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An artificial protein L for the purification of immunoglobulins and Fab fragments by affinity chromatography

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Abstract

The development and characterization of an artificial protein L (PpL) for the affinity purification of antibodies is described. Ligand 8/7, which emerged as the lead from a de novo designed combinatorial library of ligands, inhibits the interaction of PpL with IgG and Fab by competitive ELISA and shows negligible binding to Fc. The ligand 8/7 adsorbent ($K_a \sim 10^4\ M^{-1}$) compared well with PpL in binding to immunoglobulins from different classes and sources and, in addition, bound to IgG_1 with κ and λ isotypes (92% and 100% of loaded protein) and polyclonal IgG from sheep, cow, goat and chicken. These properties were also reflected in the efficient isolation of immunoglobulins from crude samples.

Keywords: Immunoglobulins; Protein L; Affinity chromatography; Biomimetic; Ligands

1. Introduction

Antibodies are an important and emergent class of biotherapeutics [1–3], diagnostics [4,5] and purification agents [6,7]. The pool of available antibody structures includes native antibodies but also monoclonal antibodies and genetically engineered related molecules, resulting from modifications of the antibody structure, and designed to ensure high specificity and functionality. The expertise for the isolation of genes encoding human variable regions, and their expression in bacterial systems allied with the introduction of phage-display technology, has simplified enormously the selection of fully human variable domains [8]. The small fragments [scFv, Fab or $F(ab')_2$], containing the antigen-determining regions, are preferred in some therapeutic or diagnostic situations [9,10], and can be used per se or reconstituted into a fully human antibody molecule.

The steps in the manufacturing process for a biotherapeutic must follow GMP guidelines, such that the final product is a "well characterised biologic", with increased probabilities of receiving full acceptance from regulatory entities, such as the US Food and Drug Administration (FDA) [11]. The diversity of antibody structures that can be engineered and the variety of expression systems that can be utilised for their production [12], complicate enormously the efficient isolation of these biotherapeutics. Therefore, the downstream processing of immunotherapeutics has challenged both research and industry, and inspired the combination and exploitation of different types of interactions and separation techniques to minimize costs and increase yields. Nowadays, the purification of antibodies is mostly performed by affinity-based techniques including affinity chromatography [11,13] and non-chromatographic techniques, such as affinity precipitation [14,15] and aqueous two-phase systems [16]. Biospecific affinity ligands, mainly Ig-binding proteins isolated from the surface of bacteria (proteins A, G and L), have been the most popular ligands for antibody purification. However, a new class of pseudobiospecific ligands emerged as a result of the

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development of combinatorial strategies allied with new computational tools for rational design [11]. These low-molecular weight biomimetics aim at being an improved version of the natural affinity ligands [17]. Synthetic affinity ligands possessing non-fissile bonds, such as the family of tailor-made triazine-scaffolded ligands [18], present several advantages over their biological templates, specially in terms of chemical resistance to cleaning-in-place (CIP) and sterilization-in-place (SIP) procedures and the low cost of production [18,19]. Protein L isolated from the surface of *Peptostreptococcus magnus* is an immunoglobulin light chain binding protein, particularly suitable for the purification of scFv, Fab and $F(ab')_2$ [20]. Protein L binds with high affinity (K_d of 1 nM) to a large number of immunoglobulins with κ1, κ3 and κ4 light chains, but not to κ2 and λ subgroups, and thus recognises 50% of human and more than 75% of murine immunoglobulins [21]. The absence of a synthetic equivalent for protein L lead to the development of an artificial protein L ligand [22,23]. Such a ligand has potential applications as an affinity adsorbent and a detection agent for immunoglobulins, and possibly as a therapeutic agent per se. The strategy followed has involved the de novo design of bisubstituted triazine ligands using as a template the interaction between the C^* protein L domain and human Fab fragment (1 HEZ.pdb) [24], the solid-phase combinatorial synthesis of a 169-membered ligand library and the assessment for binding to human IgG and selectivity for the Fab fragment [23,25]. Ligand 8/7 was selected as the lead due to its unique specificity towards the Fab fragment of immunoglobulins, and its chemical structure has been analysed and confirmed by ^1H and ^{13}C NMR and mass spectrometry. In the present study we further characterise the interaction between ligand 8/7 and immunoglobulins, compare the synthetic ligand with protein L, and also evaluate its potential as an affinity adsorbent to purify antibodies from crude extracts.

2. Experimental

2.1. Materials

All materials were of the highest purity available and all the antibodies purchased were of reagent grade. Polyclonal antibodies (human, bovine, chicken, goat, mouse and sheep), human myeloma immunoglobulin (Ig) G_1 with κ and λ light chains, IgM from serum and recombinant protein L were obtained from Sigma (Dorset, UK). Human IgA from plasma, human $F(ab')_2$ and human Fc from serum were purchased from Calbiochem (Nottingham, UK). Human Fab was obtained from MP Biomedicals (Eschwege, Germany). EZ-Link Activated Peroxidase and ImmunoPure Immobilised Protein L were purchased from Pierce Biotechnology (Cheshire, UK). Protein L domain (wild type) was a kind gift from Dr. Michael Gore from the University of Southampton, UK. Crude extracts (goat serum and human sera with high contents of IgM-κ) and semi-purified preparations with human IgA were kind contributions from Eugen Kopp (LMU, Institute für Immunologie, Munich, Germany). The marker used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was the full range rainbow marker from Amersham BioSciences (Buckinghamshire, UK). SDS–PAGE was performed in an X-Cell SureLock from Novex Mini-Gel, Invitrogen (Renfrew, UK) and controlled by a Bio-Rad model 200/0.2 power supply apparatus.

2.2. Preparation of ligand 8/7 adsorbents

Preparation of adsorbents with different alkane-spacer arms (0–6 carbons) and with different ligand concentrations (0–15 μmol/g moist mass gel) was performed as in [26] and the synthesis of ligand 8/7 followed the procedure described previously in [23].

2.3. Effect of pH and ionic strength of the binding buffer

Ligand 8/7 immobilised on to hexanediamine-modified agarose (15 μmol/g moist weight gel) was equilibrated in different binding buffers. For the study of the effect of pH, solutions of phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl) with different pH were prepared—pH 6.0, 6.5, 7.0, 7.4 and 8.0. For the study of effect of the ionic strength, solutions of PBS (10 mM sodium phosphate, pH 7.4) with different salt concentrations were prepared—150 mM NaCl, 300 mM NaCl, 500 mM NaCl and 1 M NaCl. Proteins tested were human IgG and human Fab, reconstituted in the equilibration buffer used in the assay. The affinity chromatography assay was then performed as described in [23].

2.4. Optimisation of the elution conditions

Human IgG and Fab (0.5 mg/ml in PBS buffer) were loaded onto columns (0.8 cm × 6 cm) with packed ligand 8/7 adsorbent (15 μmol/g moist weight gel) and the affinity chromatography was performed according to [23], except that the elution buffers were varied. Several parameters have been studied and the elution buffers included. (i) Effect of low pH 0.1 M glycine·HCl, pH 2.0; 0.1 M glycine·HCl, pH 3.0; 0.05 M citrate buffer, pH 3.0; 0.1 M acetate buffer pH 4.0, 4.5 and 5.0; 0.05 M citrate and 0.1 M sodium phosphate buffer, pH 2.4 and 1 M propionic acid, pH 2.1. (ii) Effect of high pH 0.15 M NH_4OH, pH 11.0 and 0.5 M TEA, pH 12.6. (iii) Effect of salt 0.1 M glycine·HCl pH 2.0 with 0.1 M, 0.5 M or 1 M NaCl. (iv) Effect of reversible protein denaturants 3 M KSCN; 6 M urea; 0.05 M citrate and 0.1 M phosphate buffer with 6 M guanidine·HCl. (v) Effect of organic solvents 50% (v/v) ethylene glycol, pH 10.5; 0.1 M glycine·HCl, pH 2.0 with 5, 15, 35 and 50% (v/v) ethylene glycol; 0.1 M glycine·HCl, pH 2.0 with 5 mM EDTA and 50% (v/v) ethylene glycol; 0.05 M citrate and 0.01 M sodium phosphate buffer, pH 2.8 with 50% (v/v) ethylene glycol; 0.05 M citrate and 0.1 M sodium phosphate buffer, pH 2.8 with 5 mM EDTA and 50% (v/v) ethylene

glycol; 50% (v/v) dimethyl sulfoxide (DMSO); 10% (v/v) dioxane, pH 3.8. (vi) Effect of detergents 0.2% (v/v) Triton X-100 and 1% (v/v) 3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS) [protein eluted quantified by the BCA Protein Assay (Pierce Biotechnology)]. (vii) Effect of temperature 0.1 M glycine·HCl, pH 2.0 by decrease of temperature (0 °C) and by increase of temperature (40 °C). (viii) Effect of column inversion 0.1 M glycine·HCl, pH 2.0 after inverting the flow through the column. %protein eluted = [(mg eluted × 100)/(mg bound)].

2.5. Affinity of ligand 8/7 adsorbents for pure immunoglobulin fractions

Ligand 8/7 immobilised in hexanediamine-modified agarose (15 μmol/g moist weight gel) was assessed by standard affinity chromatography [23] for binding to purified fractions of human Fab, $F(ab')_2$, Fc, IgA and IgM and also polyclonal IgG from human, cow, mouse, rabbit, sheep, goat and chicken. The ligand 8/7 adsorbent was equally tested for binding to human myeloma IgG_1 proteins with κ and λ light chains by microscale affinity chromatography and quantitative enzyme-linked immunosorbent assay (ELISA) [23]. In parallel, ImmunoPure Immobilised Protein L was also evaluated for binding to human IgG, $F(ab')_2$, Fab and Fc, mouse and cow IgG and human myeloma IgG_1 proteins with κ and λ light chains, following the same procedure as for ligand 8/7 adsorbent.

2.6. Partition coefficient analysis of the interaction between ligand 8/7 adsorbent and human IgG and Fab

Partition equilibrium experiments were performed as described in [17,26]; 0.2 ml of human IgG solutions in PBS (3–0.4 mg/ml) and 0.02 g of immobilised ligand 8/7 (15 μmol/g moist weight gel); and 0.1 ml of human Fab solutions in PBS (3–0.1 mg/ml) and 0.01 g of immobilised ligand 8/7 (15 μmol/g moist weight gel). The amount of free protein was measured spectrophotometrically at 280 nm, and the amount of protein bound to the ligand in the equilibrium calculated. The adsorption phenomena followed Langmuir type isotherms and was represented by the rectangular hyperbolic relationship:

$$q = \frac{Q_{\max} K_a C}{1 + K_a C} \quad (1)$$

where q is the bound and C the unbound protein, $Q_{\max}$ corresponds to the maximum concentration of matrix sites available to the partioning solutes (which can also be defined as the binding capacity of the adsorbent), and K_a the association constant. The adsorption data derived from the isotherms was linearised into the Scatchard plots [27]:

$$\frac{q}{C} = K_a Q_{\max} - K_a q \quad (2)$$

The data were further transformed to Hill plots that assign numerical values to the degree of cooperativity of the system [28], where n_H symbolises the Hill coefficient:

$$\log\left(\frac{q}{Q_{\max} - q}\right) = \log K_a + n_H \log C \quad (3)$$

2.7. Competitive ELISA

Human IgG and human Fab were conjugated to EZ-Link Activated Peroxidase (HRP) according to the supplier instructions (Pierce Biotechnology). The wells of an ELISA microplate were coated with 100 μl of protein L or protein L domain (10 μg/ml) in coating buffer (0.05 M sodium carbonate–hydrogen carbonate, pH 9.6) overnight at 0–4 °C. After three washing steps with PBST (PBS-Tween 20; 0.05%, v/v), the plate was blocked with PBST (200 μl/well) and incubated for 1 h at room temperature. The plate was extensively washed with PBST and 100 μl of PBST added to each well except the first row. For the determination of the inhibition of ligand 8/7 in the interaction between protein L and protein L domain with IgG and Fab, 200 μl of ligand 8/7 solution (82 μM) was added to the first row and diluted (1:2) by transferring 100 μl from well to well along the plate. Protein conjugated to HRP (hlgG-HRP, 1:1000; hFab-HRP, 1:500 in PBST) (100 μl) was added to all wells and the plate incubated for 2 h at room temperature. After incubation, the plates were carefully and extensively washed with PBST. Substrate solution [5 mM Na_2HPO_4, 2 mM citric acid, 1.85 mM *o*-phenylenediamine dihydrochloride (Merck) and 0.04% (v/v) H_2O_2] was freshly prepared and 100 μl added to the wells. The plates were incubated at room temperature in the dark. For the protein L coated plates the incubation time was 10 min (hlgG-HRP) or 30 min (hFab-HRP). For the protein L domain coated plates the incubation period was 30 min (hlgG-HRP) or 60 min (hFab-HRP). After the incubation period, 50 μl of H_2SO_4 (2 M) were added to each well and the absorbance read at 490 nm. The control wells contained (i) no protein-HRP; (ii) no protein-HRP and no ligand; (iii) protein-HRP and no ligand (corresponding to 100% binding—inhibition data were calculated relative to this value). For the determination of the affinity constant between protein L or protein L domain and IgG and its fragments, two strategies were considered: in the first row, instead of ligand 8/7 solution, a protein L or protein L domain solution (1 μM) or human IgG or human Fab solutions (1 μM) were added and the methodology described above was followed.

The Cheng–Prusoff equation expressed by:

$$\frac{1}{K_2} = \frac{ED_{50}}{1 + pK_1} \quad (4)$$

relates the affinity constant K_2 (association constant of the interaction inhibitor L_2 and L_1) with the ED_{50}, having as constants p (concentration of labelled ligand L_1) and K_1 (association constant for L_1-receptor) [29]. The last parameter may be also determined by the Cheng–Prusoff equation

where unlabelled molecule L_1 is considered as the inhibitor L_2, and therefore it is evaluated by the displacement of labelled L_1 by itself. As an alternative it is also possible to use the receptor in solution as the inhibitor L_2.

2.8. Purification of immunoglobulins from crude samples

Crude extracts (goat serum and human serum with high contents of IgM-κ) and a semi-purified preparation with human IgA-λ, were diluted 1:3 in PBS buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) and 0.2 ml of each loaded onto two pre-equilibrated columns, one containing 0.2 g of ligand 8/7 adsorbent (15 μmol/g moist weight gel) and the other 0.2 g of ImmunoPure Immobilised protein L. The resins were washed extensively with PBS until the absorbance at 280 nm of the collected samples reached ≤0.005. Bound protein was eluted with 0.1 M glycine·HCI pH 2.0, whence 1 ml fractions were collected and immediately neutralised with 100 μl of 1 M Tris·HCl pH 9.0. Loading, washing and elution samples (10 μl of each) were denatured by adding 5 μl of sample buffer (2 ml glycerol, 2 ml 10% (w/v) SDS, 0.25 mg bromophenol blue, 0.5 ml β-mercaptoethanol and 2.5 ml of stacking gel 4×, for a 10 ml solution; stacking gel 4× (100 ml solution): 6.06 g Tris base, 4 ml SDS 10% (w/v) adjusted to pH 6.8 with 12 M HCl) and boiling for 5 min. These samples were then applied to the wells of Novex Tris–glycine precast gels (4–20%) (Invitrogen), previously placed in the X-Cell SureLock (lnvitrogen, Renfrew, UK) filled with running buffer (1 l solution: 3 g Tris base, 14.4 g glycine, 1 g SDS). The gels were run at 160 V and then stained with SimplyBlue Safe Stain (Invitrogen, Renfrew, UK). Ultra-pure water (UHP) (50 ml) was added to the gels on a glass petri dish, microwaved for 50 s, incubated for 1 min at room temperature with orbital agitation and the water removed. This procedure was repeated twice. Staining solution (40 ml) was added, microwaved for 1 min, incubated for 5 min at room temperature with orbital agitation and the solution removed. UHP water (100 ml) was added and incubated with the gels at room temperature for 15 min. The gels were destained with 20 ml of 20% (w/v) NaCl solution, overnight at room temperature, with orbital agitation, and then dried and scanned. The relative intensities of each band analysed with Scion Image software (www.scioncorp.com).

3. Results and discussion

Specific recognition elements studied in the X-ray crystaliographic structure of the complex between protein L from *Peptostreptococcus magnus* (PpL) and human Fab (Fig. 1) prompted the rational design and construction of a 169-membered solid-phase ligand library, synthesised by a combinatorial modified "mix-and-split" procedure [23]. Upon assessment for binding to human IgG and selectivity for the Fab fragment [23,25], ligand 8/7 (Fig. 2) has been

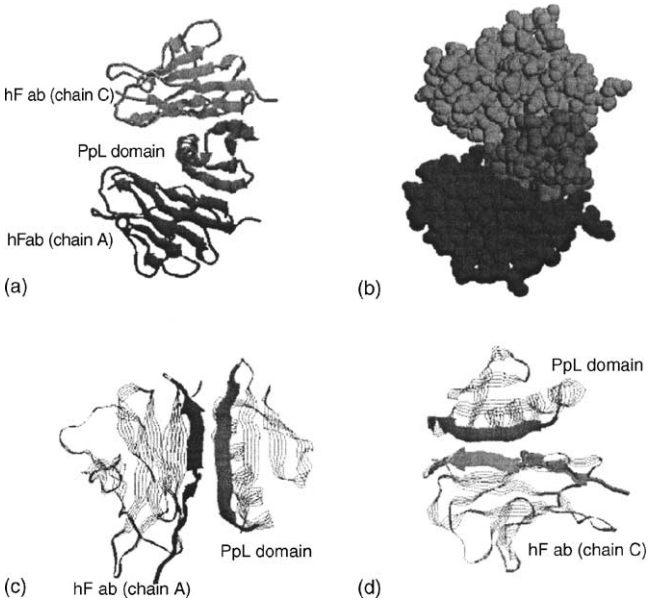

Fig. 1. Complex between human Fab 2A2 and C^* domain from PpL_{WT}. Representation of the asymmetric complex between a single C^* PpL_{WT} domain and two V_L domains (chains A and C; 1 HEZ.pdb): (a) secondary structures of the partners involved in the complex; (b) structures in which each atom is represented as a sphere of van der Waal's radius; (c) first binding interface showing that the β-strands from V_L (chain A) and C^* domain are anti-parallel; (d) second binding interface showing that β-strands from V_L (chain C) and C^* domain are parallel.

selected as the lead ligand for mimicking the interaction between protein L and Fab fragments [23]. This molecule, contains aromatic and aliphatic moieties with polar substitutents, comprising of 4-aminobenzamide and 4-amino butyric acid (Fig. 2). The previously reported artificial protein A, ligand 22/8 [17], has a more hydrophobic nature than ligand 8/7, since it comprises a triazine-scaffold substituted with 3-aminophenol and 4-amino-1-naphthol.

These differences reflect the type of amino acid residues that are involved in the interaction of protein A from *Staphylococcus aureus* (SpA) and PpL with immunoglobulins: In SpA, the hydrophobic Phe132-Tyr133 motif was shown to be

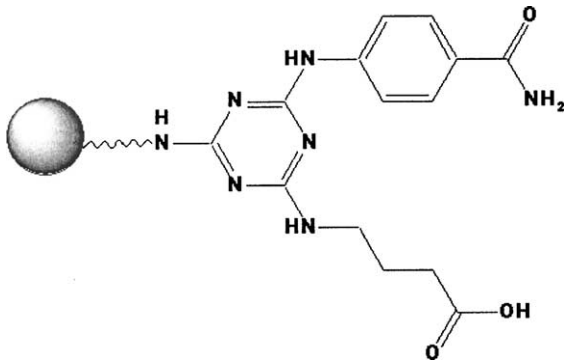

Fig. 2. The ligand 8/7 absorbent. Representation of ligand 8/7 (4-{4-(4-carbamoyl-phenylamino)-6-chloro-[1,3,5]triazin-2-ylamino}butyric acid) immobilised in a solid support.

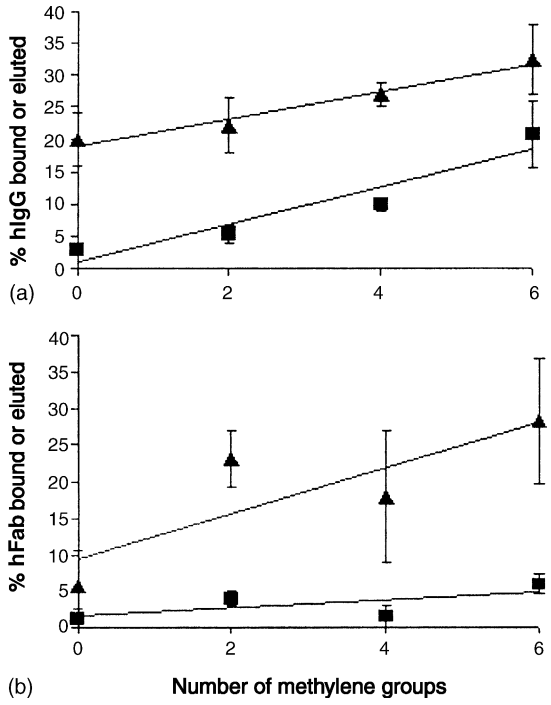

Fig. 3. The effect of the number of methylene groups of the spacer arm (n) on adsorbents comprising Iigand 8/7 in the percentages of human IgG (a) and Fab (b) bound (▲) and eluted (■). Adjusted linear regression of the data: %hIgG bound vs. n ($R^2 = 0.9701$); %hIgG eluted vs. n ($R^2 = 0.9004$); %hFab bound vs. n ($R^2 = 0.6921$); %hFab eluted vs. n ($R^2 = 0.4399$). %bound = [(mg bound × 100)/(mg loaded)] and %eluted = [(mg eluted × 100)/(mg loaded)]. Number of replicates: 3.

relevant in the interaction with the Fc fragment [30], whereas in PpL, a series of predominantly hydrophilic amino acid residues and hydrogen bonds are the basis of the complex with the light chains [24,31]. In order to further characterise and optimise the interaction between ligand 8/7 and human IgG and Fab, the effect of different parameters in the binding and elution of target proteins was analysed, and the suitability of this ligand for the isolation of immunoglobulins or fragments from crude samples evaluated.

3.1. Effect of the spacer arm length

The effect of different alkane-spacer arms interposed between ligand 8/7 and the solid support was studied. It is known that spacer arms increase the steric accessibility of the immobilised ligand and also diminish steric hindrance effects between the ligand and the agarose beads [32]. The spacer arms comprised of alkane chains with 0–6 methylene groups, since it has been suggested that longer chains, due to their extended flexibility, may fold back in hydrophilic environments [33]. It was observed that ligand 8/7 immobilised in hexanediamine-modified agarose was the best affinity adsorbent for binding and eluting the target proteins, human IgG and Fab (Fig. 3). In general, there was an increase in the percentages of proteins bound and eluted with an increase in the number of methylene groups, this effect being more pronounced for the human IgG system. It was suggested from these results that the 6-carbon hydrophobic spacer increases the effective ligand concentration available to interact with the target protein and, at the same time, has a positive contribution in the binding of ligand 8/7 to human IgG and Fab.

3.2. Effect of ligand concentration

The concentration of ligand is also an important factor for the efficiency of an affinity adsorbent. The different epoxy-content of the agarose beads was achieved by varying the reaction time with epichlorohydrin. Saturation on the epoxy-content was observed after 100 min of reaction, probably due to the limitation on the number of free reactive hydroxyl groups in the agarose beads, whilst until saturation, there was a linear increase in the epoxy-content with the reaction time (data not shown). The resins containing different epoxy densities were allowed to react exhaustively with hexanediamine for the introduction of the 6-carbon spacer arm (final amination content similar to epoxy-content) and then coupled to ligand 8/7. The ligand density was proportional to the epoxy-content of the original resins. Adsorbents containing different concentrations of ligand 8/7 were further examined for binding and eluting human IgG and human Fab, by affinity chromatography (Fig. 4). There is a general

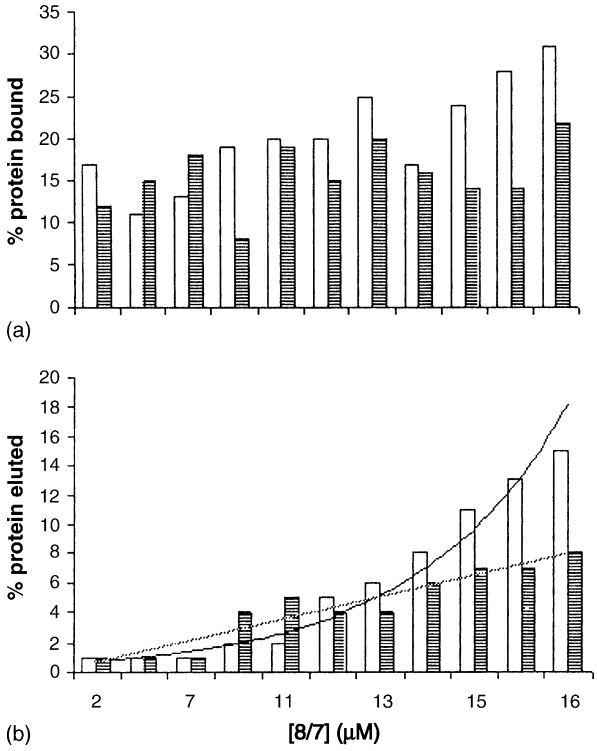

Fig. 4. Amount of human IgG (□) and human Fab (▤) bound (a) and eluted (b) as a function of the Iigand 8/7 concentration (μmol/g) in the affinity adsorbents. (b) Represented the adjusted exponential regression of the data %hIgG eluted vs. [8/7] ($R^2 = 0.958$), bold line; and the adjusted linear regression of the data %hFab eluted vs. [8/7] ($R^2 = 0.901$), dashed line. %bound = [(mg bound × 100)/(mg loaded)] and %eluted = [(mg eluted × 100)/(mg loaded)].

tendency towards an increase in protein bound with an increase in density of immobilised ligand (Fig. 4a). However, in terms of the percentages of human IgG and Fab eluted, there was an increase in protein recovery for higher ligand concentrations showing exponential and linear regressions for the %IgG eluted and %Fab eluted, respectively (Fig. 4b). Several features may contribute for these observations. First, only a small percentage (0.1–10%) of the resin capacity is effective for binding the target protein. Secondly, the ligand concentration and the distribution of ligands in the agarose matrix are not homogeneous. Thirdly, for low ligand concentrations, it is likely that nonspecific interactions may occur between the protein and the free amino groups or the alkane chain of the spacer arm at the surface of the matrix. Fourthly, the proteins studied differ in size and avidity; IgG presents a higher probability of cross-linking with ligand molecules. These conclusions also support the hypothesis that ligand and spacer arm may act in conjunction on binding to human IgG and Fab, and that higher concentrations of ligand 8/7 are necessary to achieve this effect. The range of attainable ligand concentrations is probably less than the density that allows the maximum binding and elution capacities, although it is clear that a minimum ligand concentration of ~10 μmol/g is required to have a percentage of protein eluted greater than 4%.

3.3. Effect of pH and ionic strength of the binding buffer

The choice of PBS buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) as the standard equilibration/binding buffer for the affinity chromatography assays with ligand 8/7 adsorbents was made according to the conditions recommended for protein L affinity chromatography [34,35]. The pH and ionic strength may influence the binding process between the ligand and complementary proteins. The pH will affect the charged state of amino acid residues and other groups that may be involved in the binding interaction, whereas an increasing in the ionic strength encourages the establishment of hydrophobic interactions [36]. Although it may difficult to interpret the influence of pH in the interaction between ligand 8/7 and IgG/Fab, because the net charges of both of molecules may change in the range of pH values studied, the data presented in Fig. 5a and b suggests that there are ionic interactions involved in the binding of ligand 8/7 to the target proteins. The range of pH values considered (6.0–8.0) is within the pH values commonly employed in affinity chromatography by maintaining the protein in a similar environment to that of biological fluids. The percentages of human IgG bound and eluted decreased linearly with the increase in pH ($R^2 = 0.98$ and 0.77, respectively) (Fig. 5a). The percentages of human Fab bound and eluted also decrease with higher pH values ($R^2 = 0.71$ and 0.94, respectively) (Fig. 5b). Regarding the effect of ionic strength, the percentage of human IgG bound decreases with the increase in salt concentration ($R^2 = 0.76$) (Fig. 5c). The percentage of human IgG eluted increases with increasing

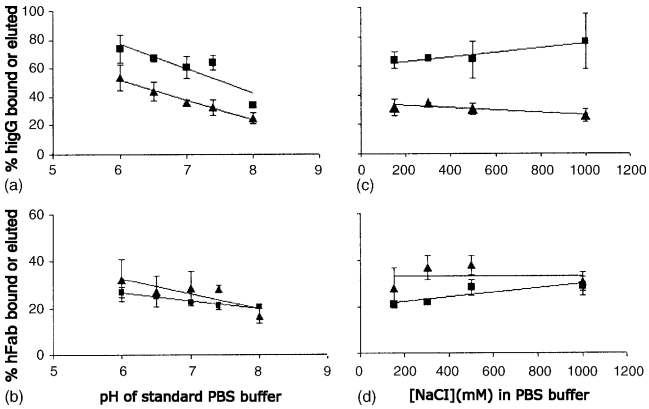

Fig. 5. Effect of pH of the standard PBS buffer on the binding (▲) and elution (■) of human IgG (a) and Fab (b) from adsorbents containing Iigand 8/7 immobilised in hexanediamine-modified agarose. Effect of salt concentration (NaCl) of the standard PBS buffer on the binding (▲) and elution (■) of human IgG (c) and Fab (d) from adsorbents containing ligand 8/7 immobilised in hexanediamine-modified agarose. %bound = [(mg bound × 100)/(mg loaded)] and %eluted = [(mg eluted × 100)/(mg bound)]. Number of replicates: 3.

salt concentrations of the binding buffer ($R^2 = 0.86$). However, the error bar associated with the percentage of human IgG eluted increases for higher ionic strength values. Different salt concentrations in the binding buffer had a negligible effect on the percentage of human Fab bound ($R^2 \sim 0$), although the amount of protein eluted is positively correlated with the salt concentration in the binding buffer ($R^2 = 0.73$) (Fig. 5c and d). When the salt concentration increases, the ionic interactions are decreased, but the hydrophobic effects become stronger: thus, it is conceivable that the overall effect reflects no change in the percentage of human Fab bound, but the elution of the Fab fragment with the glycine buffer (pH 2.0) is facilitated. It can be envisaged that, for both proteins, there is a contribution of electrostatic and hydrophobic interactions when binding to ligand 8/7 adsorbents.

3.4. Optimisation of elution conditions

The role of the elution buffer is to disrupt interactions between the ligand and target proteins by reducing the binding affinity between them and forcing the protein back into the mobile phase, preferably in an active form, making the design of an elution system a trial and error approach that can be the stumbling block in an affinity chromatographic process [36]. The results obtained for the elution of human IgG by different eluants have shown that, in low pH buffers (5.0–2.0), the elution of human IgG is inversely related to the pH of the buffer; therefore, elution with the standard elution buffer (0.1 M glycine·HCl pH 2.0) presented the best results (64%) (Fig. 6). Elution of protein with high pH buffers (47%) is slightly lower than for low pH. Changes in the ionic strength of the eluant by imposing different concentrations of NaCl

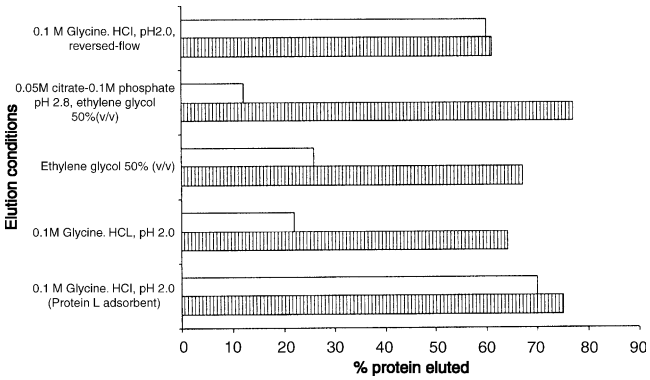

Fig. 6. Human IgG (▥) and human Fab (□) eluted [%eluted = (mg eluted) × 100/(mg bound)] off ligand 8/7 adsorbents with different elution conditions; comparison with the percentage of proteins eluted from a commercial protein L adsorbent using the standard elution buffer (0.1 M glycine–HCl pH 2.0).

reduced the elution with increasing salt concentrations (20% elution for 0.1 M NaCl and 7% elution for 1 M NaCl). As for the utilisation of organic solvents and detergents, ethylene glycol 50% (v/v) allowed the best recovery yields, especially when associated with citrate-phosphate buffer (77% elution) (Fig. 6). Chaotropic salts and reversible denaturants eluted less effectively than the standard elution buffer. The optimal elution conditions for the recovery of human IgG from immobilised ligand 8/7 were applied to the elution of human Fab. The elution of human Fab from ligand 8/7, in standard conditions (0.1 M glycine·HCl pH 2.0), corresponds to about 22% of bound protein (Fig. 6). The low pH citrate-phosphate buffer, ethylene glycol 50% (v/v) and the combination of the latter with the standard glycine buffer yielded percentages of human Fab eluted between 26% and 28%. When the temperature was increased from 25 °C to 40 °C, the percentages of Fab eluted were equivalent to elution with standard buffer at room temperature (25%), whereas decreasing the temperature to 0 °C resulted in a lower protein recovery (14%). The best recovery of human Fab occurred for the reversed-flow affinity process with standard elution buffer (60%) (Fig. 6). Human Fab is a small molecule with a low avidity, when compared to IgG, but may be readily retained at the top of the adsorbent. It is known that inversion of the flow during the elution step has also been used as an alternative to facilitate elution of proteins that saturate the top portion of the resin [37]. The percentages of protein eluted (0.1 M glycine·HCl pH 2.0) from a commercial immobilised protein L affinity column were 75% and 70% for human IgG and Fab, respectively (Fig. 6). In conclusion, using the standard elution buffer (0.1 M glycine·HCl, pH 2.0), 64% of bound human IgG is recovered and, if ethylene glycol is added, this value may raise up to 77%. Regarding the elution of human Fab, up to 60% of protein is recovered by using the standard elution buffer in a reversed-flow. It is conceivable that other elution conditions, not tested in these studies, might prove more efficient.

Table 1
Affinity constants (K_a) and equilibrium binding capacity (Q_{max}) estimated for the interaction between ligand 8/7 or PpL and the human proteins IgG and Fab

Method	Ligand	Protein	K_a (M^{-1})	Q_{max} (mg protein/g resin)
Partition equilibrium[a]	8/7	Human IgG	5.5×10^4	1.2
		Human Fab	7.2×10^4	0.7
Competitive ELISA[b]	8/7	Human IgG	2.3×10^2	–
		Human Fab	4.5×10^3	–
	PpL	Human IgG	2.3×10^7	–
		Human Fab	1.4×10^7	–
	PpL Domain	Human IgG	1.7×10^6	–
		Human Fab	3.3×10^6	–

[a] The affinity constants and Q_{max} were calculated from Scatchard plots; K_a values estimated by both Scatchard and Hill plots were of the same order of magnitude (data not shown).
[b] Affinity constants estimated with the Cheng–Prusoff equation [29].

3.5. Determination of affinity constants by partition equilibrium experiments

Scatchard plots indicate whether the interaction between the protein and ligand is (i) reversible and unimolecular (a 1:1 ratio where the protein binds to a single ligand population and vice versa); (ii) derived from a positive cooperative binding process between equivalent binding sites; or (iii) is due to heterogeneous binding sites/negative cooperativity effects. Accordingly, the shape of the Scatchard plot will be linear, convex or concave. The values of K_a (obtained from Scatchard and Hill plots; adjusted linear correlations $R^2 \geq 0.957$), for the interaction of 8/7 with both IgG and Fab, were in the order of $10^4\ M^{-1}$ (Table 1). The Hill coefficient (n_H) assumed the value 1.0, indicating a reversible and unimolecular interaction between the proteins and the ligand. This coefficient is not only an indication of the number of binding sites, but also an index of the degree of positive ($n_H > 1$) or negative ($n_H < 1$) cooperativity of the systems [38]. The value of Q_{max}, directly taken from the Scatchard equations, comes as moles ligand/volume resin (M); considering the density of Sepharose CL-6B (1.02 g/ml), Q_{max} can be expressed as moles ligand/g resin. The values of Q_{max} (8.2 nmol/g and 14.3 nmol/g resin) are 10^3 lower than the measured ligand density (15 μmol/g resin), indicating that the amount of ligand that is able to interact strongly with the target proteins is 0.1% of the total ligand concentration measured analytically. The fact that the capacity of affinity adsorbents is often much lower than that predicted from the number of ligands bound to the matrix has been noticed previously in affinity chromatography [36]. It is argued that proteins bind primarily to ligands immobilised on the outer surface of the solid support and that these adsorbed proteins will block access to sites that are buried in the three-dimensional lattice of the beads. Additionally, the heterogeneity in the distribution of adsorption sites on a matrix results in local deviations of

ligand density from the theoretically calculated values. As the interaction between human IgG or Fab and immobilised ligand 8/7 is unimolecular, the maximum number of moles of ligand available to interact with the protein will be theoretically equivalent to the maximum number of moles of protein that can be adsorbed into the resin. Therefore, Q_{max} can also be expressed as the equilibrium binding capacity of immobilised ligand 8/7 and will be expressed as mg protein/g resin (Table 1). The maximum adsorption capacity for the proteins, estimated from the Scatchard plot, is higher than the determined capacity by frontal analysis (0.5 mg IgG/g resin and 0.2 mg Fab/g resin) [39]. This fact has already been noticed in other situations involving triazine bisubstituted affinity adsorbents. The theoretical maximum capacity of immobilised ligand 22/8 to human IgG was ~151.9 mg IgG/g gel, whereas the observed capacity was ~51.9 mg IgG/g gel [17].

3.6. Determination of affinity constants by competitive ELISA

The affinity constants between ligand 8/7 and human IgG and Fab, estimated by competitive ELISA, were within the range 10^2–10^3 M^{-1} (Table 1 and Fig. 7). These values are three to six orders of magnitude lower than the corresponding K_a values for the interaction between PpL and PpL domain and the human proteins, which in turn were lower than those expected from the literature (10^7–10^9 M^{-1}) [40,41]. As expected, the affinity constants estimated for a single PpL domain reduce one order of magnitude when compared to the entire protein. The competitive ELISA allows the relative comparison of the strength of binding of free ligands (PpL and 8/7) and the target proteins, and gives evidence that ligand 8/7 can compete with PpL in binding to IgG and Fab. The estimated affinity constants for the interaction between immobilised-ligand 8/7 and human IgG/Fab (10^4 M^{-1}) were one to two orders of magnitude higher than those estimated when the ligand was free in solution. This difference may be related to the low solubility of ligand 8/7 in aqueous solutions and also to the influence of the solid support in maintaining the orientation, local concentration and avidity of the immobilised ligand.

3.7. Binding of ligand 8/7 to pure immunoglobulin fractions

Ligand 8/7 has an affinity and specificity towards the human Fab fragment of immunoglobulins analogous to PpL (Table 2) [23]. However, a comparison of ligand 8/7 and PpL showed that 8/7 is a more versatile ligand; while maintaining the specificity for the human Fab moiety, it also binds to the most abundant human heavy chain isotypes (binding 0.40 and 0.55 of IgA and IgM (mg protein/g resin), respectively). In addition, ligand 8/7 binds both human IgG_1 λ and human IgG_1 κ (1.00 and 0.92 (mg protein/g resin), respectively), the former showing no interaction with PpL. Furthermore,

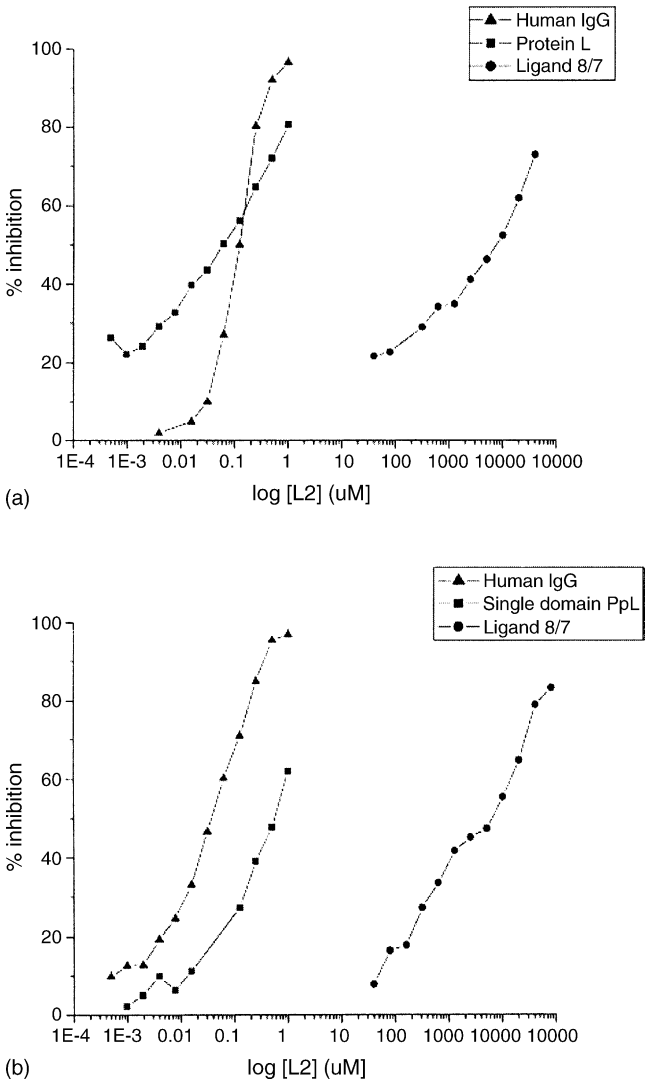

Fig. 7. Inhibition curves of the competitive ELISA assays performed with protein L (a) or PpL single domain (b) coated microtitre plates. The inhibitors were unlabelled human IgG (▲); solutions of PpL or PpL domain (■); or solutions of ligand 8/7 (●).

of particular relevance, is the binding of ligand 8/7 to polyclonal IgG from a wide range of different species, including those that bind to PpL, such as mouse IgG [ligand 8/7 bound 0.43 mg and PpL 0.41 mg (per gram of resin)], and also those that do not interact with PpL, such as sheep (0.30), cow (0.30), goat (0.25) and chicken (0.20) IgG (values in mg protein/g resin).

3.8. Purification of immunoglobulins from crude samples

Ligand 8/7 and protein L adsorbents were evaluated for their capability of enriching the immunoglobulin content from various crude samples (Fig. 8). In goat serum, the immunoglobulin fraction corresponded to 44% (M_r 50 000 and 25 000 bands), whereas albumin was estimated to represent

Table 2
Comparison of the binding of immunoglobulins to ligand 8/7 and protein L

	Ligand 8/7	Protein L
Human		
IgG	++	++
IgA	++	++[a]
IgM	++	++[a]
F(ab')$_2$	++	++
Fab	++	++
Fc	–	–
κ light chains	+++	++
λ light chains	+++	–
Polyclonal IgG		
Mouse	++	++
Sheep	++	–[a]
Bovine	++	–
Rabbit	++	+
Goat	++	–
Chicken	++	–[a]

Table constructed on the basis of affinity chromatographic assays undertaken for both ligand 8/7 and PpL adsorbents (+++: binding ≥80%; ++: binding 20–80%; +: binding 0–20%; –: no binding).

[a] Immunoglobulins not tested by affinity chromatography, data from literature [34,41].

31% of the total protein by weight (densitometry analysis). This contaminant was largely eliminated during the washing steps from both ligand 8/7 and PpL adsorbents, the elution fractions being verified to contain <8% of albumin (Fig. 8a). While the amount of immunoglobulin eluted from the PpL adsorbent was negligible, as expected from the literature [41], the content of immunoglobulins in the fraction eluted from the ligand 8/7 adsorbent was estimated as 66%. Apart from the immunoglobulin bands at M_r 50 000 and 25 000, others at 200 000, 100 000, 75 000 and 35 000 were also visible in the 8/7 elution lane (∼30% of total protein), although bands at M_r 100 000 and 75 000 may be also attributable to the immunoglobulin fraction. Ligand 8/7 and PpL adsorbents were effective at isolating immunoglobulins from human serum with a high content of IgM (κ-type) (Fig. 8b), achieving immunoglobulin preparations with purities of 91% and 95%, respectively. The contaminants present in both elution fractions were <10%, with again the absence of the albumin band noted. The isolation of human IgA (λ-type) from a semi-purified fraction was only possible with the ligand 8/7 adsorbent, since no immunoglobulin was recovered from the PpL affinity column (Fig. 8c). The estimated immunoglobulin content in the loading sample was 60%, which was increased to 93% in the eluted fraction from immobilised ligand 8/7. The ligand 8/7 adsorbent was significantly better than immobilised PpL for the isolation of goat immunoglobulins and the human immunoglobulins IgM-κ and IgA-λ. The synthetic ligand yielded immunoglobulin fractions with up to 93% purity in a single step. These experiments represent preliminary studies, undertaken under standard, but non-optimised conditions for each different system, but show the potential of ligand 8/7 to isolate immunoglobulins directly from crude samples.

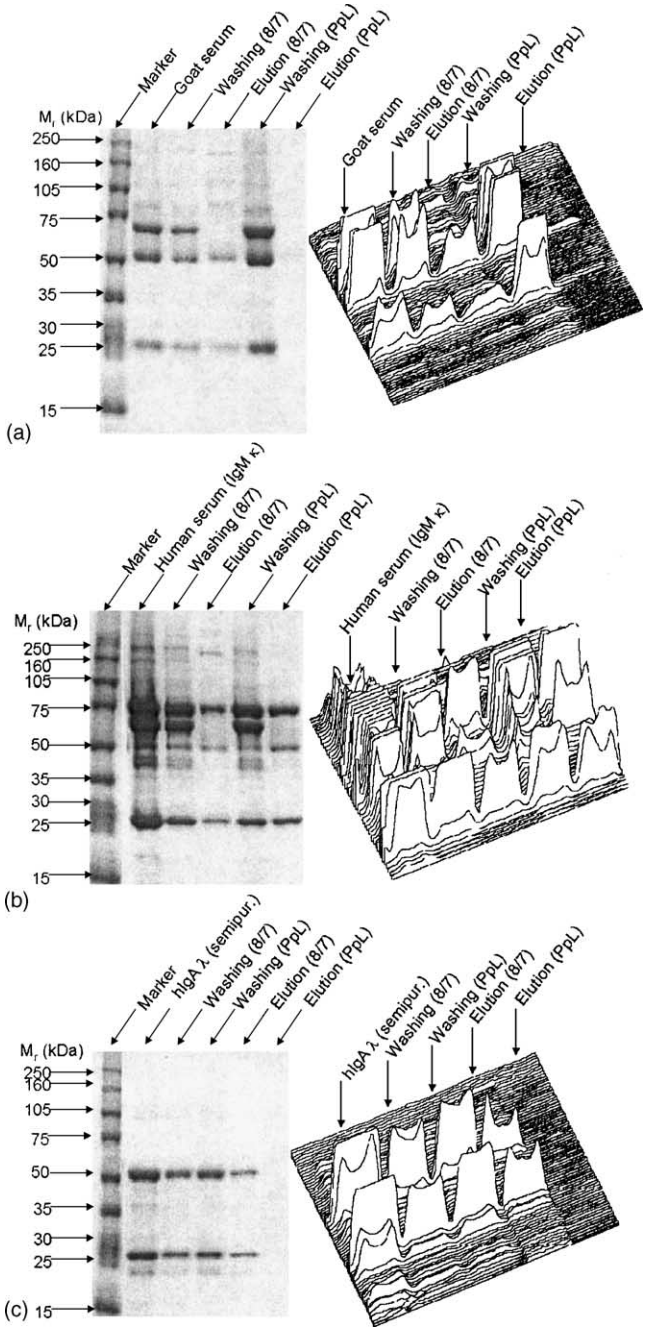

Fig. 8. Application of ligand 8/7 adsorbents in the purification of immunoglobulins. Analysis of protein loaded, washed and eluted off ligand 8/7 adsorbent and PpL adsorbent by SDS–PAGE (under reducing conditions and stained with SimplyBlue SafeStain) and correspondent densitometric analysis: (a) SDS–PAGE and densitometric plot of the purification of immunoglobulins from goat serum; (b) SDS–PAGE and densitometric plot of the purification of immunoglobulins from human serum with a high content of IgM (κ); (c) SDS–PAGE and densitometric plot of the purification of human IgA (λ) from a semi-pure sample. kDa: kilodalton.

4. Concluding remarks

To date, there is no synthetic affinity ligand with specificity for small antibody fragments (scFv, Fab or F(ab′)$_2$) [23], which are particularly relevant in some therapeutic [42]

and diagnostic [9] situations. We have previously described the rational design and synthesis of a combinatorial solid phase triazine-scaffolded library of affinity ligands, used for the rapid lead discovery of a PpL mimic [23]. The research work in this paper describes in more detail the interaction between ligand 8/7 and the target proteins (IgG and Fab) and evaluates the application of this ligand as an affinity adsorbent for the one-step purification of immunoglobulins. Variation of the spacer length indicated that a 6-carbon aliphatic arm, interposed between the ligand and the agarose beads, confer to the adsorbent the best performance in binding and eluting the target proteins. Studies on the effect of pH and ionic strength of the binding buffer, as well as the evaluation of different eluants for protein recovery, suggested that both ionic and hydrophobic interactions contribute to the binding events of ligand 8/7 and IgG or Fab. Association constants for the interaction of the ligand with the protein in the range 10^3–10^8 M^{-1} are normally the most suitable for purification purposes [43], since very high affinities, such as those observed in native IgG-binding bacterial proteins, are not ideal for affinity purification [20]. While the interaction between a single PpL domain and a Fab fragment has a large contact surface, it was predictable that the low-molecular-mass ligand 8/7 would present reduced apparent K_a values compared to the native protein it mimics. This fact has already been observed on the previously reported ligand 22/8 [17,44]. The ligand 8/7 adsorbent was able to bind in a similar manner to the immunoglobulins and fragments that interacted with PpL; in addition, it was able to bind proteins that PpL does not bind to, namely human IgG with λ chains, which represents an improvement over wild type PpL. Ligand 8/7 also binds to polyclonal IgG from sheep, cow, goat and chicken (which PpL does not interact with), that may be of interest for the purification of antibodies from these animal sources. The data here presented demonstrates that ligand 8/7 is comparable with PpL, can compete with PpL in binding to human IgG and Fab, and represents a more versatile and universal immunoglobulin binding ligand. Like other synthetic triazine-based ligands, ligand 8/7 is inexpensive to produce and is expected to be highly resistant to SIP and CIP treatments [17,26,44]. Ligand 8/7 was designed to be used for the affinity chromatographic purification of immunoglobulins, although it can be used for other applications, such as an affinity ligand in affinity precipitation [15] or aqueous two-phase systems, or as a diagnostic or therapeutic agent.

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