ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 12 (2): 74-80, 2013
ISSN 1682-296X / DOI: 10.3923/biotech.2013.74.80
© 2013 Asian Network for Scientific Information

Efficient Chromatographic Processes for Elevated Purification of Antibody Fragment (Fab D1.3) from Crude *Escherichia coli* Culture

Reza Jalalirad
School of Chemical Engineering, College of Engineering and Physical Sciences,
The University of Birmingham, Edgbaston, B15 2TT, UK

Abstract: Purification of antibody fragments (Fabs) from *Escherichia coli* crude extracts is more difficult than purification of whole molecule of antibody, owing to lack of the Fc constant regions in Fabs. This study compares the capacity and performance of different chromatographic processes for purification of Fab D1.3 from *Escherichia coli* culture broth samples. Affinity and ion exchange chromatography techniques were utilized as purification techniques. In the first purification experiment, direct loading of the culture broth sample, having high conductivity of ~40 m sec cm^{-1}, on a HiTrap protein G column led to a low Fab D1.3 binding (<1%). Chicken Egg White Lysozyme (HEWL) was coupled with CNBr-sepharose 4 Fast Flow matrix and employed for capturing the Fab from culture broth sample. By doing this chromatography process, 17% of the existing Fab in the sample was captured on the lysozyme column and 48% Fab purity was yielded. Further purification from the lysozyme column's eluates on HiTrap protein G yielded 97% purity. The overall yield of the target Fab in the sequential lysozyme affinity-protein G affinity chromatography route was 17%. The performance of this sequential method was compared to the sequential CEX-protein G affinity chromatography process. Despite lower purity achieved (14%) in the eluates of the column, capture of the Fab from the culture broth sample was very efficient (>97%) by CEX chromatography, in comparison to the HEWL-sepharose column. The overall yield of the target Fab in the sequential CEX-protein G affinity chromatography route was 83% which was much higher than the figure (18-33%) achieved from the sequential lysozyme affinity-protein G affinity chromatography.

Key words: Antibody fragment production, *Escherichia coli*, affinity chromatography, lysozyme-coupled CNBr-sepharose, cation exchange chromatography, purification yield

INTRODUCTION

Antibody fragments (Fabs), lacking the glycosylated Fc constant regions, have the advantage over whole antibodies in applications requiring rapid tissue penetration and rapid clearance from the blood or kidney (Yokota *et al*., 1992). Antibody fragments are useful in diagnostic applications, tumour therapy and tumour imaging as a result of their small size (Holliger and Hudson, 2005; Wu and Senter, 2005; Babaei *et al*., 2002). In contrast to some of the heavily engineered antibody fragments, the Fab has native sequence; subsequently it is less likely for the Fab to be immunogenic when used as therapy. Due to these features, the production of antibody fragments is industrially highly demanded and the antibody fragment pipeline is expanding with three therapeutic Fabs approved by US Food and Drug Administration (two of which, namely Ranibizumab and Certolizumab pegol, are manufactured in the periplasm of *E. coli*) and many in the active clinical pipeline and preclinical research (Reichert, 2012; Nelson, 2010; Nelson and Reichert, 2009).

Affinity Chromatography (AC) which separates proteins based on reversible interaction between a protein and its particular ligand coupled to a chromatography matrix, is successful when an appropriate ligand is accessible for the protein of interest (Hage, 1999; Cuatrecasas and Anfinsen, 1971).

Purification of antibody fragments (Fabs) from the feedstock originated from *E. coli* crude extracts is more challenging in comparison to the purification of whole molecule of antibody due to lack of the Fc constant regions in Fabs. There are few bacterial receptors as ligands for antibodies. These ligands have been employed in packed bed chromatography for purification of Immunoglobulins. Protein A, a 42 kDa cell surface protein of *Staphylococcus aureus*, is a ligand only applicable for the purification of whole molecule of Immunoglobulin

Corresponding Author: Reza Jalalirad, Production and Research Complex, Pasteur Institute of Iran, Tehran-Karaj Highway, 31599 Karaj, Iran Tel: +98 263 6102999 Fax: +98 263 6102900

purification. The primary binding site of protein A on IgG is situated at the junction between the C_H2 and C_H3 domains on the Fc fragment (Lindmark *et al*., 1981) which are absent in antibody fragments (Fabs). Protein G, a ~60 kDa cell surface protein from group C and G *Streptococci*, can also bind to IgG with high affinity.

C_H2 and C_H3 regions located on the Fc fragment are mostly responsible for the interaction with protein A and protein G, while C_H1 domain only interacts weakly with protein G (Derrick and Wigley, 1994; Bjorck and Kronvall, 1984). Therefore, Fabs lacking C_H2 and C_H3 regions have only a weak affinity to protein G.

Ion Exchange Chromatography (IEC) is a technique which is used for purification of charged biomolecules including proteins. IEC is on the basis of reversible interactions between oppositely charged biomolecules and chromatography resins (Staby *et al*., 2000). Negatively and positively charged exchangers have positively and negatively charged counter-ions and are termed cationic and anionic exchangers (CEXs and AEXs), respectively. The differences in charge properties of biological compounds are often substantial and IEC is able to separate species with very minor differences in properties, e.g. two proteins differing by only one charged amino acid.

In this work, purification of the antibody fragment Fab D1.3 which was expressed and produced in *E. coli* cultivation using fed-batch fermentation, was investigated using single and combination of different chromatographic techniques (i.e., affinity and CEX chromatography methods). The efficiency of chromatography techniques for the purification of Fab was examined, the purity of the obtained Fab after each purification process was measured and the best combination of chromatographic procedures for the purification of Fab D1.3 was introduced.

METHODS

Fed-batch fermentation process: *Escherichia coli* W3110 (ATCC 27325) transformed with a plasmid containing the gene for the anti-lysozyme Fab D1.3 (Hodgson *et al*., 2007) was propagated overnight on nutrient agar supplemented with 15 mg L^{-1} tetracycline. Starting cultures were prepared by inoculating 100 mL aliquots of Luria Bertani (LB) broth containing 15 mg L^{-1} tetracycline with a fresh single colony of *E. coli* and shaking at 37°C and 200 rpm for 13 h.

Fermentation was conducted using a 10 L Electrolab fermentor (Tewkesbury, UK) and the complex medium reported previously (Jalalirad, 2013). After 11 h induction with 0.1 mM IPTG, the culture broth was separated from biomass by centrifugation (9390 g for 600 sec) and the supernatant containing Fab D1.3 was utilized in subsequent purification processes, as mentioned below.

Fab D1.3 purification

Fab D1.3 purification on HiTrap protein G column: For this purpose, 1 mL HiTrap protein G was equilibrated with 20 mM sodium phosphate buffer, pH 7.0. A 21 h culture broth sample (120 mL) from fed-batch cultivation (11 h post induction), centrifuged at 9390 g for 600 sec, was clarified by filtration through 0.45 μm syringe filters (Millipore, MA, USA). The sample (pH 7.0) was directly put through the HiTrap protein G column using an AKTA explore system (GE Healthcare, Life Sciences, Buckinghamshire, UK) at a flow velocity of 1 mL^{-1} min. After loading, the column was extensively washed with 20 mM sodium phosphate buffer, pH 7.0 and elution was made with 0.1 M glycine-HCl, pH 2.7 at the same flow rate. The eluate was instantaneously neutralized with 1 M Tris-HCl, pH 9.0 and stored at 4°C. The amount of Fab D1.3 in various fractions was measured using ELISA.

Fab D1.3 purification on chicken egg white lysozyme (HEWL)-activated CNBr-sepharose 4 Fast Flow matrix/ HiTrap protein G column: Coupling HEWL to CNBr activated sepharose was performed according to the manufacturer instruction (GE Healthcare, Uppsala, Sweden). Briefly, 1 g CNBr-sepharose 4FF matrix was washed with 15 medium volumes of cold (4°C) 1 mM HCl. Small wash portions (1 medium volume, 5 mL) was used and the mixture left 120 sec for equilibration during each washing step. The washed medium was then added to 5 mL solution containing 0.1 M $NaHCO_3$ (pH 8.3), 0.5 M NaCl and 10 mg HEWL for overnight shaking at 4°C. The reaction was followed by measuring UV-absorbance (at the wavelength 280 nm). In the next step, CNBr-sepharose was washed with 5 medium volumes (5×5 mL) of coupling buffer to wash away HEWL. HEWL-coupled CNBr sepharose was contacted with 0.1 M Tris-HCl, pH 8.0, for 2 h to block non-reacted groups on the medium. The coupled medium was eventually washed for 4 cycles using alternate low and high pH (0.1 M acetate buffer, pH 3-4, containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.3, containing 0.5 M NaCl), in which 15 ml of each buffer applied and each buffer wash took 120 sec.

After developing HEWL-activated CNBr-sepharose 4 Fast Flow matrix, the purification work was conducted. Purification from the culture broth sample was performed on HEWL-activated CNBr-sepharose 4FF column (5 mL). For this purpose, 480 mL culture broth was filtered through 0.45 μm syringe filters and loaded on the column at a flow velocity of 3.8 mL^{-1} min. Afterwards, the column

was washed with 20 mM phosphate buffer, pH 7.0. Elution was made with 50 mM diethylamine, pH 11.5. Eluted fractions containing Fab D1.3 from chromatography on the lysozyme-sepharose 4FF were pooled and desalted on PD10 columns prior to manual loading onto 1 mL HiTrap protein G column pre-equilibrated with 20 mM sodium phosphate, pH 7.0. After extensive washing with the equilibration buffer, bound material was eluted with 0.1 M glycine-HCl, pH 2.7. The eluate was neutralized immediately with 1 M Tris-HCl, pH 9.0 and stored at 4°C. Various fractions were analysed using SDS-PAGE, Western blotting and ELISA.

Fab D1.3 purification on HiTrap SP sepharose XL/ HiTrap protein G column: Purification from cell-free culture broth sample (after 11 h post induction with 0.1 mM IPTG) using this process was performed as previously described (Jalalirad, 2013). Various fractions were analysed using SDS-PAGE, Western blotting and ELISA.

Analytical methods

SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) and Western blotting: SDS-PAGE was performed on 15% gels at 20 mA for 2.5 h using Laemmli electrophoresis and runningbuffers. For immunodetection, non-specific binding sites on the membrane were blocked using 3% (w/v) BSA made in PBS after band transfer and the membrane was incubated with anti-IgG (Fab specific)-peroxidase antibody for 1 h. The enzyme substrate (TMB membrane peroxidise substrate system (3-C)) solution was added to the blot and incubated at room temperature until the desired band intensity was achieved.

Sandwich enzyme-linked immunosorbent assay (ELISA): For this purpose, 96-well microtiter plate was coated with 100 µL of 0.01% (w/v) Henn Egg White Lysozyme (HEWL), made in coating buffer (1.59 g L^{-1} Na_2CO_3 and 2.93 g L^{-1} $NaHCO_3$, pH 9.6), overnight at 4°C. Blocking was made with 200 µL PBS-BSA (1 PBS tablet and 1 g BSA/100 mL distilled water) per well, with shaking (500 rpm) in a microplate incubator shaker for 1 h, at 37°C. The wells were then washed three times with 300 µL wash buffer (1 PBS tablet and 100 µL Tween 20/100 mL distilled water) per well. Samples (100 µL) were transferred to the washed ELISA plate, incubated at 37°C, 500 rpm for 1 h and washed again with the wash buffer three times. The plate was then loaded with 100 µL anti-IgG (Fab specific)-peroxidase antibody, incubated at 37°C, 500 rpm for 1 h and then washed three times with the wash buffer. Peroxidase substrate (100 µL) was added to each well and the reaction was stopped after 600 sec by adding 100 µL of 1 M H_3PO_4. The plate was read at the wavelength of 450 nm.

Total protein quantification: The amount of total protein present in the samples was measured using bicinchoninic acid assay (BCA assay kit, Thermoscientific, Rockford, IL, USA).

RESULTS AND DISCUSSION

Fab D1.3 purification on HiTrap protein G column: Though protein G has strong affinity for the Fc regions of IgG molecule, it also shows weak affinity towards the C_H1 domain of Fab (Humphreys *et al.*, 1997). Purification of various Fab molecules from Chinese Hamster Ovary (CHO) cell supernatant and the periplasmic fraction of *E. coli* on immobilised protein G has been reported in literature (Kwack, 2000; Humphreys *et al.*, 1997; Proudfoot *et al.*, 1992). Proudfoot *et al.* (1992) exploited this low-affinity interaction for the purification of chimeric mouse-human B72.3 Fab and F(ab')$_2$ fragments and showed that chimeric B72.3 Fab bound weakly to the protein G-sepharose so that it could be separated from F(ab')2 and eluted with a pH 7.0 wash, whereas B72.3 F(ab')$_2$ required elution at pH 2.0. In the present work, purification of Fab D1.3 using protein G affinity chromatography was experimented. The ELISA figure relevant to purification of the Fab from culture broth directly on HiTrap protein G column has been illustrated in Fig. 1. As it is evident from the ELISA data, most (>99%) of the Fab applied to the column failed to bind and diminutive quantities of the Fab were detected in the wash and elution fractions. In the works performed by other scientists on direct purification of various Fab fragments on protein G columns, no detailed data (e.g., chromatography/conductivity values) had been given; therefore it was impossible to compare the experimental conditions (e.g., conductivity) in this work with theirs.

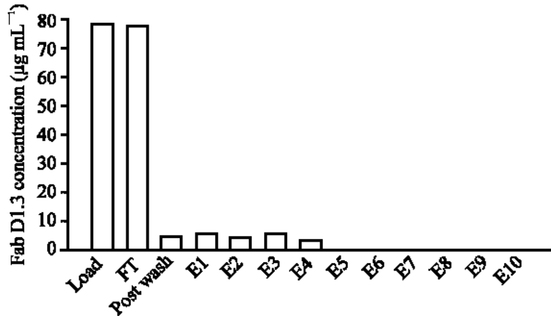

Fig. 1: ELISA data for the purification of Fab D1.3 from a 21 h culture broth sample on a HiTrap protein G column

Fab D1.3 purification on HEWL-activated CNBr-sepharose 4 Fast Flow matrix/ HiTrap protein G column: Protein and other molecules containing primary amino groups can be coupled directly to the CNBr-activated sepharose gel. Basic amino acids in HEWL, i.e., twelve arginine residues and six lysine residues, could react with cyanate ester groups in CNBr-activated sepharose (Sasaki *et al.*, 2003). HEWL interacts extensively with V_K and V_H domains in Fab D1.3, forming three hydrogen bonds to the V_K domain and nine hydrogen bonds to the V_H domain (Ward *et al.*, 1989). It has been shown that amongst various avian and human lysozymes, D1.3 monoclonal antibody can specifically bind HEWL (Harper *et al.*, 1987). Purification of heavy and light chain variable domains (Fv) of the anti-lysozyme D1.3 from culture broth and the periplasmic fraction of *E. coli* has been done on HEWL-sepharose column (Winter *et al.*, 2003; Holmes *et al.*, 1998; Cumber *et al.*, 1992); however, no detailed data (i.e., chromatogram and purity of the eluted target protein) has been reported.

In the present work, purification of Fab D1.3 from culture broth on a lysozyme-sepharose affinity column was also investigated. The chromatogram, SDS-PAGE and Western blotting results relevant to this experiment have been shown in Fig. 2. Analysis of the elution fractions by SDS-PAGE confirmed the present of the Fab in the eluates and showed that impurities accompanied with the Fab.

In the purification works done by other scientists using lysozyme-sepharose column which discussed earlier in this section, no chromatogram or SDS-PAGE/Western blotting figures had been presented; therefore, the purity of the purified antibody fragment in those works was not manifest.

As the Fab in the elutions originated from the lysozyme-sepharose column was accompanied with impurities, an extra step of purification on HiTrap protein G was performed following desalting the eluates on PD10. By doing this highly pure Fab D1.3 was obtained, as indicated in SDS-PAGE results (Fig. 3).

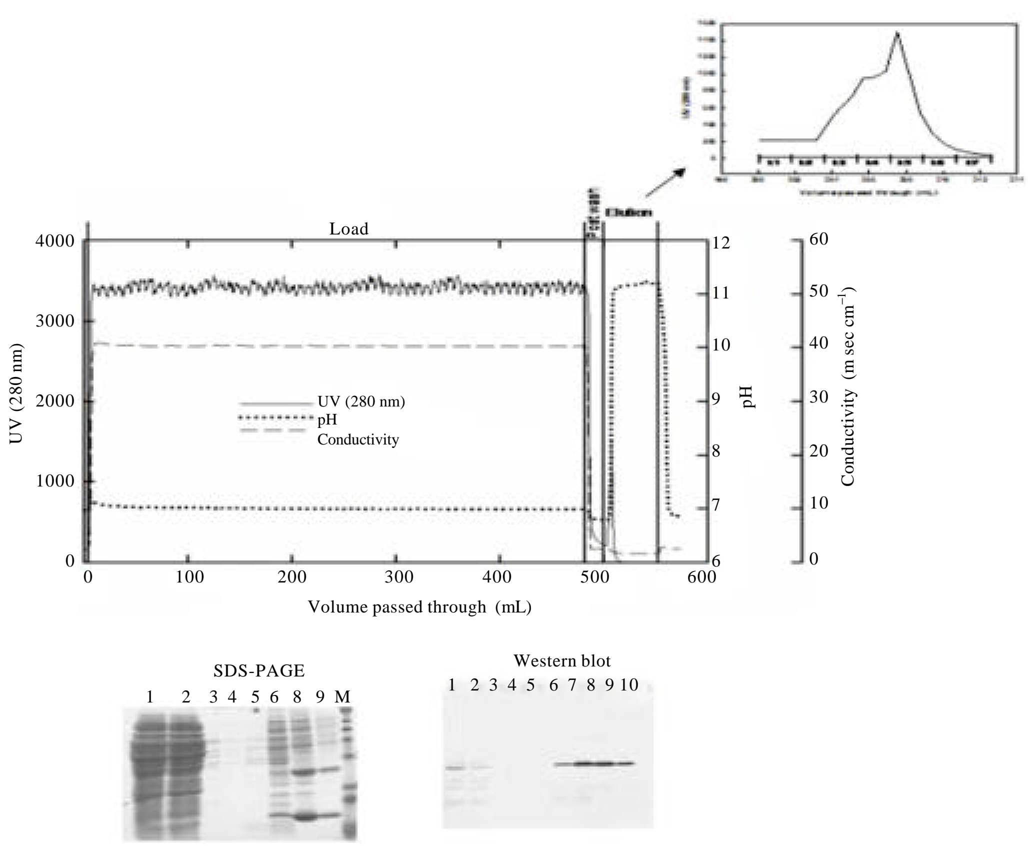

Fig. 2: Chromatogram (top), SDS-PAGE (bottom left) and Western blot (bottom right) analyses for the purification of Fab D1.3 from culture broth (21 h culture) on a lysozyme-activated sepharose 4FF column, Phosphate buffer (20 mM, pH 7.0) was employed for equilibration and the post wash and 50 mM diethylamine, pH 11.5 was used as the eluent, Lane: 1-load, 2-flow through, 3-post wash, 4 to 10-elution fractions, M-molecular weight markers

Table 1: Chromatography data for purification of Fab D1.3 from culture broth (21 h culture) on sequential lysozyme-activated sepharose 4FF-desalting gel filtration (GF)-protein G affinity chromatography and sequential CEX-GF-protein G affinity chromatography processes

	Sequential lysozyme affinity-GF-Protein G affinity chromatography route								Sequential CEX-GF-Protein G affinity chromatography route[f]							
	Lysozyme-sepharose column				HiTrap protein G column				CEX column				HiTrap protein G column			
Step	Load	FT[a]	Wash	Elution	Load	FT	Wash	Elution	Load	FT	Wash	Elution	Load	FT	Wash	Elution
Volume (mL)	480	480	20	12	3.5	3.5	5	4	480	480	25	7	7	7	5	2.5
Fab D1.3 (mg)	19.58	15.55	0.49	3.43	3.43	0.04	0.01	3.40	8.3	0.16	0.85	7.19	7.19	0.23	0.04	6.85
Total protein (mg)	1888.2	1864.8	16.53	7.03	7.0	0.21	0.02	3.5	1303.4	1235	17.5	50.2	50.2	35	0.1	7
Target yield (%)[b]	100	79.42	2.5	17.52	17.5	0.2	0.03	17.36	100	1.8	10.2	86.7	86.7	2.8	0.6	82.5
Total protein yield (%)[c]	100	98.76	0.88	0.37	0.37	0.01	0	0.19	100	94.8	1.3	3.9	3.9	2.7	0	0.5
Purity (%)[d]	1.04	0.83	2.96	48.79	49	19.05	25	97.14	0.6	0	4.9	14.3	14.3	0.7	46	97.9
PF[e]	1			47.05				93.63	1			22.5				153.7

[a]Flow through, [b]Calculated by dividing amount of Fab D1.3 in each fraction by amount of Fab D1.3 in the load multiplied by 100, [c]Calculated by dividing amount of total protein in each fraction by amount of total protein in the load multiplied by 100, [d]Calculated by dividing amount of Fab D1.3 by amount of total protein in each fraction multiplied by 100, [e]Is purification factor calculated by dividing targetyield by total protein yield, [f]Data from Jalalirad (2013)

Fig. 3: SDS-PAGE gel selected fractions from the purification of partially purified Fab D1.3 on HiTrap protein G. Eluted fraction from chromatography on lysozyme sepharose 4FF (Fig. 2) was desalted on a PD10 column prior to loading onto a HiTrap protein G column. M- molecular weight markers and lane 1 is elution fraction 1

According to the chromatography data (Table 1) the purification by lsozyme-sepharose column yielded 48.7% Fab purity for purification from culture broth sample due to non-specific binding of other proteins to the lysozyme-activated sepharose column, as proved by SDS-PAGE analysis. Fab D1.3 eluted from the protein G column was substantially purified, i.e., reaching>97%, demonstrating that the protein G column is very effective for removal of bulk impurities and polishing stage of a purification process for antibody fragment. However, the overall yield of the target Fab in this sequential process was low (17.3%) due to low capacity of the lysozyme-activated CNBr-sepharose.

Fab D1.3 purification on HiTrap SP sepharose XL/ HiTrap protein G column: Cation Exchange (CEX) chromatography can be used as a very helpful technique for concentrating proteins of interest in the beginning of a purification process. For optimal sorption of the Fab on the cation exchange matrix, the pH and conductivity of the load were adjusted to 5.0 and 5.0 m sec cm^{-1}, respectively. To obtain high pure Fab D1.3, a further step of purification on HiTrap protein G was performed using eluates desalted on PD10 column.

The chromatography data related to the Fab purification from culture broth sample by a sequential SP sepharose Xl Cation Exchange (CEX)-desalting Gel Filtration (GF)-protein G affinity chromatography route have been also illustrated in Table 1. As it is shown in this table, the Fab capture from the culture broth sample on the cation exchanger was very successful (>97% capture) and the pooled eluate from this CEX step yielded a ca. 14% Fab purity and a 22.5-fold purification factor. Further Fab purification from desalted CEX eluates on protein G column resulted in a highly pure Fab (with>97% purity). The overall yield and purification factor in this 'CEX-GF -protein G affinity' route were 83% and 153-fold, respectively, being significantly higher than that obtained previously using the 'lysozyme affinity-GF-protein G affinity' procedure (Table 1).

CONCLUSION

Streptococcal protein G can bind strongly to the Fc region (between C_H2 and C_H3 domains) and weakly to C_H1 domain. Therefore, protein G can be used for purification of whole IgG molecules and some reports have shown that it is a potential ligand for purification of antibody fragments as well (Derrick and Wigley, 1994; Bjorck and Kronvall, 1984). In this work purification of Fab D1.3 by various chromatography routes has been investigated. Fab D1.3 binds very weakly (<1%) to protein G column, when the culture broth sample (with conductivity ~40 m sec cm^{-1} and pH 7.0) is directly loaded on the protein G column.

Amongst avian and human lysozymes, D1.3 antibody can specifically bind HEWL (Harper *et al*., 1987). In this work it has been demonstrated that Fab D1.3 can be purified from culture broth on a HEWL-activated CNBr-sepharose column with 48% purity. The Fab with such purity can bind (>97%) to protein G when the pooled HEWL column eluate is desalted on PD10 column using 20 mM sodium phosphate, pH 7.0. As a result, Fab D1.3 can be purified >97% using coupled HEWL and protein G affinity columns. However the overall yield of such chromatography step is low (~18%) due to low binding capacity of the lysozyme column.

Cation exchangers have a very good capacity for capturing and concentrating target proteins in the early stage of purification process. HiTrap SP sepharose XL binds and captures Fab D1.3 (>97%) from 480 mL culture broth (with pH 5.0 and conductivity 5.0 m sec cm^{-1}) and as a result concentrated target protein (with 14% purity) is achieved. The Fab with such purity can bind greatly to protein G when the pooled CEX column eluate is desalted on PD10 column using 20 mM sodium phosphate, pH 7.0. Therefore, Fab D1.3 can be purified >97% using coupled HiTrap SP sepharose XL and protein G columns. The overall yield of coupled HiTrap SP sepharose XL and protein G columns is considerably high (83%) and therefore appears more suitable than coupled HEWL and protein G affinity columns for purification of Fab D1.3.

ACKNOWLEDGMENTS

The author would like to thank Owen Thomas and Eirini Theodosiou (The University of Birmingham, UK) for their valuable assistance. Financial support received from the Ministry of Health and Medical Education of Iran for this study is highly appreciated.

REFERENCES

Babaei, M.H., R. Farshid Far and R. Najafi, 2002. Evaluation of tumor targeting with radiolabeled F(ab)2 fragment of a humanized monoclonal antibody. DARU J. Pharm. Sci., 10: 49-53.

Bjorck, L. and G. Kronvall, 1984. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J. Immunol., 133: 969-974.

Cuatrecasas, P. and C.B. Anfinsen, 1971. Affinity chromatography. Annu. Rev. Biochem., 40: 259-278.

Cumber, A.J., E.S. Ward, G. Winter, G.D. Parnell and E.J. Wawrzynczak, 1992. Comparative stabilities *in vitro* and *in vivo* of a recombinant mouse antibody FvCys fragment and a bisFvCys conjugate. J. Immunol., 149: 120-126.

Derrick, J.P. and D.B. Wigley, 1994. The third IgG-binding domain from Streptococcal protein G: An analysis by X-ray crystallography of the structure alone and in a complex with Fab. J. Mol. Biol., 243: 906-918.

Hage, D.S., 1999. Affinity chromatography: A review of clinical applications. Clin. Chem., 45: 593-615.

Harper, M., F. Lema, G. Boulot and R.J. Poljak, 1987. Antigen specificity and cross-reactivity of monoclonal anti-lysozyme antibodies. Mol. Immunol., 24: 97-108.

Hodgson, I., C.D.J. Lennon and B.V. Kara, 2007. Expression system. Patent, International Publication Number WO 2007/088371 A2.

Holliger, P. and P.J. Hudson, 2005. Engineered antibody fragments and the rise of single domains. Nat. Biotechnol., 23: 1126-1136.

Holmes, M.A., T.N. Buss and J. Foote, 1998. Conformational correction mechanisms aiding antigen recognition by a humanized antibody. J. Exp. Med., 187: 479-485.

Humphreys, D.P., A.P. Chapman, D.G. Reeks, V. Lang and P.E. Stephens, 1997. Formation of dimeric Fabs in *Escherichia coli*: Effect of hinge size and isotype, presence of interchain disulphide bond, Fab' expression level, tail piece sequences and growth conditions. J. Immunol. Methods, 209: 193-202.

Jalalirad, R., 2013. Selective and efficient extraction of recombinant proteins from the periplasm of *Escherichia coli* using low concentrations of chemicals. J. Ind. Microbiol. Biotechnol., 40:1117-1129.

Kwack, K.B., 2000. A new purification method for the Fab and F(ab)2 fragment of 145-2C11, hamster anti-mouse CD3e antibody. J. Biochem. Mol. Biol., 33: 188-192.

Lindmark, R., C. Biriell and J. Sjoquist, 1981. Quantitation of specific IgG antibodies in rabbits by a solid-phase radioimmunoassay with 125I-Protein A from Staphylococcus aureus. Scand. J. Immunol., 14: 409-420.

Nelson, A.L. and J.M. Reichert, 2009. Development trends for therapeutic antibody fragments. Nat. Biotechnol., 27: 331-337.

Nelson, A.L., 2010. Antibody fragments: Hope and hype. MAbs., 2: 77-83.

Proudfoot, K.A., C. Torrance, A.D.G. Lawson and D.J. King, 1992. Purification of recombinant chimeric B72.3 Fab and F(ab)2 using streptococcal protein G. Protein Expr. Purif., 3: 368-373.

Reichert, J.M., 2012. Marketed therapeutic antibodies compendium. MAbs., 4: 413-415.

Sasaki, C., A. Kristiansen, T. Fukamizo and K.M. Varum, 2003. Biospecific fractionation of chitosan. Biomacromolecules, 4: 1686-1690.

Staby, A., I.H. Jensen and I. Mollerup, 2000. Comparison of chromatographic ion-exchange resins: I. strong anion-exchange resins. J. Chromatogr. A, 897: 99-111.

Ward, E.S., D. Gussow, A.D. Griffithd, P.T. Jones and G. Winter, 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature, 341: 544-546.

Winter, G.P., E.S. Ward and D. Gussow, 2003. Single domain ligands, receptors comprising said ligands, methods for their production and use of said ligands and receptors. US patent No 6248516. http:// www. google.com/patents/US6545142

Wu, A.M. and P.D. Senter, 2005. Arming antibodies: Prospects and challenges for immunoconjugates. Nat. Biotechnol., 23: 1137-1146.

Yokota, T., D.E. Milenic, M. Whitlow and J. Scholm, 1992. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res., 52: 3402-3408.