodeoxynucleotide linker encoding two asparagine–glycine liaisons was designed and also cloned into pMRD1 to give pMRD6 (Fig. 1). Recombinant plasmids were transformed into M182.

Preparation and analysis of crude extracts

Overnight cultures (50 ml or 2 l) of strains were prepared in Lennox broth containing 80 μg/ml ampicillin. Bacteria were harvested by centrifugation and resuspended in 9 ml of sterile phosphate-buffered saline pH 7.3 (PBS: 0.14 *M* NaCl, 1.47 m*M* KH_2PO_4, 7.96 m*M* Na_2HPO_4 and 2.68 m*M* KCl in distilled water), and 1 ml of 10 m*M* dithiothreitol. After French pressing and recentrifuging, the supernatants were decanted and 0.1 ml of 9 μ*M* phenylmethylsulfonyl fluoride was added. Total protein was determined by the Bradford method [17] using a bovine serum albumin standard. β-Galactosidase activity was determined by standard methods [18] and referenced to the activity of purified *E. coli* β-galactosidase (Sigma). One activity unit was defined as μmol hydrolysed per min per 0.1 ml of cell extract.

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing conditions [19]. Typically, 10 μg total protein were loaded per lane and the gels were calibrated with molecular mass markers (Sigma).

Enzyme-linked immunosorbent assay (ELISA)

A capture ELISA was performed to screen a panel of murine anti C_k Mabs for candidates to establish immunoaffinity matrices. Microtitre plate wells were sensitized with each candidate Mab (approximately 5 μg/ml) in coupling buffer pH 9.6 (CB: 15 m*M* Na_2CO_3 and 34.9 m*M* $NaHCO_3$ in distilled water). Wells were washed with PBS–Tween (0.05%, v/v) and 0.1-ml samples containing C_k–β-galactosidase fusions (100 μg total protein) in PBS–Tween were added. Following incubation (37°C, 2 h), the plates were rewashed and developed using the β-galactosidase assay substrate, ONPG. Colour was read in an ELISA plate recorder (Anthos) at $A_{405\ nm}$.

In order to evaluate potential column elution conditions, a Bence Jones kappa (BJK) protein [20] was purified and used to sensitize the wells of a microtitre plate in CB at 5 μg/ml. The plate was washed in PBS–Tween and selected purified Mabs were incubated in PBS–Tween at 10 μg/ml. Different-potential eluting agents were then incubated in the wells for 20 min at room temperature, and the plates were rewashed with PBS–Tween. Mabs remaining on the well walls were quantified using polyclonal anti-mouse IgG (Fc) peroxidase conjugate (The Binding Site, Birmingham, UK). The plates were developed using *o*-phenoldiamine and the absorbance read at 492 nm.

Adsorbent preparation, performance assay and use

Approximately 20 mg of selected murine Mabs were purified from ascitic fluids by precipitation with ammonium sulphate and DE-52 cellulose anion-exchange chromatography (Whatman). Five different matrices were prepared by reacting 15–20 mg of selected purified Mabs with 5-ml lots of CNBr-activated Sepharose 4B (Pharmacia). The remaining active groups were blocked with 1 *M* ethanolamine (pH 9). Mab binding to the matrix was estimated from difference analysis of supernatant $A_{280\ nm}$ readings before and after reaction. Matrices were washed in 25 ml of PBS–NaN_3–KSCN (3 *M*) prior to use. Immunoaffinity adsorbents were stored at 4°C in PBS–NaN_3.

To estimate the immunoadsorbent performance characteristics, 1 ml of different purified BJK concentrations (0.2, 0.15, 0.1 and 0.05 mg/ml PBS–NaN_3) were mixed with 0.1-ml batches of settled immunoaffinity matrix. The mixture was agitated for 16 h at 4°C. The amount of uncomplexed BJK was estimated from difference analysis of supernatant $A_{280\ nm}$ readings before and after incubation, and the quantity of BJK bound by the Mabs was calculated. The Mab–BJK complex dissociation constant (K_d) and maximum adsorption capacity (q_m) were estimated using the methods of Chase [21], as modified by Desai and Lyddiatt [22]. Affinity matrices were also tested as fixed beds (2 ml) with saturating concentrations of BJK, recycling for 16 h at 4°C. The column saturation capacity (C) and productivity (P) were calculated from spectrophotometric analysis at 280 nm.

Cell extracts (20 ml) from 2-l cultures of M182 pMRD6 were diluted to 400 ml in PBS–NaN_3 and recycled (30 ml/h) through a 4-ml bed of each immunoaffinity matrix for 16 h at 4°C. The bed was washed in PBS–NaN_3 and then in the same buffer supplemented with 1 *M* NaCl. Specifically bound protein was eluted from the matrices in PBS–NaN_3–KSCN (3 *M*) or 50% ethylene glycol–0.1 *M* glycine–NaOH (pH 10.5). The elution flow-rate was 15 ml/h, and recovered fractions were immediately desalted on 12 ml of G-25 Sephadex (Pharmacia). Total protein and β-galactosidase activity were determined (as already explained) of the recovered fractions.

Hydroxylamine treatment and analysis

Aliquots of pMRD6 fusion protein, which had been eluted with 50% ethylene glycol–0.1 *M* glycine–NaOH (pH 10.5), were dialysed extensively against distilled water and freeze-dried. One vial of the freeze-dried protein was treated with 2 *M* hydroxylamine in 0.2 *M* Tris-base (pH 9) for 4 h at 45°C [23]. A second control vial was similarly treated in buffer without hydroxylamine. Samples were desalted, freeze-dried and resuspended in PBS–NaN_3. Resuspended material was transferred to nitrocellulose from an SDS–PAGE (15%) gel by Western blotting. To detect released C_k (12 kDa) from the fusion (130 kDa), blots

were screened with polyclonal anti-human kappa chain peroxidase conjugate (The Binding Site).

RESULTS AND DISCUSSION

Production and analysis of fusion proteins

The starting point of this work was plasmid pAA204 which carries the first four amino acids of *gal*E fused to *lac*Z, the *gal*E–*lac*Z fusion being under control of the constitutive *gal*P2 promoter [16]. This plasmid was exploited to clone a fragment carrying a *gal*–C_k fusion. The *gal*–C_k fragment was fused to *lac*Z to give the *gal*–C_k–*lac*Z fusion in pMRD1. Cells carrying pMRD1 express the C_k–β-galactosidase fusion protein, illustrated in Fig. 1, which is recognised with low affinity by the murine Mabs specific for C_k (see later).

During the construction of pMRD1 a unique *Bam*HI site was introduced at the C_k–*lac*Z junction. To attempt to increase Mab affinity for the fusion protein, multiples of a 12-base pair polylinker were inserted at the *Bam*HI site on pMRD1 to give pMRD2, -3, -4 and -5 (Fig. 1). ELISAs were used to estimate recognition of the fusions by selected Mabs demonstrated as specific for C_k by Lowe *et al.* [24]. The insertion of linker sequences giving pMRD2-5 results in an increased affinity for the C_k fusion proteins by the Mabs (Fig. 2). In most cases the best recognition was observed with pMRD4 which carries the 24-base pair insert, resulting in the introduction of eight amino acids between C_k and β-galactosidase.

From the results in Fig. 2 we deduced that the introduction of spacing between C_k–*lac*Z promotes recognition by the Mabs. The best recognition required insertion of 8–12 amino acids (compare pMRD4 and pMRD5). A cleavable nine-amino acid linker was, therefore, designed carrying two hydroxylamine target sites. The 27-base pair oligodeoxynucleotide was inserted between C_k and *lac*Z of pMRD1 to give pMRD6 (Fig. 1). Fig. 3 shows the expression of C_k–β-galactosidase fusion proteins in extracts from cells carrying pMRD6, pMRD1 or the starting plasmid pAA204. The gel shows the increased molecular mass of the fusions compared to the galE–β-galactosidase fusion expressed from pAA204 (130 kDa compared to 116 kDa). Insertion of the cleavable linker in pMRD6 does not alter the expression of the fusion. Interestingly, β-galactosidase expression in cell extracts carrying pMRD1-6 (16 activity units per ml) was reduced compared to extracts from cells containing pAA204 (254 activity units per ml). Western blot analysis demonstrated that the fusion proteins were stable in the *E. coli* host. Further, since the β-galactosidase fusions were active we deduced

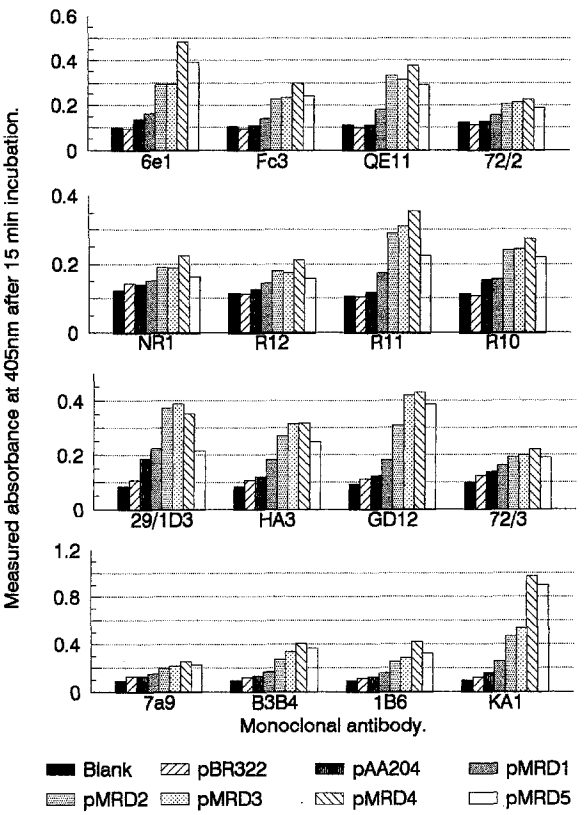

Fig. 2. Screening of fusion proteins with different Mabs. Extracts of cells carrying different plasmids were incubated in ELISA wells sensitized with sixteen different Mabs. The different samples used with each Mab are as follows: Blank: PBS–Tween buffer only; pBR322: crude extracts of M182 expressing no *lac*Z; pAA204: crude M182 extracts expressing *gal*E–*lac*Z fusion; pMRD1: crude M182 extracts expressing *gal*–C_k–*lac*Z fusion; pMRD2 and -3: derivatives of pMRD1 with 12 base pairs between C_k and *lac*Z; pMRD4: derivative of pMRD1 with 24 base pairs between C_k and *lac*Z; pMRD5: derivative of pMRD1 with 36 base pairs between C_k and *lac*Z.

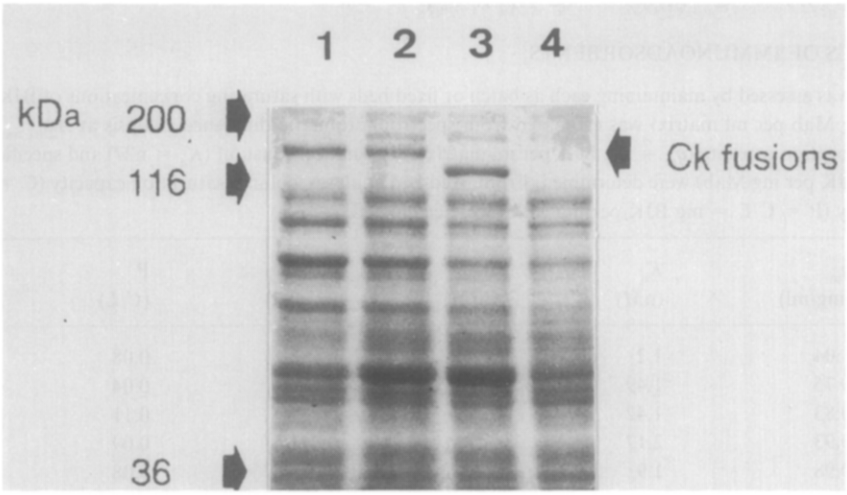

Fig. 3. SDS-PAGE analysis of fusion protein. Crude extracts of M182 carrying the following plasmids were analysed on a 7.5% SDS-PAGE gel. Lane 1: pMRD6 carrying a *gal–C_k–lac*Z fusion with a 27 base pair cleavable linker between C_k and *lac*Z; lane 2: pMRD1 carrying a *gal–C_k–lac*Z fusion; lane 3: pAA204 carrying *gal*E–*lac*Z fusion; lane 4: pBR322 carrying no *lac*Z.

that natural tetrameric aggregation had occurred [25]. Eight out of the sixteen Mabs used in the experiment shown in Fig. 2 were selected and tested in ELISAs with the pMRD6 fusion protein. The results (Fig. 4) show that two Mabs B3B4 and KA1 are particularly active in capturing the C_k–β-galactosidase fusion. From these results the two strongest candidates (B3B4 and KA1) and 6e1, QE11 and R11 Mabs were selected for development of immunoaffinity adsorbents. The latter three Mabs, undistinguished by performance, were selected on grounds of availability.

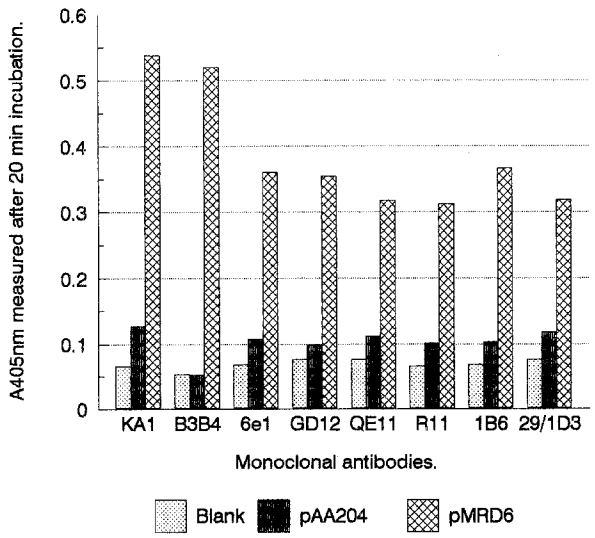

Fig. 4. ELISA of selected Mabs with pMRD6 fusion protein. ELISA wells were sensitized with different Mabs, specific for C_k, and incubated with either PBS–Tween (blank) or crude extracts of M182 carrying pAA204 or pMRD6. Capture of C_k–β-galactosidase protein was revealed using the β-galactosidase assay.

Performance assay of columns

Immunoaffinity adsorbents were prepared with five selected Mabs, and purified BJK was incubated with each matrix to permit the estimation of their performance characteristics. From the data in Table I, the potential maximum capacity of the immunoaffinity matrices can be calculated using the average ligand concentration (2.6 mg Mab per ml matrix) and the stoichiometry of binding. With ideal Mab orientation on the Sepharose matrix, variable regions of 1 mol of Mab (150 kDa) are free to interact with 2 mol of BJK (24 kDa). The average concentration of immobilised Mab is approximately 17 nmol/ml (2.55 mg), and this potentially will bind, and yield, a maximum q_m of 34 nmol/ml of BJK (0.8 mg), if quantitative recovery is assumed. Specific binding activity (SBA), the maximum possible

TABLE I

PERFORMANCE CHARACTERISTCS OF IMMUNOADSORBENTS.

Performance of the immunoadsorbents was assessed by maintaining each as batch or fixed beds with saturating concentrations of BJK protein. Ligand concentration (L = mg Mab per ml matrix) was estimated from spectrophotometric difference analysis at $A_{280\ nm}$. From batch analyses, the maximum adsorption capacity (q_m = mg BJK per ml matrix), dissociation constant (K_d = nM) and specific binding activities (SBA = q_m/L = mg BJK per mg Mab) were determined. From fixed-bed analyses, column saturation capacity (C = mg BJK per ml matrix) and productivity (P = C/L = mg BJK per mg Mab) were calculated.

Matrix	L (mg/ml)	q_m (mg/ml)	K_d (nM)	SBA (qm/l)	C (mg/ml)	P (C/L)
6e1	2.3	1.04	1.2	0.45	0.18	0.08
QE11	2.6	0.75	1.49	0.29	0.11	0.04
KA1	2.6	0.83	1.42	0.32	0.28	0.11
B3B4	2.7	0.93	2.17	0.35	0.25	0.09
R11	2.7	0.98	1.9	0.36	0.22	0.08

mass ratio of BJK/Mab per ml of matrix at saturation, is therefore 0.3.

From batch performance analysis, the q_m and SBA values approximate to the ideal calculated above (0.8 and 0.3, respectively). The low dissociation constants (K_d) demonstrate that the Mabs have strong affinity for C_k. In contrast fixed bed operations provide C and P values reduced by three- to eight-fold from the values calculated using the imunoadsorbent ligand density. Consistently though two Mabs (KA1 and B3B4) bound antigen more strongly both in ELISA and fixed-bed characterizations. However, since C and P values relate to the performance of fixed beds, they are most useful when considering the adsorbent matrices operated in this study.

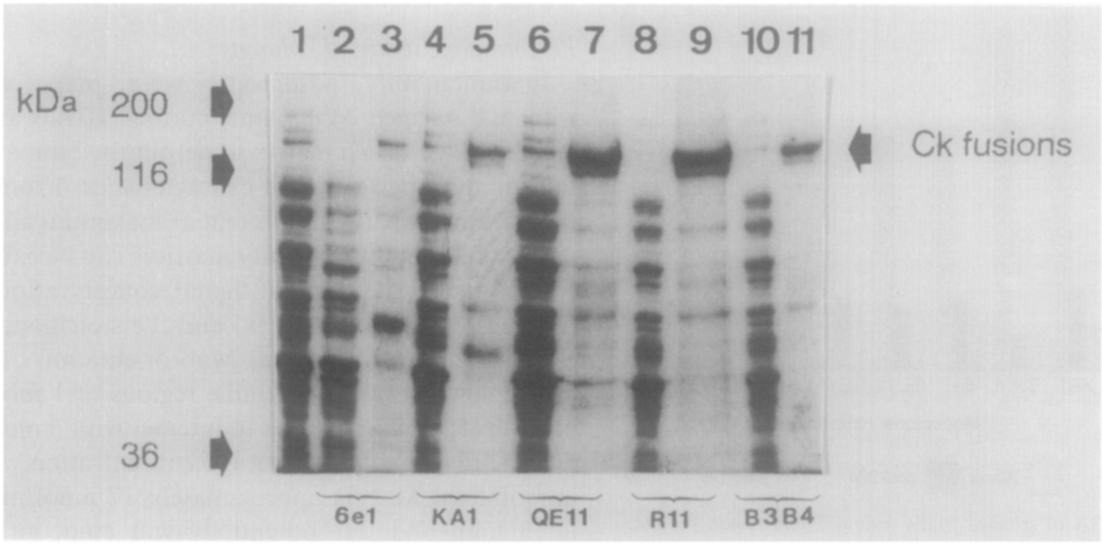

Fig. 5. Immunoaffinity purification of pMRD6 fusion protein. Cell extracts of M182 containing pMRD6 were adsorbed onto, and eluted from, each prepared adsorbent (labelled below the gel). Samples were analysed by SDS-PAGE. Lane 1: starting protein; lanes 2, 4, 6, 8 and 10: unbound protein; lanes 3, 5, 7, 9 and 11: eluted protein.

Use of immunoaffinity columns

The immunoaffinity adsorbents were tested in 4 ml of fixed bed with extracts of M182 containing excess pMRD6 fusion protein, recycling overnight at 4°C. Once unbound and non-specifically bound protein had been displaced, specifically bound protein was eluted with 3 *M* KSCN, desalted and analysed by SDS-PAGE. The results in Fig. 5 show that, surprisingly, the best yields of purified fusion were obtained using QE11 and R11. Unfortunately lower molecular mass contaminants are also present. The total eluted protein from the QE11 fixed bed was 0.11 mg and from the R11 adsorbent, 0.16 mg of protein was prepared. Since the expected capacity of the matrices was approximately 34 nmol of product per ml, the ideal maximum yield of fusion (520 kDa) would have been 17.68 mg/ml. Even accounting for the capacity reduction (three- to eight-fold) for fixed beds observed with pure BJK, these adsorbents apparently under perform by at least 50-fold. Clearly the fusion/Mab stoichiometry is far from ideal and is probably complicated by the molecular mass and shape of the tetrameric native C_k–β-galactosidase fusion protein expressed from pMRD6.

Evaluation of elution conditions

During testing of selected immunoadsorbents we found that the 3 *M* KSCN used for elution of fusion protein led to loss of β-galactosidase activity. We tested sixteen alternative potential elution conditions for effects on β-galactosidase activity and destabilisation of Mab–epitope complexes. Purified β-galactosidase, rather than pMRD6 fusion protein, was used to screen the alternative elution conditions to eliminate complications arising from other proteins present in the crude extract. Hence purified β-galactosidase was incubated in the sixteen different conditions and, following dialysis, the remaining activity was quantified (see bottom of Fig. 6). In order to test the effects of the sixteen conditions on the stability of Mab–epitope complexes, each of the five selected, purified Mabs were immobilized on ELISA plate well walls that had been sensitized with BJK. The five Mab–BJK complexes were then incubated

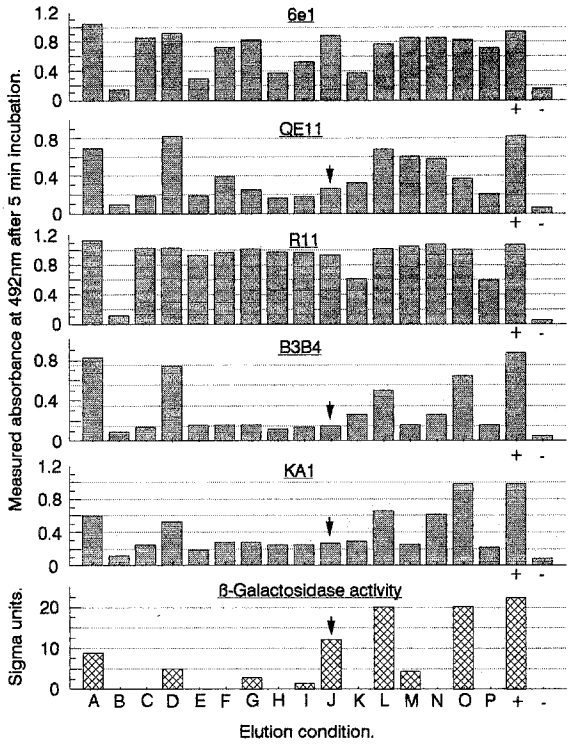

Fig. 6. Elution of different Mabs from immobilised BJK. Sixteen different potential elution conditions (labelled A–P) were tested for their effect on Mab–BJK complexes and on β-galactosidase activity. Potential eluting agents screened are: (A) 0.1 *M* potassium acetate–HCl pH 4; (B) 1 *M* acetic acid–ammonium acetate pH 2.6; (C) 0.1 *M* $NaHCO_3$–1 *M* NaCl pH 10.8; (D) 0.1 *M* sodium acetate–1 *M* KCl pH 4; (E) 0.1 *M* glycine–HCl pH 2.5; (F) 4 *M* $MgCl_2$ pH 7; (G) 0.15 *M* NH_4OH pH 10.8; (H) 1 *M* NH_4OH; (I) 50% ethylene glycol–NaOH pH 10.5; (J) 50% ethylene glycol–0.1 *M* glycine–NaOH pH 10.5; (K) 0.5 *M* acetic acid–ammonium acetate pH 3.5; (L) 0.01 *M* EDTA; (M) 0.1 *M* Na_2CO_3 pH 10.6; (N) 0.1 *M* glycine–HCl pH 3.3 and 0.5 *M* NaCl; (O) 0.02 *M* K_2HPO_4–KH_2PO_4 buffer pH 7 and 0.75 *M* NaCl; (P) 0.25 *M* NH_4OH pH 11.2 and 0.15 *M* NaCl; (+) positive controls, no elution condition was incubated; (−) negative controls.

with each of the sixteen potential elution conditions. The wells were washed and remaining murine Mabs were measured using polyclonal anti-mouse IgG (Fc) peroxidase conjugate (see top of Fig. 6). The 50% ethylene glycol–0.1 *M* glycine–NaOH (pH 10.5) eluting conditon (J), led to retention of 50% β-galactosidase activity and dissociated three Mabs (QE11, B3B4 and KA1) from the BJK immobilised on ELISA well walls. Purification of pMRD6 fusion protein was then

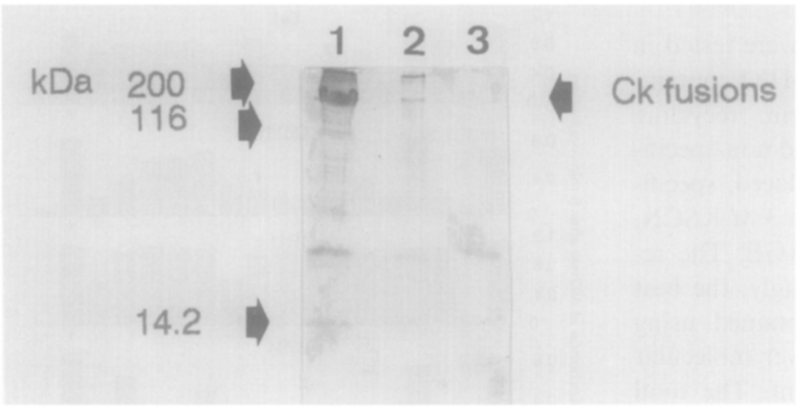

Fig. 7. Western blot of purified pMRD6 fusion protein. pMRD6 fusion protein, eluted with condition J (see Fig. 6) from the QE11 adsorbent, was treated with hydroxylamine and analysed by Western blot using polyclonal anti-C_k peroxidase conjugate. Lane 1: eluted pMRD6 fusion protein; lane 2: pMRD6 fusion protein treated in buffer without hydroxylamine; lane 3: pMRD6 fusion protein treated in buffer with hydroxylamine.

repeated with the QE11 column using condition J for elution.

Use of QE11 and hydroxylamine treatment

The C_k–Cl.lnk–β-galactosidase fusion protein was purified from extracts of cells carrying pMRD6 on 4 ml of the QE11 affinity matrix. Unbound and non-specifically bound protein was washed away. Specifically bound protein was eluted under condition J, no β-galactosidase activity was detected. Fig. 7 shows a Western blot analysis of the eluted products using a polyclonal anti-C_k peroxidase conjugate. Lane 1 shows the presence of a 130 kDa protein corresponding to the fusion protein, but also some lower molecular mass contaminants. From control Western blots (data not shown) the lower-molecular-mass species appear to result from fusion breakdown and cross-reacted *E. coli* proteins. Significant contaminating proteins were co-purified from crude extracts despite washing with 1 *M* salt prior to elution. Recovered product, treated with hydroxylamine, is shown in lane 3 whilst lane 2 has the same eluted product treated with cleavage conditions in the absence of hydroxylamine. Lanes 1 and 2 reveal the C_k fusion protein at 130 kDa. Lane 3 shows the pMRD6 fusion protein is completely cleaved by hydroxylamine as the 130 kDa band disappears and a unique band of C_k appears at 12 kDa, compared to lanes 1 and 2.

CONCLUSIONS

It is possible to establish constructs which facilitate the selective isolation of a wide range of elusive bioproducts using techniques of molecular biology and protein engineering. However, this approach when applied to affinity chromatography is not without its problems. In this example, β-galactosidase expression is reduced when C_k is fused to the N-terminus. The protein fusion can lose stability under the particular conditions of elution and cleavage. The distancing experiment demonstrated that Mab–C_k interactions could be increased by the inclusion of spacers, but spacer length was not the sole determinant: positioning of C_k domains clearly has an additional, significant role. In future work attention should be concerned with optimising column adsorbent capacity and minimising non-specific binding. Upstream processes of ion-exchange adsorption of $(NH_4)_2SO_4$ precipitation prior to immunoadsorption may reduce product contamination, but would inevitably diminish overall yields. The measured recovery of fusion protein from each adsorbent was over 50-fold less than

theoretical values calculated for fixed beds. This reflected the problem of relating matrix performances, established with purified proteins (*i.e.* BJK), to complex crude extracts containing a large tetrameric fusion protein. If this technique is to succeed as a general approach to purification of the widest range of biopharmaceuticals, then detailed optimisation is clearly required. Maintaining the activity of large proteins is clearly problematic even through single-step purification processes.

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REFERENCES

1 P. O'Carra, in R. Epton (Editor), *Chromatography of Synthetic and Biological Polymers, Vol. 2, Hydrophobic, Ion exchange and Affinity Methods*, Ellis Horwood, Chichester, 1978, Ch. 11, p. 131.
2 G. W. Jack, R. Blazek, K. James, J. E. Boyd and L. R. Micklem, *J. Chem. Tech. Biotechnol.*, 39 (1987) 45.
3 E. Sada, *J. Chem. Eng. Jpn.*, 23 (1990) 259.
4 H. M. Sassenfeld, *TIBTECH*, 8 (1990) 88.
5 S. J. Brewer and H. M. Sassenfeld, in E. L. V. Harris and S. Angal (Editors), *Protein Purification Applications: A Practical Approach*, IRL Press, Oxford, 1990, Ch. 6, p. 91.
6 P. A. Hammond, T. Atkinson, R. F. Sherwood and M. D. Scawen, *Pharm. Technol. Int.*, April (1991) 24.
7 H. A. Chase, *Chem. Eng. Sci.*, 39 (1983) 1099.
8 B. Uhlén, B. Nilsson, B. Guss, M. Lindberg, S. Gatenbeck and L. Philipson, *Gene*, 23 (1983) 369.
9 B. Löwenadler, B. Nilsson, L. Abrahmsén, T. Moks, L. Ljungqvist, E. Holmgren, S. Paleus, S. Josephson, L. Philipson and M. Uhlén, *EMBO J.*, 5 (1986) 2393.
10 T. P. Hopp, K. S. Prickett, V. L. Price, R. T. Libby, C. J. March, D. P. Cerretti, D. L. Urdal and P. J. Conlon, *Bio/Technology*, 6 (1988) 1204.
11 B. Hammarberg, P. A. Nygren, E. Holmgren, A. Elmblad, M. Tally, U. Hellman, T. Moks and M. Uhlén, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 4367.
12 B. Nilsson and L. Abrahmsén, *Methods Enzymol.*, 185 (1990) 144.
13 M. J. Casadaban and S. N. Cohen, *J. Mol. Biol.*, 138 (1980) 179.
14 F. Bolivar, R. L. Rodriguez, P. J. Greene, M. C. Betlock, H. L. Heyneker, H. W. Boyer, J. H. Crosa and S. Falkow, *Gene*, 2 (1977) 95.
15 T. Maniatis, E. F. Fritsch and J. Sambrook, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1982.
16 A. H. A. Bingham and S. Busby, *Mol. Microbiol.*, 1 (1987) 117.
17 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
18 J. H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, New York, 1972, p. 352.
19 U. K. Laemmli, *Nature*, 227 (1970) 680.
20 M. Schiffer, R. L. Girling, K. R. Ely and A. B. Edmundson, *Biochemistry*, 12 (1973) 4620.
21 H. A. Chase, *J. Chromatogr.*, 297 (1984) 179.
22 M. A. Desai and A. Lyddiatt, *Bioseparation*, 1 (1990) 43.
23 T. Moks, L. Abrahmsén, E. Holmgren, M. Bilich, A. Olsson, M. Uhlén, G. Pohl, C. Sterky, H. Hultberg, S. Josephson, A. Holmgren, H. Jörnvall and B. Nilsson, *Biochemistry*, 26 (1987) 5239.
24 J. Lowe, D. Hardie, R. Jefferis, N. R. Ling, P. Drysdale, P. Richardson, C. Raykundalia, D. Catty, P. Appleby, R. Drew and I. C. M. Maclennan, *Immunology*, 42 (1981) 649.
25 I. Zabin, *Mol. Cell Biochem.*, 49 (1982) 87.