Babol University of Medical Sciences
IJMCM
International Journal of Molecular and Cellular Medicine
p-ISSN: 2251-9637 o-ISSN: 2251-9645
Cellular and Molecular Biology Research Center

Biological Activity of Mouse OX40L-Igg Purified With Two Novel Resins

Hossein Rezvan[1*], Selman A Ali[2], Sahar Hamoonnavard[1], Robert Rees[3]

1. Department of Pathobiology, Faculty of Veterinary Sciences, Bu-Ali Sina University, Hamedan, Iran.
2. School of Science, Nottingham Trent University, Clifton Lane, Clifton, Nottingham, NG11 8NS, UK.
3. Department of Life Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK.

Article type: Original Article

Received: 2023.06.10
Revised: 2024.02.27
Accepted: 2024.04.07

ABSTRACT

One of the important stimulating molecules for the function of T lymphocytes is tumor necrosis factor receptor OX40 (CD134), activated by its cognate ligand OX40L (CD134L, CD252). OX40L interactions have been proposed as a potential therapeutic target for treating infectious and non-infectious diseases. The main purpose of this study was to determine the potency of two novel resins MBI and MEP for the purification of OX40L-IgG fusion protein and the biological activities of this OX40L-IgG fusion protein. The biological activity of the OX40L-IgG purified by these resins compared with protein A sepharose resin. Mice treated with the same doses of the OX40L purified by the three resins showed a significant delay in tumor growth compared to the controls injected with PBS. Mice treated with the OX40L purified by MBI resin showed a significant delay in tumor cell (CT26) growth compared with mice injected with OX40L purified by other resins.

Keywords: OX40L-IgG, purification, MBI, MEP

Cite this article: Rezvan H, *et al*. Biological Activity of Mouse OX40L-IgG Purified With Two Novel Resins. *International Journal of Molecular and Cellular Medicine*. 2023; 12(4):335-349. **DOI:** 10.22088/IJMCM.BUMS.12.4.335

*Corresponding: Hossein Rezvan
Address: Department of Pathobiology, Faculty of Veterinary Sciences, Bu-Ali Sina University, Hamedan, Iran.
E-mail: h.rezvan@basu.ac.ir

Introduction

OX40 ligand (OX40L) was first identified at the low-level expression on T cells activated with an EBV-transformed B cell line in 1994 (1). OX40L is a type II transmembrane protein that reacts with OX40 molecules with high affinity and slow dissociation. Both OX40L mRNA and protein are markedly expressed in human T-cells infected with type-1 leukaemia virus; cloning of this protein leads to the proliferation of T lymphocytes followed by effective stimulation (2).

Initial cloning and first sequencing revealed that there is close proximity between OX40 and OX40L to other TNF family molecules encoded by TNFRSF4 and TNFSF4, which are members of TNF and TNF receptor superfamilies on chromosomes1 (3).

It has been shown that OX40-OX40L engagement is capable of signaling both the cells on which they are expressed and has a positive regulatory effect on division, survival, effecter function, and the number of T cells at the peak of immune responses (4). This interaction induces a strong co-stimulatory signal, which promotes the activation and memory development of the T cells (4). OX40L is preferentially expressed on activated B cells, macrophages, DCs, and endothelial cells at the site of inflammation. DCs express the OX40L constitutively and the expression of this protein becomes further up regulated by LPS (5). In early cognate interaction between the B and T cells, OX40-OX40L engagement also triggers an OX40L reverse signal that enhances IgG production of B cells and promotes the maturation of DCs (6).

It is now clear that the main purpose of protein purification is the isolation of the given protein with maximum yield and highest purity while the protein holds its chemical and biological integrity. As the structure and characteristics of proteins are different, the purification method of proteins also varies. Many studies have been carried out to develop methods by which proteins can be purified with a high yield and optimum biological activity. Most protein purification methods are based on differences in the biochemical properties of proteins such as overall charge, size, and hydrophobicity between the protein of interest and contaminants. Some common methods used to purify proteins are precipitation with ammonium sulfate (NH4)2SO4, ultracentrifugation, and chromatography. The basic procedure in chromatography relies on separating the protein passage through a column packed with different resins, which can interact with the protein of interest. These resins normally consist of a ligand, which binds to the protein of interest, a matrix, which is a solid phase to immobilize the ligand, and a spacer arm, which is normally included in between the matrix and the ligand (7).

The main objective of this study was to determine the potency of two novel resins named MBI and MEP for purification of OX40L-IgG fusion protein and compare them with protein A Sepharose. The OX40L-IgG fusion protein, which was produced using protein A-Sepharose, was transfected into B9B8E2 cells. B9B8E2 cells are hamster kidney cells transfected with both mouse OX40L and mouse IgG1 capable of producing and releasing mouse OX40L-IgG fusion protein, the cell culture supernatant. For the purification of OX40L-IgG from B9B8E2 culture supernatants, two novel resins named MBI and MEP were used. In this study, the purification conditions of B9B8E2 cell produced OX40L-IgG for both MBI and MEP resins were first optimized and then the biological activity of the OX40L purified by these resins was compared with protein A-Sepharose resin.

Materials and methods

Cells- OX40L

OX40L-IgG transfected B9B8E2 cells were gifted by Xenova Ltd. Purification of OX40L-IgG was obtained from collecting B9B8E2 culture supernatants. The OX40L-IgG-producing cell line (B9B8E2) was cultured according to the protocol supplied by Xenova plc. Briefly, the cell culture media was renewed every 5 to 7 days and the supernatants were collected and kept in -80 °C until required.

Optimization and purification of mouse OX40L-IgG by MBI resin

The structure of 2-mercapto-5-benzimidazole sulfonic acid is based upon the presence of a heterocyclic, a sulphur atom, and an aromatic ring supporting a strong acidic group, which is negatively charged over the whole range of working pH (Figure 1, Table 1). According to the information presented by the manufacturer, antibodies are adsorbed to the resin in physiological ionic strength whereas the elution occurs at bufferic pHs. MBI, a novel resin designed for the purification of antibodies, was used to purify OX40L-IgG from the B9B8E2 cell supernatant. To optimize the purification conditions, sodium acetate buffer and sodium acetate buffer plus NaCl at pH 4, 4.5, 5, 5.2, 5.5, 6 or 6.5 were used as loading buffers, and tris buffer at pH 9 was used for elution. Fractions were collected and total protein was determined by measuring absorbance at 280 nm using a spectrophotometer and also for selected fractions using BCA kit. The presence of OX40L was determined in all fractions by dot blotting and the concentration of OX40L was marked as + to +++ according to the size of spots, mOX40-mIgG1 (MM1) was used as the control.

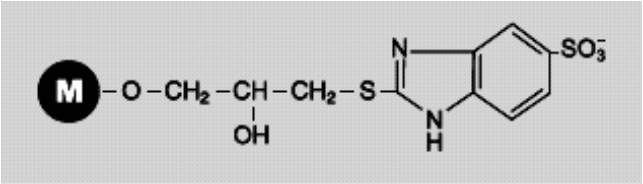


Fig. 1. Structure of the MBI Ligand (Adapted from MBI Hypercel product note). MBI ligand has a sulfonate group present on the aromatic ring.

Table 1. Key features of MBI Hypercel (Adapted from MBI Hypercel product note).

Particle size	80-100µm
Dynamic binding capacity for hu IgG	20-40 mg/mL
Ligand	2-mercapto-5-benzimidazole sulfonic acid
Working pH	Adsorption: pH 5.0-5.5 Elution: pH 8.0-9.5
Cleaning pH	3-14
Pressure resistance	<44 psi
Typical working pressure	<14psi

Purification of mouse OX40L-IgG fusion protein by MEP Hypercel resin

The structure of 4-mercapto-ethyl-pyridine is based on the presence of a pyridine ring (Figure 2). MEP is attached to a hydrophilic matrix through a hydrophobic spacer arm. The hydrophobic spacer arm provides enhanced selectivity for the adsorption of proteins, particularly antibodies. The presence of a pyridine ring is also shown to enhance protein selectivity (8, 9). MEP has an isoelectric pH point of 4.8 and hence is

uncharged in neutral conditions. The resin becomes positively charged when the pH is below 5 due to repulsive forces between the resin and the positively charged antibody. Pyridine rings associated with sulphur atoms are relatively well documented as being able to separate immunoglobulins from complex mixtures such as serum proteins (10). The main properties of the MEP resin are shown in (Table 2). Because in MEP, resin different proteins are eluted at different pHs, elution buffers at different pHs were applied continuously in decreased order from high to low. So, to optimize the conditions of the resin for the purification of the OX40L-IgG fusion protein, a constant pH of 8 for loading and a set of pHs of 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.7, 4.5, 4.3, 4.0 and 3.0 for elution were applied. The flow of each buffer at each pH was continued until the absorbance at 280 nm was stable at 0.01. All fractions were collected and tested for OX40L by western blotting using anti-mOX40L antibodies.

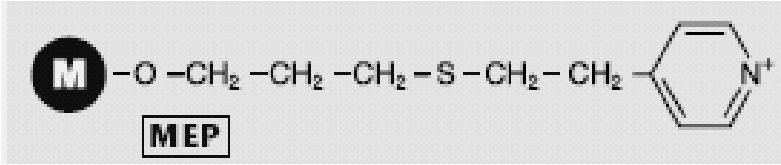


Fig. 2. Structure of the MEP Ligand (Adapted from MEP Hypercel product note).

Table 2. Key features of MEP Hypercel (Adapted from MEP Hypercel product note).

Particle size	80-100 µm
Dynamic binding capacity for hu IgG	≥ 20 mg/mL
Ligand	4-Mercapto-Ethyl-Pyridine
Ligand density	70-125 µmol/mL
Working pH	3-12
Cleaning pH	3-14
Pressure resistance	< 44 psi
Typical working pressure	<14 psi

Fraction absorption at 280nm for samples loaded at pH4 using sodium acetate or sodium acetate+ NaCl for loading buffer

Two milliliters of B9B8E2 cell culture supernatant was mixed up with the same volume of 100 mM sodium acetate or 100 mM sodium acetate + 150 mM NaCl at a set of pHs from 4 to 6.5. After loading the samples, the OX40L was eluted with Tris buffer at pH 9. All samples were collected in fractions of 6 mL. The absorbance of fractions was measured at 280 nm by spectrophotometer.

mOX40- mIgG1 (MM1) was used as a control study, B9B8E2 cells, donated by Xenova plc, were transfected with the murine OX40L and IgG1 plasmids and were used to produce the mOX40-mIgG1 (MM1).

Biological activity of OX40L-IgG

The biological effect of the OX40L purified by MBI and MEP on the growth of CT26 tumor cells was assessed and compared with that purified by protein A sepharose resin. Female BALB/c mice were purchased from Harlan Olac (Oxon, UK) and maintained in accordance with the ethical codes (Code Number: IR. BASU.REC.1398.053) of practice for housing and care of animals (Nottingham Trent

University, Nottingham, UK). Four groups of 10 female BALB/c mice were implanted S.C. with 2×10^4 CT26 tumor cells on the right flank. On days 3 and 7, three groups were injected I.P. with 500 mg/100 µL/mouse OX40L-IgG purified by MBI, MEP, and protein A Sepharose accordingly. The fourth group was injected with 100 mL PBS and used as a control.

Statistical analysis

Data analysis was done using SPSS Version 25 using the Student's t-test.

Results

The highest level of total protein was obtained in elution fractions when the samples were loaded at pH 4 (Fig and Table 3). There was no significant difference between sodium acetate and sodium acetate plus NaCl at 280nm absorbance; however, the amount of protein in the eluting fractions was slightly higher in the presence of NaCl (Figure 3).

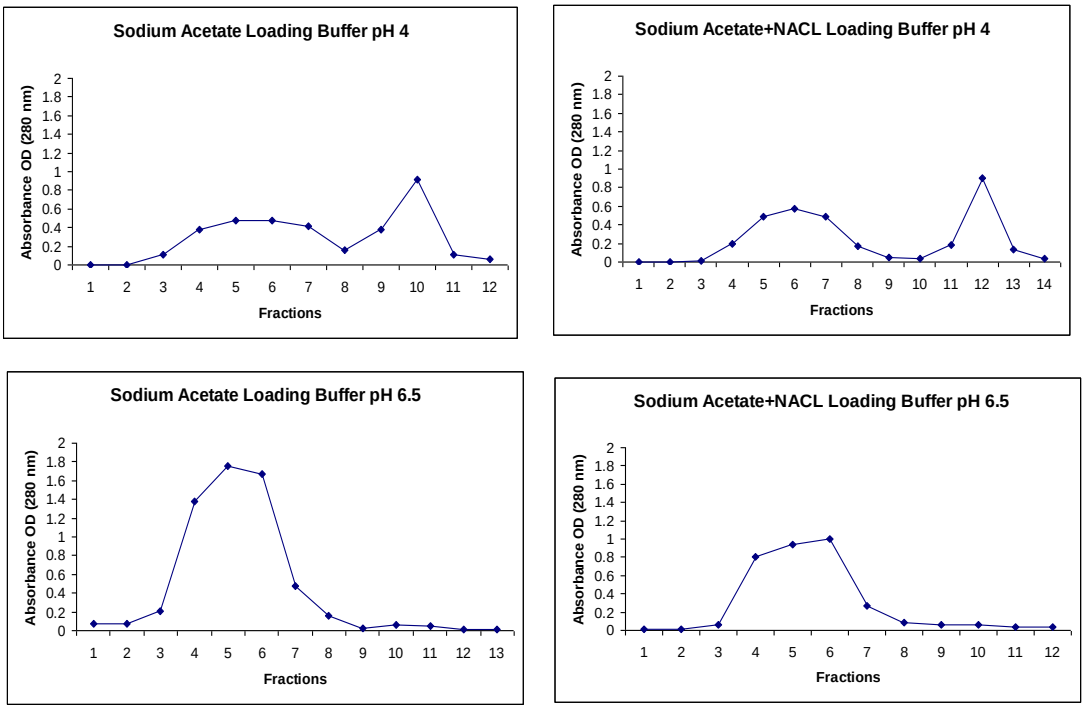


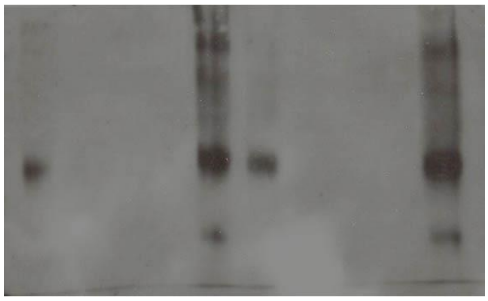
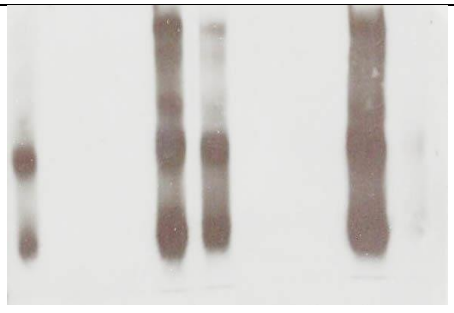
Fig. 3. Fraction absorption at 280 nm for samples loaded at pH 4 using sodium acetate or sodium acetate+ NaCl for loading buffer. 2 mL of B9B8E2 cell culture supernatant was mixed up with same volume of 100 mM sodium acetate or 100 mM sodium acetate + 150 mM NaCl at a set of pHs from 4 to 6.5. After loading the samples, the OX40L was eluted with Tris buffer at pH 9. All samples were collected in fractions of 6 mL. The absorbance of fractions was measured at 280 nm by spectrophotometer.

To confirm the presence of OX40L-IgG in the fractions and determine the purity of the samples, selected fractions were probed with anti-mouse OX40L, anti-mouse IgG and bovine IgG antibodies using Western blotting. mOX40-mIgG1 (MM1) produced by Xenova plc was used as positive control (Figure 4).

Table 3. Total protein and detection of OX40L in sample fractions purified by MBI resin.

Buffer. Sodium Acetate				**Buffer. Sodium Acetate+NaCl280nm**			
Loading pH	4	Elution Fractions	10, 11	Loading pH	4	Elution Fractions	11, 12
Fraction	**Protein mg/ml**	**Fraction**	**Dot Blotting Results**	**Fraction**	**Protein mg/ml**	**Fraction**	**Dot Blotting Results**
1	0.120419	1	-	2	0.120013	3	-
2	0.119877	2	-	5	0.169938	4	-
5	0.159926	4	-	10	0.124884	5	-
7	0.153432	6	-	12	0.469833	8	-
9	0.19754	10	+++			11	+++
10	0.458671	11	++			12	+++
11	0.134625						

To optimize the pH for the elution buffer, Tris buffer at pH 7, 7.5, 8, 8.5, 9, 9.5, and 10 was used together with sodium acetate plus NaCl at pH 4 as loading buffer. The results clearly showed that increasing the pH of the elution buffer from 7 to 10 increased the total protein in the eluted fractions. The highest protein measurement was for the fractions eluted at pH 9.5, which was finally selected as the optimum elution pH (Figure 5).

A: Probed by anti-mouse OX40L antibody	
50 KD →  **22 KD** → **1 2 3 4 5 6 7 8 9**	1- Unpurified sample 2, 3- Loading fractions (sodium acetate pH=4) 4, 5- Elution fractions (tris pH 9.5) 6, 7- Loading fractions (sodium acetate + NaCl pH=4) 8, 9- Elution fractions (tris pH 9.5)
B: Probed by anti-mouse IgG antibody	
50 KD →  **22 KD** → **1 2 3 4 5 6 7 8 9**	1- Unpurified sample 2, 3- Loading fractions (sodium acetate pH=4) 4, 5- Elution fractions (tris pH 9.5) 6, 7- Loading fractions (sodium acetate + NaCl pH=4) 8, 9- Elution fractions (tris pH 9.5)
C: Probed by anti-bovine IgG antibody	

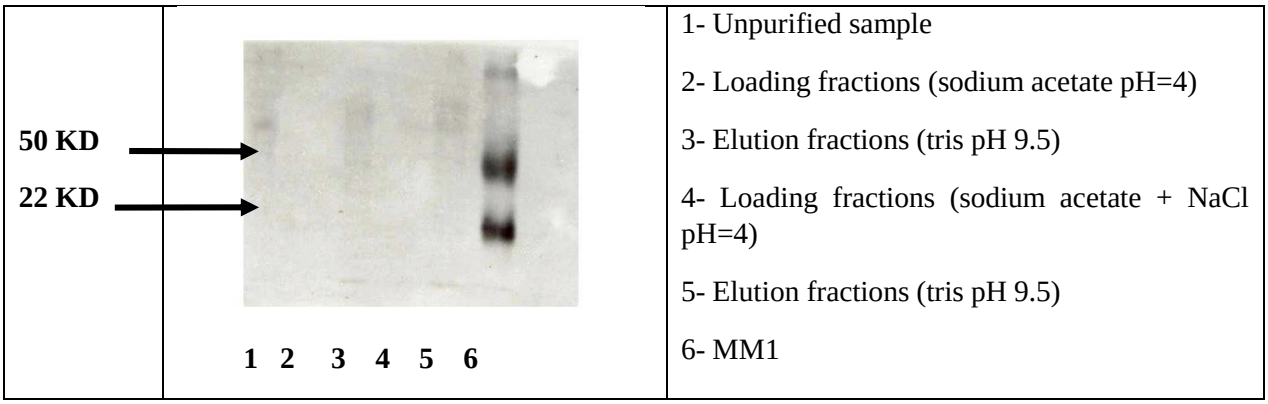


Fig. 4. Detection of OX40L purified by MBI resin using Western blotting. B0B8E2 cell culture supernatants were collected and purified by MBI resin. The presence of OX40L was detected in the sample fractions collected during loading and elution phases by gel electrophoresis and using anti-mouse OX40L antibodies. The presence of mouse IgG and bovine IgG were also determined by using anti-mouse or bovine IgG antibodies.

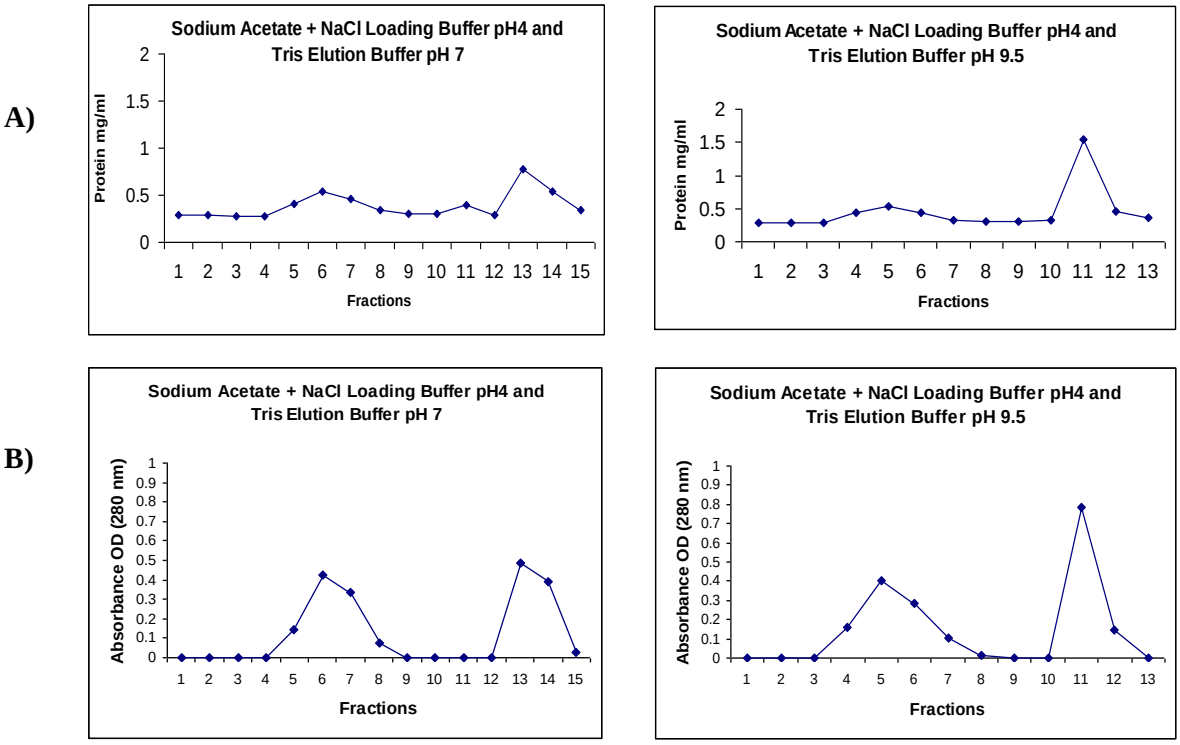


Fig. 5. Total protein of sample fractions evaluated by protein assay or measuring the absorbance at 280 nm. 2 mL of B9B8E2 cell culture supernatant was mixed up with same volume of 100mM sodium acetate buffer containing 150mM NaCl and the pH was adjusted at 4. The OX40L was eluted with Tris buffer at a set of pHs from 7 to 10. All samples were collected in fractions of 6 mL. **A)** The total protein was assessed by BCA kit according to the manufacturer's instructions. **B)** Absorbance was also measured at 280 nm by spectrophotometer.

Due to the presence of FCS in the cell culture media of B9B8E2 cells, the sample fractions loaded at pH 4 and pH 5.2 were collected and checked for bovine serum albumin (BSA) by Western blotting. The results clearly showed that the amount of BSA as detected by Western blotting in the elution fraction was much higher at pH 4 in comparison to pH 5.2 (Figure 6).

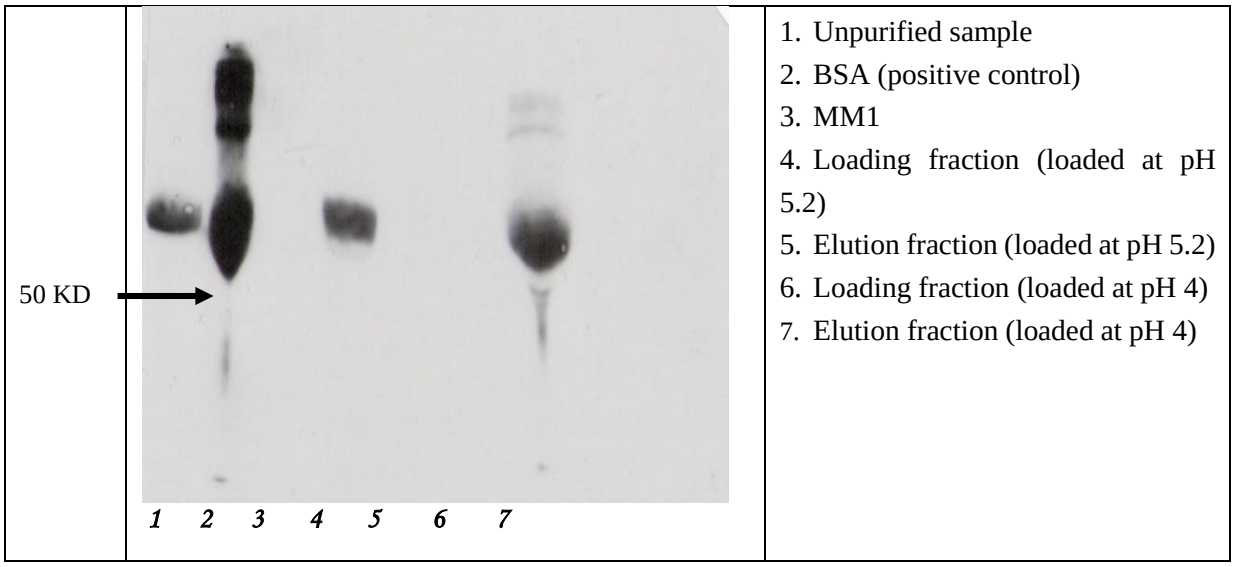


Fig. 6. Detection of BSA in OX40L samples purified by MBI resin at pH 4 and pH 5.2. B9B8E2 cell culture supernatants were purified by MBI resin. The samples were loaded at a set of pH from 4 to 7 and eluted at pH 9.5. The purified samples were analyzed for BSA by Western blotting using anti-BSA antibodies.

Purification of mouse OX40L-IgG fusion protein by MEP Hypercel resin

MEP has an isoelectric pH point of 4.8 and hence is uncharged in neutral conditions. The resin becomes positively charged when the pH is below 5 due to repulsive forces between the resin and the positively charged antibody. Pyridine rings associated with Sulphur atoms are relatively well documented as being able to separate immunoglobulins from complex mixtures such as serum proteins (10).

To optimize the conditions of MEP resin for purification of the OX40L-IgG fusion protein, a constant pH of 8 for loading and a set of pHs of 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.7, 4.5, 4.3, 4.0, and 3.0 for elution were applied. Because in MEP resin different proteins are eluted at different pHs, elution buffers at different pHs were applied continuously in decreased order from high to low. The flow of each buffer pH was continued until the absorbance at 280 nm was stable at 0.01 (Figure 7). All fractions were collected and tested for OX40L by Western blotting using anti-mOX40L antibodies. The Western blotting results clearly showed that the OX40L-IgG fusion protein bound to MEP resin starts eluting at pH below 5, which peaked at pH 4.5 (Figure 8).

To optimize the conditions of MEP resin for purification of the OX40L-IgG fusion protein, a constant pH of 8 for loading and a set of pHs of 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.7, 4.5, 4.3, 4.0, and 3.0 for elution were applied. Because in MEP resin different proteins are eluted at different pHs, elution buffers at different pHs were applied continuously in decreased order from high to low. The flow of each buffer pH was continued until the absorbance at 280 nm was stable at 0.01 (Figure 7). All fractions were collected and tested for OX40L by Western blotting using anti-mOX40L antibodies. The western blotting results clearly showed that the OX40L-IgG fusion protein bound to MEP resin starts eluting at pH below 5, which peaked at pH 4.5 (Figure 8).

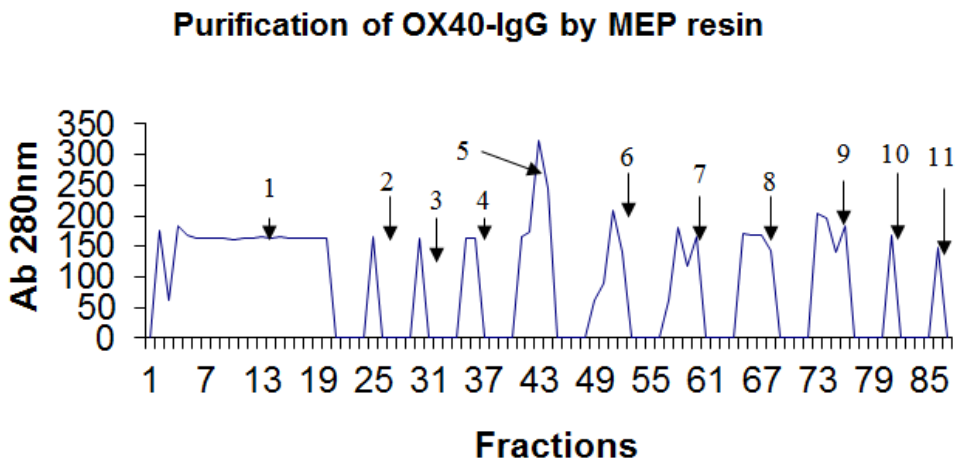


Fig. 7. Optimization of elution pH for purification of mouse OX40L-Ig fusion protein by MEP resin. 2 mL B9B8E2 cell culture supernatant was mixed with the same volume of 50 mM tris buffer and adjusted at pH 8. After loading the sample, the loading buffer was run continuously until the absorbance at 280 was stabled at 0.01. Elution buffer (50 mM sodium acetate) at a set of pH was applied for elution. 1: pH 8.0, 2 to 11: pH 6.0, 5.8, 5.6, 5.4, 5.2, 5.0, 4.7, 4.5, 4.3, 4.0, and 3.0, respectively.

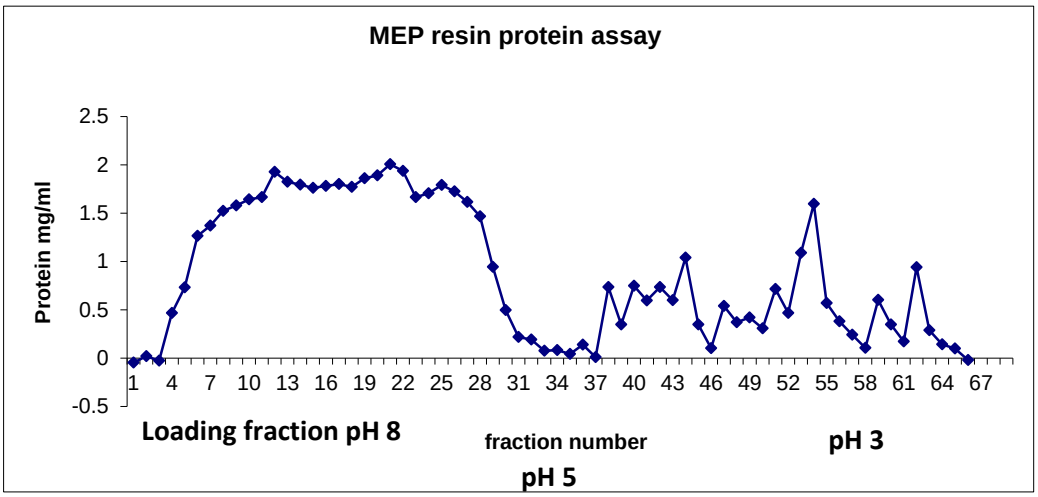
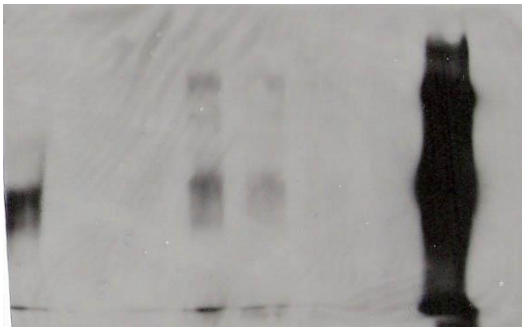
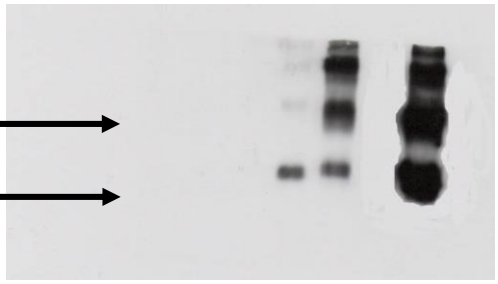


Fig. 8. Improved method of purification of OX40L-IgG by MEP resin. B9B8E2 cell culture supernatants were mixed up with same volume of the loading buffer (50 mM tris buffer pH 8) and then loaded onto MEP resin after adjusting the pH to 8. The column was washed with the loading buffer until the absorbance at 280 was stabled at 0.01. The column was first eluted with the elution buffer (50 mM sodium acetate pH) at pH 5 followed by pH 4.5 and pH 3.

In the second step, to confirm the conditions of the purification, the samples were loaded at pH 8. Then, the resin was eluted, after washing with the loading buffer, with the elution buffers of pH 5, 4.5, and 3 continuously (Figure 8). Using this method, the OX40L-IgG fusion protein was successfully purified at pH 4.5. The presence of OX40L in the elution fractions was confirmed by western blotting using anti-OX40L antibodies (Figure 9A). To evaluate the purity of the OX40L, the MEP purified samples were also checked for the presence of mouse IgG, bovine IgG, and bovine albumin (Figure 9B, C, D).

<table>
<tr><td colspan="3">A: OX40L purified by MEP resin probed by anti-OX40L antibody</td></tr>
<tr><td>50 KD

22 KD</td><td> 
1 2 3 4 5 6 7 8</td><td>1. Unpurified sample
2. Loading fraction
3. Fractions eluted at pH 5
4. Fractions eluted at pH 4.5
5. Fractions eluted at pH 4.5
6. Fractions eluted at pH 3
7. Fractions eluted at pH 3
8. MM1</td></tr>
<tr><td></td><td>B: Mouse IgG</td><td></td></tr>
<tr><td>50 KD

22 KD</td><td> 
2 3 4 5 6 7 8</td><td>1. Loading fraction
2. Loading fraction
3. Fractions eluted at pH 5
4. Fractions eluted at pH 5
5. Fractions eluted at pH 4.5
6. Fractions eluted at pH 4.5
7. Fractions eluted at pH 3
8. MM1</td></tr>
<tr><td></td><td>C: Bovine IgG</td><td></td></tr>
<tr><td>50 KD

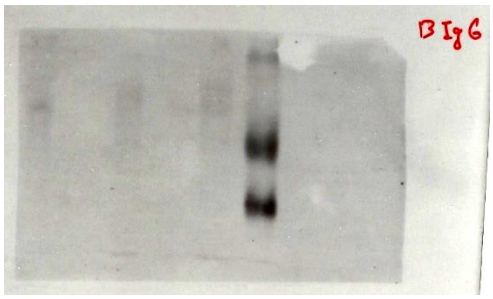
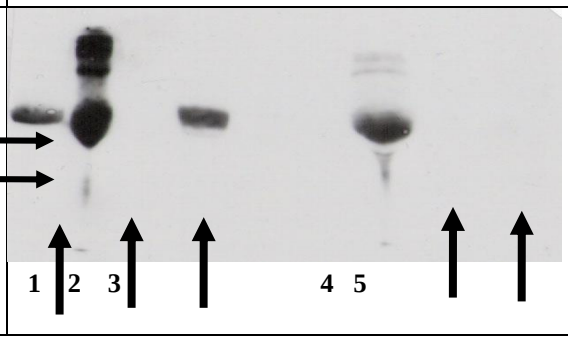
22 KD</td><td> 
2 3 4 5 6</td><td>1.Unpurified fraction
2. Loading fraction
3. Fractions eluted at pH 5
4. Fractions eluted at pH 4.5
5. Fractions eluted at pH 3
6. MM1</td></tr>
<tr><td></td><td>D: BSA</td><td></td></tr>
<tr><td>50 KD
22 KD</td><td> 
1 2 3 4 5</td><td>1. Unpurified fraction
2. BSA (positive control)
3. MM1
4. Loading fraction
5. Fractions eluted at pH 4.5</td></tr>
</table>

Fig. 9. Detection of OX40L, mouse IgG, bovine IgG and BSA in samples purified by MEP resin. B9B8E2 cell culture supernatants were loaded at pH 8 (see Materials and Methods) and eluted at pH 5, 4.5 and 3 continuously. The presence of OX40L, mouse IgG, bovine IgG and BSA was checked in the fractions by Western blotting using relevant antibodies.

Purification of OX40L-IgG fusion protein by protein A Sepharose resin

As a standard method for the purification of antibodies, the Protein A Sepharose column was used to purify the OX40L-IgG fusion protein from the B9B8E2cell culture supernatant (Figure 10). B9B8E2 cell supernatant was loaded on the column at pH 7, washed with PBS, and eluted at pH 3. The fractions were analyzed for the presence of OX40L by Western blotting and compared with that of Xenova OX40L (Figure 11). The results clearly showed that purification was affected by the stringency and washing speed between the loading and elution (data not shown). Therefore, loading time, the stringency of wash, and elution were modified to optimize conditions for OX40L purification (data not shown).

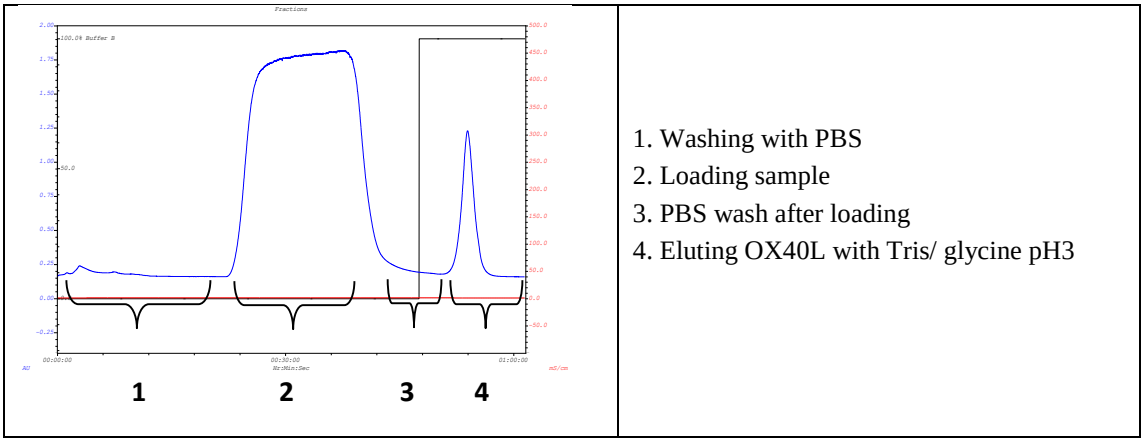


Fig. 10. OX40L purification using Protein A Sepharose Column. Protein A Sepharose resin was washed with PBS and B9B8E2 cell culture supernatants were loaded onto the column. The column was given another PBS wash to remove all unbound proteins. The OX40L bound to the column was then eluted with tris/glycin at pH 3.

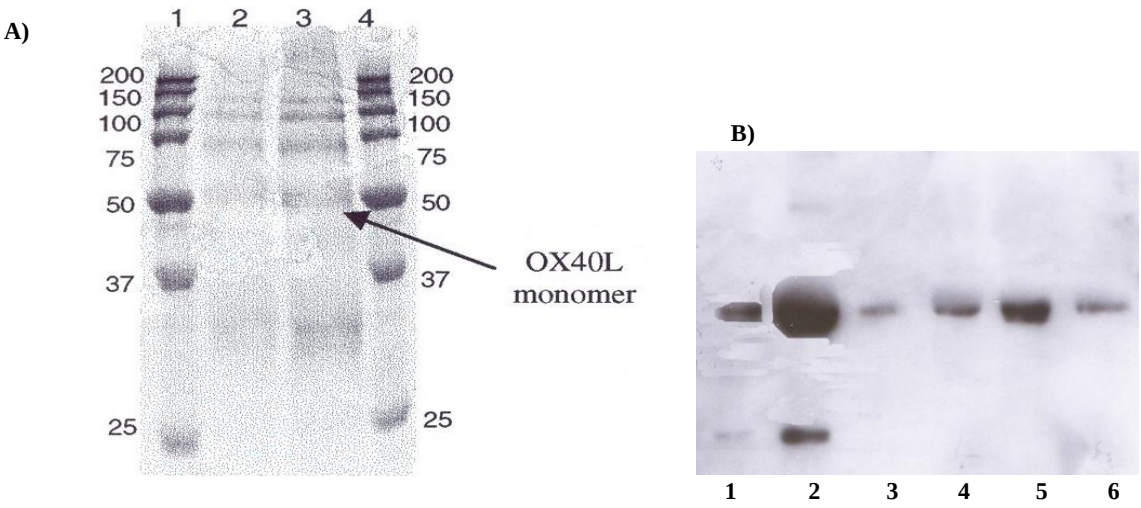
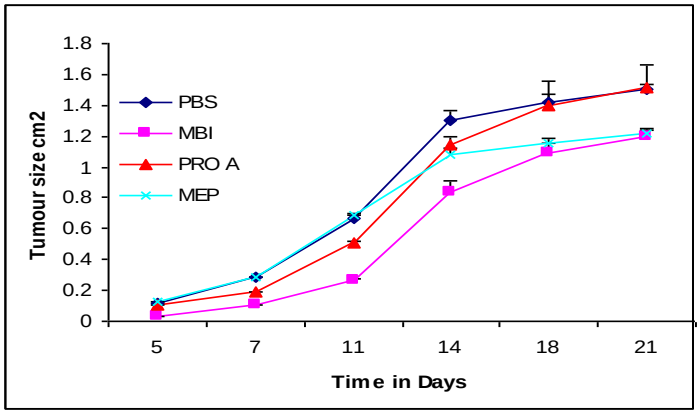


Fig. 11. Purification of OX40L-IgG with protein A Sepharose. A) 9B8E2 cell supernatants were purified by protein A Sepharose resin (see materials and methods). B) Alteration of the time or speed of the wash between loading and elution largely affected the concentration of the OX40L in the purified.

Biological activity of OX40L-IgG

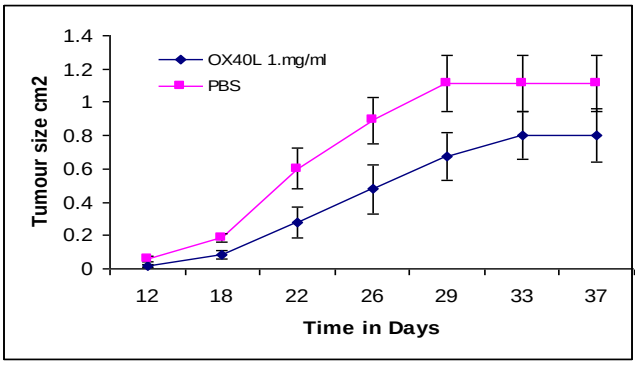
The biological effect of the OX40L purified by MBI and MEP against tumor cell (CT26) growth showed that a significant delay in tumor growth occurred when mice were injected with the OX40L regardless of the resin used for purification. However, tumor progression in mice injected with the OX40L-IgG purified by MBI resin was significantly slower than those injected with the OX40L purified by the other resins. No significant difference was observed between the biological activities of the OX40L purified by MEP and protein A Sepharose resins (Figure 12). The MBI-purified OX40L-IgG was also effective at a concentration of 1.3 mg and 40µg/100 µl/mouse (Figure 13 A & B respectively).



P-value MBI, PBS	0.0020
P-value MEP, PBS	0.047
P-value Pro A, PBS	0.03
P-value MBI, Pro A	0.0026
P-value MBI, MEP	0.018
P-value MEP, Pro A	0.261

Fig. 12. Efficacy of OX40L purified by MBI, MEP or protein A Sepharose resin against the growth of CT26 tumour cells *in vivo*. Four groups of 10 BALB/c mice were implanted S.C. with 2×10^4 CT26 tumor cells and injected I.P. with 500µg per mouse of OX40L-IgG purified by MBI, MEP and protein A Sepharose resins. The fourth group was given 100 µL per mouse PBS. The tumour growth was monitored regularly twice a week. Student t-test was used to analyze the data. The graph represents one experiment.

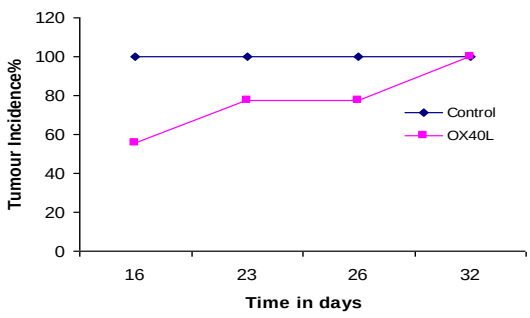
A



P-value 0.0012

1/9 mice tumour free

B



P-value= 0.024

Fig. 13. Effect of OX40L-IgG purified by MBI resin on CT26 tumour cell growth *in vivo*. Two groups of 10 BALB/c mice were injected S.C. with 2 $\times10^4$ CT26 cells. One group was treated intraperitoneally with 1.3 mg (graph A) and 40µg (graph B) OX40L-IgG fusion protein purified by MBI column. The OX40L-IgG protein was injected twice on day 3 and 7. Animals were monitored regularly for tumor growth. Student t-test was used to analyze the data. Bars represent S.E. n=10.

Discussion

Optimal purification for antibodies and other proteins has always been an objective for biomedical researchers. Finding a specific ligand, which can be used for purification and making a fusion protein is used for purification of these proteins using specific resins such as protein A Sepharose. The therapeutic efficacy of the MM1 fusion protein has previously been described in murine tumor models (11). In the present study, two novel resins (MBI & MEP) were used for the first time for the purification of OX40L-IgG fusion protein. To obtain optimum purification, the conditions of loading and elution were optimized and the purity was determined by Western blotting using anti-murine OX40L antibodies. The product's biological activity was also assessed against tumor growth and compared with that of the conventional methods (protein A Sepharose), which were previously described by Xenova plc (Xenova plc MM1 product leaflet).

MBI Hypercel is a resin designed for specific chromatographic capture of antibodies [MBI Hypercel product note]. In a study carried out by Brenac, MBI resin was successfully used for the purification of antibodies from a cell culture supernatant and it was shown that the protein uptake increased when the pH decreased (12). Ye and et al. stated that MBI has a high adsorption effect on antibody protein binding because the degree of regeneration afforded by NaOH 0.1 M was 99.7%. The interaction between IgG and Hydrophobic charge-induction chromatography (HCIC) ligands was investigated by QCM-D (dissipative quartz crystal microbalance) and indicated platform based on the surface of SAMs (self-assembled monolayer) proved to be a powerful tool for studying structures of protein adsorption layers and ligand–protein interactions (13). Our results confirmed that lowering the pH increases the binding of proteins to the resin and as the pH increases, the binding of the OX40L-IgG to the resin is reduced. The maximum binding of proteins including the OX40L-IgG fusion protein to the resin occurred at pH 4; decreased amount of unbound OX40L-IgG was detected during the loading of the sample at pH 4. This was in contradiction with the results of Brenac *et al*. where the complete absorption of their antibody occurred at pH 5. The optimum pH for desorption of OX40L-IgG from the resin was pH 9.5, similar to Brenac *et al*. (12). These results indicated that for the purification of each type of protein, conditions of the purification by MBI resin need to be optimized.

The purity of the purified product was determined by testing representative samples for contamination with BSA and bovine IgG. When samples were loaded at pH 4, the BSA was completely bound to the resin and there was no unbound BSA released during the loading phase (as determined by Western blotting). When the loading pH was increased to 5.2, no BSA was bound to the resin. Therefore, loading the samples at pH 5.2 resulted in purer OX40L-IgG fusion protein, although compared to pH 4, adsorption of OX40L-IgG fusion protein at pH 5.2 was slightly less. This specific pH adsorption (5.2) was in agreement with the results obtained by Brenac *et al*. (5.0 to 6.0) and Girot *et al*. (5.0 to 5.5) (12, 14). No bovine IgG binding was detected under the same conditions. According to the data sheets produced by Xenova plc, MM1 has been produced through the purification of the cell supernatant by protein A-Sepharose resin. Thus, there may be a selectivity for binding of different proteins to MBI resin where it is not for protein A Sepharose resin, however, this aspect requires further investigation. MEP Hypercel resin is a hydrophobic charge interaction chromatography resin designed for the purification of antibodies. It is shown that MEP Hypercel

has a negative charge at pH 7 or above and a positive charge at lower pH; therefore, proteins bind to the resin at pH above 7 whereas they start eluting at pH below 7. This resin has successfully been used for the purification of monoclonal antibodies (9, 15). In the present study, MEP resin was used for the purification of the OX40L-IgG fusion protein. To optimize the elution pH, a range of different pHs were tested, showing that the OX40L-IgG fusion protein is eluted only at pH 4.5. Testing the product for BSA and bovine IgG revealed that the MEP-purified OX40L was free of both BSA and bovine IgG, neither of which was detected in the purified samples by Western blotting indicating selectivity in the purification.

Additionally, OX40L-IgG was purified from B9B8E2 cell culture supernatant by protein A Sepharose column. Purification by protein A-Sepharose resin works based on the affinity of the FC portion of the fusion protein for protein A. It was shown that increasing the course or the speed of the wash after loading the sample caused the loss of the majority of the OX40L in samples. This clearly shows that protein A Sepharose resin has the potential to bind to the OX40L-IgG molecules but the binding is very weak. Therefore, the speed of sample flow through the resin or increasing the course of wash results in a reduction in the number of purified OX40L-IgG molecules. It has been shown that the OX40L can enhance T cells immunity (11), however, the effect of the method of purification on the biological activity of the OX40L has not yet been fully investigated. This study was also to investigate the effect of the method of purification on the biological activity of the OX40L-IgG fusion protein.

The biological activity of the OX40L purified by MBI, MEP, and protein A Sepharose resins were compared in a tumor growth inhibition experiment in a BALB/c mouse model. Mice treated with the same doses of the OX40L purified by the three resins showed a significant delay in tumor growth compared to the controls injected with PBS. Mice treated with the OX40L purified by MBI resin showed a significant delay in tumour growth compared with mice injected with OX40L purified by other resins suggesting a greater biological activity for the product purified by using the MBI resin. Better biological activity for the OX40L purified by MBI may be due to less harsh conditions of purification; in MBI resin since the samples were loaded at pH 5.2 (less acidic) and eluted at pH 9.5 (basic) whereas in MEP and protein, A Sepharose resins the samples were loaded at pH 8 or 7 and eluted at 4.5 or 3 (highly acidic). It has been observed that proteins at low pH are denatured and their tertiary or even secondary structures are changed (16). Another study performed for the purification of proteins by MBI resin showed that this resin has a very high binding capacity, which should be a result of its higher ligand density and dynamic binding capacity for IgG compared with other commercial membrane adsorbers (17). Then, the elution of the OX40L at very low pHs may have negative effects on its biological activity by altering the molecular structure of the protein. Further studies are needed to confirm these findings.

Acknowledgements

We would like to thank BU-Ali Sina University and the Nottingham Trent University for their financial support and supplying the materials.

References

1. Godfrey WR, Fagnoni FF, Harara MA, et al. Identification of a human OX-40 ligand, a costimulator of CD4+ T cells with homology to tumor necrosis factor. J Exp Med 1994;180:757-62.

2. Croft M, So T, Duan W, et al. The significance of OX40 and OX40L to T-cell biology and immune disease. Immunol Rev 2009;229:173-91.

3. Lane PJ, McConnell FM, Anderson G, et al. Evolving strategies for cancer and autoimmunity: back to the future. Front Immunol 2014;5:154.

4. Gramaglia I, Jember A, Pippig SD, et al. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. J Immunol 2000;165:3043-50.

5. Barrios CS, Johnson BD, D. Henderson J J, et al. The costimulatory molecules CD80, CD86 and OX40L are up-regulated in Aspergillus fumigatus sensitized mice. Clin Exp Immunol 2005;142:242-50.

6. Wang Q, Chen Y, Ge Y, et al. Characterization and functional study of five novel monoclonal antibodies against human OX40L highlight reverse signalling: enhancement of IgG production of B cells and promotion of maturation of DCs. Tissue Antigens 2004;64:566-74.

7. Weimer BC, Walsh MK, Wang X. Influence of a poly-ethylene glycol spacer on antigen capture by immobilized antibodies. J Biochem Biophys Methods 2000;45:211-9.

8. Bak H, Thomas OR. Evaluation of commercial chromatographic adsorbents for the direct capture of polyclonal rabbit antibodies from clarified antiserum. J Chromatogr B Analyt Technol Biomed Life Sci 2007;848:116-30.

9. Mowry MC, Meagher M, Smith L, et al. Production and purification of a chimeric monoclonal antibody against botulinum neurotoxin serotype A. Protein Expr Purif 2004;37:399-408.

10. Nopper B, Kohen F, Wilchek M. A thiophilic adsorbent for the one-step high-performance liquid chromatography purification of monoclonal antibodies. Anal Biochem 1989;180:66-71.

11. Ali SA, Ahmad M, Lynam J, et al. Anti-tumour therapeutic efficacy of OX40L in murine tumour model. Vaccine 2004;22: 3585-94.

12. Brenac V, Ravault V, Santambien P, et al. Capture of a monoclonal antibody and prediction of separation conditions using a synthetic multimodal ligand attached on chips and beads. J Chromatogr B Analyt Technol Biomed Life Sci 2005;818:61-6.

13. Ye J, Zhang Y, Meng J. Protein–Ligand interactions for hydrophobic charge-induction chromatography: A QCM-D study. Appl Surf Sci 2022;572:151420.

14. Girot P, Averty E, Flayeux I, et al. 2-Mercapto-5-benzimidazolesulfonic acid: an effective multimodal ligand for the separation of antibodies. J Chromatogr B Analyt Technol Biomed Life Sci 2004;808:25-33.

15. Dux MP, Barent R, Sinha J, et al. Purification and scale-up of a recombinant heavy chain fragment C of botulinum neurotoxin serotype E in Pichia pastoris GS115. Protein Expr Purif 2006;45:359-67.

16. Poklar N, Lah J, Salobir M, et al. pH and temperature-induced molten globule-like denatured states of equinatoxin II: a study by UV-melting, DSC, far- and near-UV CD spectroscopy, and ANS fluorescence. Biochemistry 1997;36:14345-52.

17. Ma N, Yao D, Yang H, et al. Surface modification of cellulose membranes to prepare a high-capacity membrane adsorber for monoclonal antibody purification via hydrophobic charge-induction chromatography. Ind Eng Chem Res 2018;57:13235-46.