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Recombinant proteins L and LG: efficient tools for purification of murine immunoglobulin G fragments

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Abstract

In order to improve antibody purification methods, recombinant proteins L and LG were tested in the purification of murine monoclonal immunoglobulin G (IgG) and its fragments. After affinity constant evaluation in different buffer systems, high-performance affinity chromatographic columns were prepared by coupling the proteins to Affi-prep 10 resin and tested with eight different murine monoclonal antibodies and their fragments of different isotypes. Affinity chromatographic experiments confirmed radioimmunoassay results showing that protein L bound 75% of the tested antibody fragments whereas protein LG had affinity for all the tested fragments. These results demonstrate that protein LG is the most powerful Ig-binding tool so far described.

1. Introduction

Several chromatographic methods have been proposed for the purification of monoclonal antibodies, such as gel permeation and ion-exchange methods [1–4]. However, with the discovery of immunoglobulin (Ig)-binding bacterial cell wall proteins, affinity chromatography has become the most successful procedure.

Protein A was the first IgG binding protein to be discovered [5]. The pH dependence and specificity of the antibody–protein A interaction have made protein A affinity chromatography one of the most commonly used methods for antibody purification.

Forsgren and Sjöquist [5] showed that protein A binds to the Fc region of the antibody molecule. The Fc domains of different classes and subclasses of antibodies are different. Thus, affinity of protein A for antibodies varies as a function of immunoglobulin class, subclass and species.

Protein A, present in the cell wall of *Staphylococcus aureus* bacteria, has affinity for most animal IgG. An exception is mouse IgG1 at physiological conditions [6]. However, in 1978, Ey et al. [7] reported binding of IgG1 to protein A by raising the pH of the binding buffer to 8.1. Under this condition, 98% of IgG1 was retained by the resin and could then be eluted at pH 6.0. This mild acidic elution was possible since the mouse IgG1–protein A interaction is still weak

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at pH 8.1 and at physiological ionic strength. In recent years, many groups have studied the interaction between Igs and protein A, and have demonstrated that in order to obtain optimum binding conditions, it was essential to raise both the pH and the ionic strength of the buffer. Protein G, another IgG-binding protein, was isolated by Björck and Kronvall [8] in 1984 from group C and G streptococci. The interest in protein G lies in the fact that the molecule has a different affinity for antibodies as compared with protein A. IgG antibodies from species or subclasses weakly interacting with protein A could in many cases be efficiently bound by protein G.

One drawback concerning protein G is that it contains a second binding site interacting with albumin. In 1986, Fahnestock et al. [9] and Guss et al. [10] separated the antibody-binding domain from the albumin domain by genetic manipulations, thus increasing the usefulness of recombinant protein G in antibody purification.

In 1988, Björck [11] isolated protein L from a strain of the anaerobial bacterial species *Peptostreptococcus magnus*. Protein L is a bacterial cell wall protein with binding specificity for the variable domain of human kappa light chain [12]. This binding specificity makes protein L a potential tool for purification of immunoglobulins regardless of their isotype. In 1992, Kastern et al. [13] expressed in *Escherishia coli* a protein containing four of the five kappa-binding protein L domains. More recently, Kihlberg et al. [14] obtained a fusion peptide, protein LG, containing the four repeats of the Ig-binding domain of r-protein L and two IgGFc-binding domain of protein G.

In this study, proteins L and LG were applied to the purification of murine monoclonal antibodies and their fragments.

2. Experimental

2.1. Immunoglobulins

Experiments were performed with eight murine monoclonal antibodies and their bivalent fragments. The selected immunoglobulins belonged to IgG1 and IgG2a isotypes and all had kappa light chains: B72.3 (IgG1,k) to TAG-72 [15], F023C5 and F043A2 (IgG1,k) to CEA [16], AR-3 (IgG1,k) to CAR3 [17], MOv-18 (IgG1,k) to M_r 38 000 folate-binding protein [18], 763.74T (IgG1,k) and 225.28S (IgG2a,k) to HMW-MAA [19] and D612 (IgG2a,k) to colorectal carcinoma [20].

All monoclonal antibodies were produced by growing hybridomas in mouse ascites and were purified by a two-step HPLC method combining protein A and hydroxyapatite chromatography [21].

IgG2a $F(ab)_2$ were obtained by conventional pepsin digestion and IgG1 $F(ab)_2$ were obtained by ficin digestion [22]. Digestion mixtures were purified by size-exclusion chromatography, using a 600 × 55 mm I.D. Bio-Sil SEC 250 column (Bio-Rad Labs., Richmond, CA, USA) previously equilibrated in 0.1 M citrate buffer (pH 5.0).

2.2. Protein L and protein LG

Protein L was a recombinant peptide produced in *E. coli* by expression of a gene fragment covering four of the five kappa-binding protein L domains [13]. Protein LG was a hybrid molecule obtained by combining the four kappa-binding domains of protein L with two IgGFc-binding domains of protein G [14]. Protein L and LG were both purified from high expression systems as described [13,14].

2.3. Radiolabelling of protein L and protein LG

Purified protein L and protein LG were radiolabelled with ^{125}I using the Iodogen method [23]. A 50–100-μg amount of protein was reacted for 10 min with 800 μCi of carrier-free $Na^{125}I$ (Amersham International, Amersham, UK) in the presence of 10 μg of Iodogen coated on a vial wall (Pierce Eurochemie, Oud-Beijerland, Netherlands). Labelled proteins were purified by ion-exchange chromatography on a Dowex AG 1-X8 resin (Bio-Rad Labs.) column (10 × 8 mm

I.D.) to remove unreacted iodine. The integrity of the radiolabelled proteins was checked by size-exclusion HPLC.

2.4. Affinity constant determination

Affinity constants of protein L and protein LG for IgG and $F(ab)_2$ were evaluated by non-linear regression analysis of radioimmunoassay data using the BIND program [24].

For the assay, Microvil 96-well plates (Dynatech Labs., Alexandria, VA, USA) were coated with 100 μl of a 10 μg/ml IgG or $F(ab)_2$ solution in phosphate-buffered saline (PBS) (pH 7.4). After overnight incubation at 4°C, the plates were rinsed with PBS containing 0.125% (v/v) Tween 20 (PS–Tween) and saturated with 250 μl per well of PBS containing 1% (w/v) gelatin (PBS-gelatin). A 50 μl aliquot of serially diluted protein L or protein LG in different buffers was added to the coated wells together with 50 μl of ^{125}I-labelled protein L or protein LG. The following buffers, each added with 2.5% (w/v) BSA, were used in the assay: (a) 1.5 *M* glycine–3.0 *M* NaCl (pH 9.0) (glycine buffer), 1.0 *M* Tris (pH 8.0) (Tris buffer) or 10 m*M* sodium phosphate–0.15 *M* NaCl (pH 7.4) (PBS) as possible binding buffers, and (b) 0.1 *M* sodium citrate (pH 3.0) (citrate buffer) as a possible elution buffer. After overnight incubation at 4°C and repeated washes with PBS–Tween, the wells were cut out and counted in a gamma counter.

2.5. Affinity chromatography

Protein L and protein LG chromatographic columns were prepared by coupling proteins with Affi-prep 10 resin (Bio-Rad Labs.). A volume of 1.5 ml of resin was washed with 10 m*M* acetate buffer (pH 4.5) and transferred into a reaction vessel with 12 ml of a 1 mg/ml solution of protein L or protein LG in 10 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) pH 8.0). The suspension obtained was gently stirred for 1 h at room temperature. After overnight incubation at 4°C, the remaining active ester groups were blocked with 1 ml of 1 *M* ethanolamine (pH 8.0) while keeping the suspension for 1 h at 4°C. The resin was then washed with 30 ml of 0.5 *M* NaCl and equilibrated in PBS (pH 7.4). The resin obtained was packed into an HR 5/5 column (50 × 5 mm I.D.) (Pharmacia, Uppsala, Sweden) using an HPLC pump system (Waters 600E; Millipore, Milford, MA, USA). Packing was performed with a flow-rate from 0.1 up to 2.0 ml/min in about 3 h, then the percentage coupling yield between Affi-prep 10 resin and protein was determined.

Affinity chromatographic runs with protein L and protein LG columns were performed on a Waters 600E chromatography injecting a 1-ml sample in the 0.5–1.0 mg/ml concentration range and setting the pumps at a flow-rate of 1.0 ml/min. The separation was monitored by on-line UV detection at 280 nm. The two peaks obtained in each chromatogram (flow-through and elution) were separately collected and dialysed against PBS to determine their concentration by the BCA method (Pierce Eurochemie).

2.6. Eluent selection

In order to select the elution buffer to be used in chromatography, Microvil 96-well plates were coated with 150 μl of a 100 μg/ml B72.3 $F(ab)_2$ solution in PBS. After incubation for 3 h at 37°C, the plate was washed with PBS-Tween and saturated with 250 μl per well of PBS–gelatin.

After washing, 10 μl (100 000 cpm) of ^{125}I-labelled protein LG in PBS containing 2.5% BSA (PBS–BSA) were added to the coated wells, together with 140 μl of the following buffers: 0.1 *M* sodium citrate (pH 3.0); 0.1 *M* sodium citrate (pH 2.0); 0.1 *M* glycine–0.1 *M* NaCl (pH 2); 0.1 *M* glycine–0.1 *M* NaCl (pH 2.5); 0.1 *M* glycine–0.5 *M* NaCl (pH 2); 0.1 *M* glycine–0.5 *M* NaCl (pH 2.5); 0.1 *M* glycine–1 *M* NaCl (pH 2.0); 0.1 *M* glycine–1 *M* NaCl (pH 2.5); 0.1 *M* sodium citrate (pH 2.5); PBS (pH 7.4).

After 3 h at 37°C and repeated washing with

PBS–Tween, the wells were cut out and the radioactivity measured in a gamma-counter.

3. Results and discussion

3.1. Protein L and protein LG affinity determination

Table 1 summarizes the general binding characteristics of the bacterial cell wall Ig-binding proteins. The binding characteristics of protein L and protein LG to murine IgG and $F(ab)_2$ were first evaluated by determining their affinity constants for the different selected murine immunoglobulins and their fragments. The values obtained are reported in Table 2.

The experiments with different buffers allowed the evaluation of the binding capacities of the two proteins to IgG and $F(ab)_2$ by measuring the binding variations at different pH and ionic strength. Protein L could bind six $F(ab)_2$ out of eight tested, as reported in Table 2. Protein L could weakly bind $F(ab)_2$ 225.28S in glycine buffer and not in other buffers; AR-3 $F(ab)_2$ showed no binding in any of the buffers.

The different buffers were chosen in order to select the best binding buffer to be used in chromatographic experiments in which citrate buffer (pH 3.0) was tested as a possible elution buffer. Among the different buffer systems tested, the highest binding values, expressed as the percentage ratio of bound cpm to total added cpm (% B/T), were obtained using glycine buffer (Fig. 1). Slightly lower binding values were obtained in PBS. As expected, the acid pH of citrate buffer gave the lowest binding values. These results are comparable to those obtained when protein L was reacted with whole IgG molecules (Table 2), although in that case the affinity constant values appeared to the lower than those obtained for $F(ab)_2$.

Protein LG gave markedly different results. In fact, protein LG could bind all $F(ab)_2$ tested, with significant improvements in affinity constant values with respect to protein L. As shown in Table 2, protein LG could also bind 225.28S and AR-3 $F(ab)_2$, which were not bound by protein L.

The affinity constant values of protein LG for IgG in glycine buffer ranged from $2.5 \cdot 10^7$ to $2.08 \cdot 10^8$ l/mol with a mean value of $(1.08 \pm 0.67) \cdot 10^8$ l/mol. The affinity constant values under the same conditions of protein L ranged from $6 \cdot 10^5$ to $1.18 \cdot 10^7$ l/mol with a mean value of $(5.5 \pm 5.1) \cdot 10^6$ l/mol. Such a difference is also evident for $F(ab)_2$ binding. In this case, the affinity constant values of protein LG ranged from $1.06 \cdot 10^7$ to $3.0 \cdot 10^8$ l/mol with a mean value of $(1.21 \pm 1.0) \cdot 10^8$ l/mol. The protein L values ranged from $1.3 \cdot 10^5$ to $3.45 \cdot 10^8$ l/mol with a mean value of $(7.8 \pm 12.05) \cdot 10^7$ l/mol.

These results indicate that protein L had a higher affinity for fragments than for whole IgG. Protein LG, however, showed the same affinity for IgG and IgG fragments. As regards binding to whole IgG, protein LG gave more homogeneous binding results. This feature makes protein LG the best candidate as a general affinity ligand.

Table 1
Characteristics of bacterial cell wall Ig-binding proteins

Protein	Source	M_r ($\times 10^{-3}$)	IgG-binding sites	Murine IgG binding (%)
A	*Staphylococcus aureus*	42	Fc + VH_{III}	50–100[a]
G	*Streptococcus* group C, G	59	Fc + Fab[b]	100
rG	*E. coli*	22	Fc	100
L	*Peptostreptococcus magnus*	76	Vk	70
rL	*E. coli*	36	Vk	70
rLG	*E. coli*	50	Vk + Fc + Fab	100

[a] Depending on IgG isotypes and binding conditions.
[b] Additional binding site for albumin.

Table 2
Protein L and protein LG affinity constants for murine F(ab)$_2$ and IgG in different binding buffers

	Affinity constant ($\times 10^{-7}$)			
	Glycine	TRIS	PBS	Citrate
Protein L, F(ab)2				
225.28S	1.66	n.d.[a]	n.d.	n.d.
AR-3	n.d.	n.d.	n.d.	n.d.
D612	12.80	2.26	3.37	n.d.
MOv-18	1.63	1.73	0.66	n.d.
763.74T	12.90	1.29	3.01	n.d.
B72.3	34.50	5.72	18.30	n.d.
F023A2	0.19	0.16	0.12	n.d.
F023C5	0.13	0.32	0.15	n.d.
K_{aff} (mean ± S.D.)	$(7.98 \pm 12.05) \cdot 10^{7}$	$(1.43 \pm 1.93) \cdot 10^{7}$	$(3.20 \pm 6.25) \cdot 10^{7}$	n.d.
Protein L, IgG				
225.28S	n.d.	n.d.	n.d.	n.d.
AR-3	n.d.	0.33	0.23	n.d.
D612	1.01	0.76	0.94	n.d.
MOv-18	1.11	0.27	0.59	0.12
763.74T	0.70	0.89	0.33	n.d.
B72.3	0.30	0.47	0.16	n.d.
F023C5	1.18	0.42	0.20	n.d.
F043A2	0.06	0.41	0.48	n.d.
K_{aff} (mean ± S.D.)	$(5.51 \pm 5.10) \cdot 10^{6}$	$(4.44 \pm 2.78) \cdot 10^{6}$	$(3.66 \pm 2.97) \cdot 10^{6}$	n.d.
Protein LG, F(ab)$_2$				
225.28S	1.06	0.79	1.94	1.48
AR-3	1.65	0.77	1.43	0.37
D612	6.64	7.57	18.60	0.32
MOv-18	21.60	9.39	20.60	0.89
763.74T	13.80	8.46	12.50	3.46
B72.3	30.00	36.00	18.80	0.53
F023C5	6.74	8.63	4.63	n.d.
F043A2	15.30	5.50	10.10	1.59
K_{aff} (mean ± S.D.)	$(1.21 \pm 1.01) \cdot 10^{8}$	$(0.96 \pm 1.11) \cdot 10^{8}$	$(1.10 \pm 0.78) \cdot 10^{8}$	$(1.08 \pm 1.11) \cdot 10^{7}$
Protein LG, IgG				
225.28S	6.10	2.95	3.25	n.d.
AR-3	2.92	3.90	4.88	1.67
D612	13.10	13.40	16.80	0.78
MOv-18	17.20	24.40	15.70	1.61
763.74T	14.60	2.20	16.10	1.61
B72.3	8.86	14.50	22.00	n.d.
F023A2	2.50	1.99	4.06	2.38
F023C5	20.80	26.30	18.60	n.d.
K_{aff} (mean ± S.D.)	$(1.08 \pm 0.67) \cdot 10^{8}$	$(1.42 \pm 1.05) \cdot 10^{8}$	$(1.27 \pm 0.94) \cdot 10^{8}$	$(1.01 \pm 0.94) \cdot 10^{7}$

[a] n.d. = Not determined.

3.2. Protein L and protein LG affinity chromatography

Protein L and protein LG chromatographic columns were prepared according to the above-described procedure. Protein L gave about a 20% coupling yield and protein LG about 18%.

The binding buffer selected for murine IgG and F(ab)$_2$ affinity chromatography on these two columns was glycine buffer (pH 9.0). The selec-

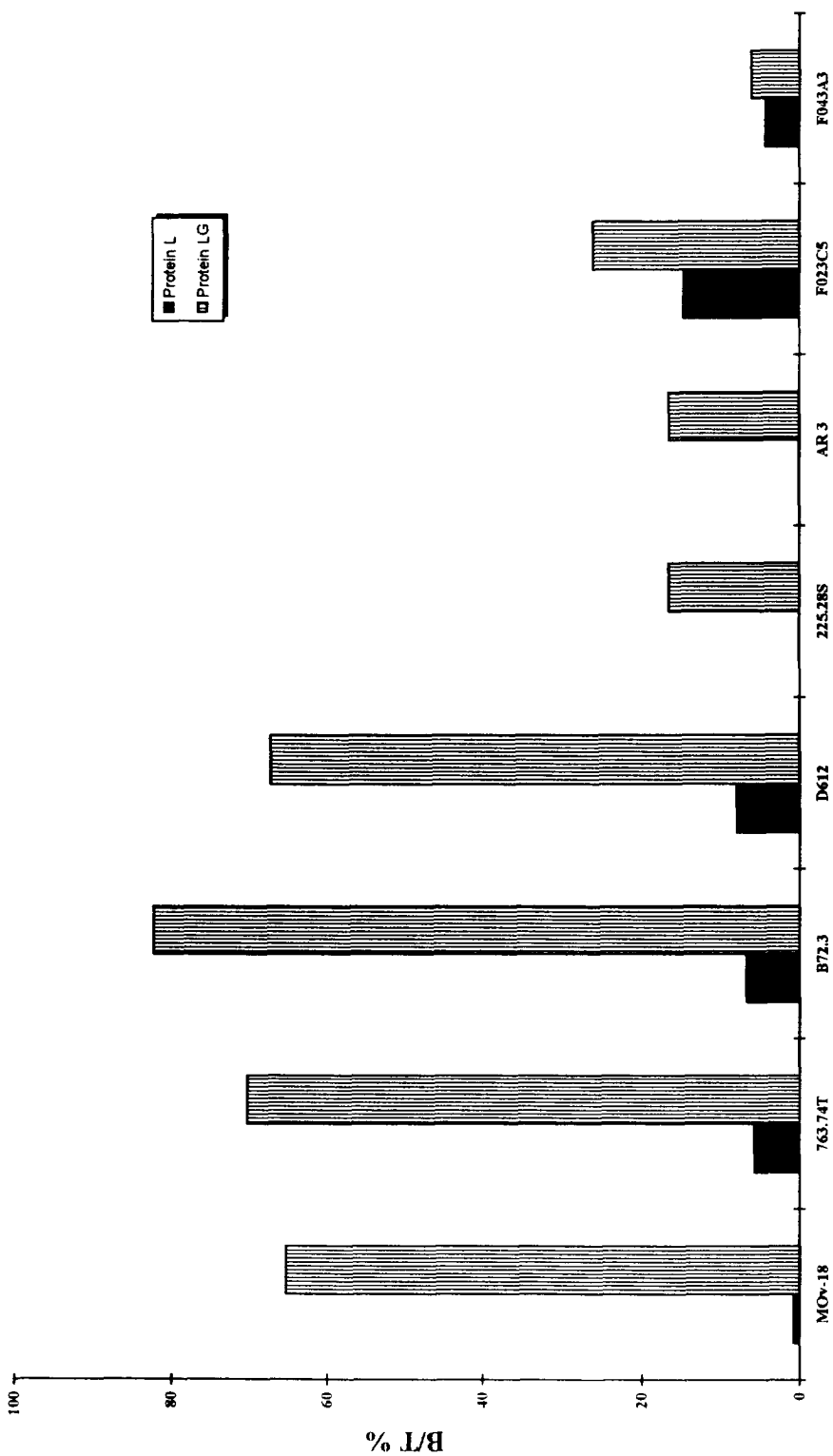

Fig. 1. Protein LG (shaded bars) and protein L (solid bars) binding to different murine antibody fragments. The histogram shows the results obtained in a radioimmunoassay experiment using glycine buffer as a binding buffer. The binding values obtained are expressed as the percentage ratio of bound cpm to total added cpm (% B/T).

tion was made on the basis of the results of affinity constant determination. On the same basis, citrate buffer (pH 3.0) could not be selected as the elution buffer as consistent binding was still recorded at that pH and ionic strength. The eluent was selected as described under Experimental and Fig. 2 illustrates the results obtained. The selected elution buffer corresponding to the lowest binding obtained was 0.1 *M* citrate (pH 2.0).

The experiments indicated that hydrophobic interactions contribute to the binding of protein LG to Ig. Thus, the binding was more affected when the ionic strength of the buffer was decreased than when the pH was lowered. Therefore, affinity chromatography of IgG and $F(ab)_2$ on protein L and protein LG columns was performed using glycine (pH 9.0) as a binding buffer and 0.1 *M* citrate (pH 2) as elution buffer.

Fig. 3 shows an example of the chromatograms obtained, and Table 3 gives the calculated recoveries and the yields. The yield was expressed as the percentage ratio of eluted IgG or $F(ab)_2$ to total IgG or $F(ab)_2$ recovered.

Protein L and protein LG affinity chromatography of murine IgG and $F(ab)_2$ confirmed the data obtained with binding experiments and affinity constant determinations. The process yields obtained with the protein LG column varied from 49.8% for 225.28S IgG up to 96.9% for D612 IgG. The protein LG column could bind all injected samples, showing yields in good agreement with radioimmunoassay data. In contrast, the protein L column failed to bind even a

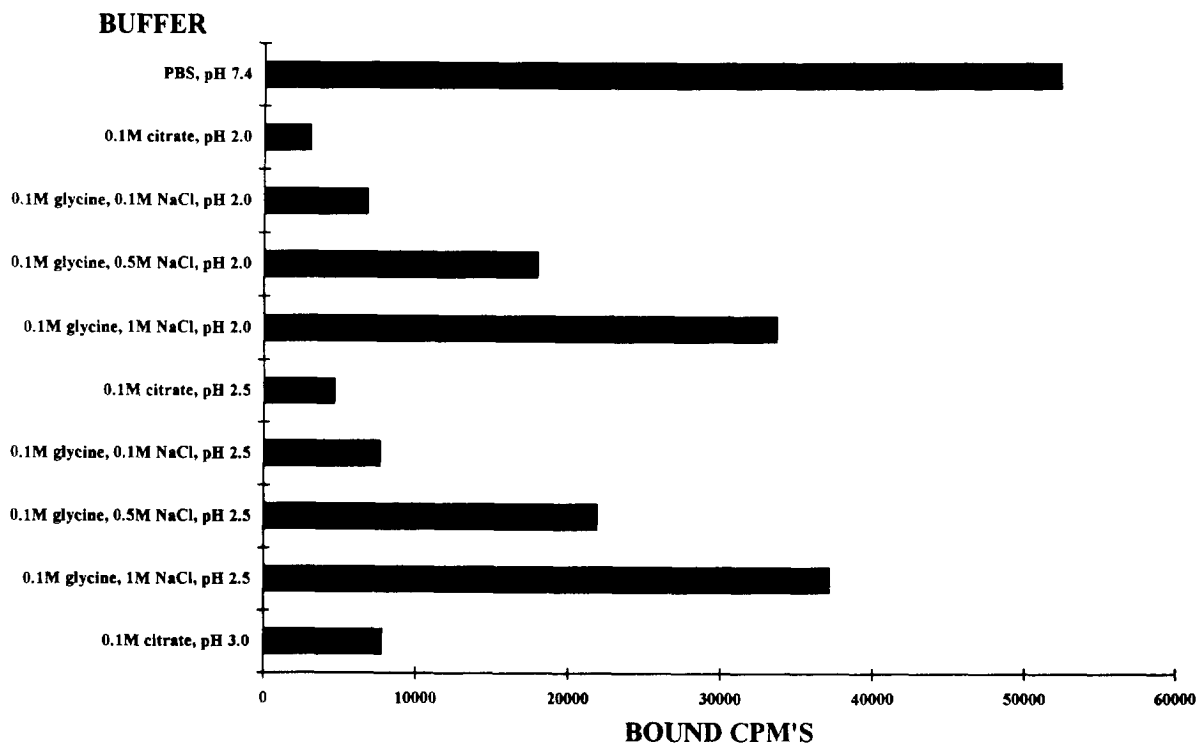

Fig. 2. Selection of elution buffer: ^{125}I-labelled protein LG binding to B72.3 $F(ab)_2$ in different elution buffers. Binding is expressed as bound cpm after 100 000 cpm addition and subsequent 3-h incubation at 37°C and repeated washing.

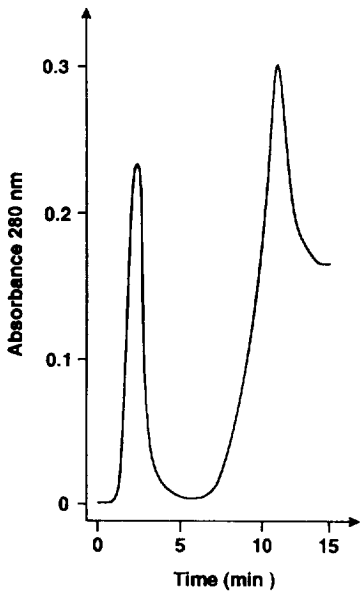

Fig. 3. Affinity chromatography of B72.3 F(ab)$_2$ on a protein LG column. The first peak (t_R = 1.17 min) is the unbound F(ab)$_2$, while the second peak (r = 13.77 min) is the eluted F(ab)$_2$. The higher baseline with the elution buffer is due to the decreased ionic strength of the buffer.

small percentage of two antibodies, and the same antibodies were also unbound in the radioimmunoassay experiments (see Tables 2 and 3). The process yields obtained with protein L columns varied from 5.8% for MOv-18 IgG up to 50.7% for F023C5 F(ab)$_2$.

In order to study the specificity of the interaction of protein LG with IgG, a series of injections were peformed on a protein LG column. A 500-μg amount of bovine serum albumin loaded on the protein LG column was not bound to the column. All albumin injected was collected in the flow-through fraction. Also, when 1 ml of murine ascitic fluid containing B72.3 IgG was injected on to the protein LG column, pure IgG were obtained in the eluted fraction, while the flow-through fraction contained all ascitic fluid impurities.

A preliminary experiment was performed to evaluate the protein LG capacity for binding recombinant fragments of murine IgG. A 500-μg amount of a purified scFv-cys anti-lysozyme (kindly provided by Dr. D. Neri, MRC, Cambridge, UK) was loaded on to the protein LG column under the same chromatography conditions as used for IgG and F(ab)$_2$. The estimates of flow-through and eluate showed that 63.7% of injected scFv was eluted from the protein LG affinity column. This result was confirmed by a solid-phase radioimmunoassay activity test of collected fractions and by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Conclusions

We have described the application of protein L and protein LG in the affinity purification of murine IgG and IgG fragments. These proteins differ from other bacterial cell wall proteins, just like protein A and protein G, because of the different binding sites to the IgG molecule. In fact, whereas the protein A and G elective binding site for IgG is in the Fc region, protein L binds the variable domain of kappa light chain selectivity. For this characteristic protein L and LG bind not only IgG, like protein A and protein G, but also F(ab)$_2$, Fab and recombinant Fv fragments. The aim of these affinity experiments was to evaluate the possibility of using protein L and LG as tools for murine antibody fragment purification.

The results show that protein L binds 75% of murine IgG and F(ab)$_2$ tested, whereas protein LG binds 100% of the preparations. Thus, protein L and LG were found to be powerful tools for the purification of murine antibodies and antibody fragments.

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Table 3
Protein L and LG affinity chromatography BCA determination of collected fractions

	Flow-through (μg)	Eluted (μg)	Total recovered (μg)	Yield (%)
(*Protein L, $F(ab)_2$*)				
225.28S	454	–	454	–
AR-3	605	–	605	–
D612	296	180	476	37.8
Mov-18	763	75	811	9.2
763.74T	410	60	470	12.7
B72.3	462	232	694	33.4
F043A2	199	281	480	58.5
F023C5	180	185	365	50.7
(*Protein L, IgG*)				
225.28S	482	–	482	–
AR-3	561	–	561	–
D612	312	204	516	39.5
MOv-18	840	52	892	5.8
763.74T	539	416	955	43.6
B72.3	395	475	870	54.6
F043A2	269	394	663	59.4
F023C5	320	105	425	24.7
(*Protein LG, $F(ab)_2$*)				
225.28S	197	257	454	56.6
AR-3	116	366	482	75.9
D612	181	314	495	63.4
Mov-18	105	416	521	79.8
763.74T	170	304	474	64.1
B72.3	116	317	433	73.2
F043A2	264	341	605	56.4
F023C5	52	606	658	92.1
(*Protein LG, IgG*)				
225.28S	304	302	606	49.8
AR-3	121	406	527	77.0
D612	15	472	487	96.9
MOv-18	241	277	518	53.5
763.74T	98	401	499	80.4
B72.3	176	348	524	66.4
F043A2	282	180	462	38.9
F023C5	107	497	604	82.3

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