

Review

Novel ligands for the affinity-chromatographic purification of antibodies

Giorgio Fassina^{*}, Menotti Ruvo, Giovanna Palombo,
Antonio Verdoliva, Maria Marino

Biopharmaceuticals, TECNOGEN, Parco Scientifico, 81015 Piana di Monte Verna (CE), Italy

Abstract

Affinity chromatography represents one of the most powerful fractionation techniques for the large-scale purification of biotechnological products. Despite its potential, the use of this methodology is limited by the availability of specific ligands for each target. Combinatorial chemistry and molecular modeling, often combined, have become interesting and innovative methods for generating novel ligands, tailored to specific biotechnological needs.

One of the greatest area of application has been the discovery of novel ligands for the purification of antibodies, which represent an emerging but very important class of innovative therapeutic agents for the treatment of a vast array of diseases. Naturally available affinity ligands, such as Protein A or G for IgG purification or lectins for IgA and IgM purification, which are obtained from microorganisms or genetically modified bacteria through complex and expensive procedures, are not well suited for large-scale purification and require moreover time-consuming analytical controls to check for the presence of contaminants which may affect the safety of the purified antibody for clinical purposes. Recent results suggest that the application of combinatorial technologies and molecular modeling for the discovery of synthetic ligands may open new avenues for the development of more efficient, less expensive and—more importantly—safer procedures for antibody purification at the industrial level. © 2001 Elsevier Science B.V. All rights reserved.

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^{*} Corresponding author. Tel.: +39-0823-612-214; fax: +39-0823-612-230.
E-mail address: fassina@tecnogen.it (G. Fassina).

1. Conventional ligands for affinity-chromatographic purification of antibodies (IgG, IgA, IgE, IgM, IgY)

Monoclonal antibodies (MAb) are becoming an important class of therapeutic agents useful for the treatment of a vast array of diseases. Many monoclonals are waiting for FDA approval and they represent almost 30% of biotechnology-derived drugs under development. Production of MAb by hybridoma technology or transgenic animals can be easily scaled up, but still immunoglobulins purification from crude feedstocks poses several problems. The main difficulties are due to the low antibody concentration in cell culture supernatants or milk of transgenic animals and the excessive amounts of contaminating proteins. The majority of monoclonals under development for clinical applications belong to the immunoglobulin G class; however, IgA, IgE, IgM and IgY are also increasingly finding application in the cure and/or diagnosis of important diseases.

Purification by affinity chromatography of monoclonal antibodies of the G class for therapy is based on the use of Protein A or G immobilized on appropriate supports [1], as a first step in capturing and concentrating the immunoglobulin from diluted feedstocks. These two proteins, which bind to the constant portion of the immunoglobulins and so can be used to purify the majority of antibodies, are obtained from microorganisms or genetically modified bacteria through complex and expensive procedures, requiring moreover time-consuming analytical controls to check for the presence of contaminants, such as viruses, pyrogens or DNA fragments, which may affect the safety of the purified MAb for clinical purposes.

Many monoclonal antibodies with potential therapeutic applications are immunoglobulins of the A class. For example, protection against an oral challenge with the invasive pathogen *Salmonella typhimurium* has been observed in mice treated with a monoclonal IgA, generated by mucosal immunization of BALB/c mice with attenuated strains of *S. typhimurium* and subsequent fusion of Peyer's patch lymphoblasts with myeloma cells [2]. Other studies have shown that monoclonal immunoglobulin A specific for *Shigella flexneri* 5a polysaccharide can protect mice against the disease [3]. Protective capacity of IgA was also found for the cholera toxin-induced chloride secretory response, in a dose-dependent manner [4]. IgA have been used for intranasal passive immunization of mice, providing protection against respiratory syncytial virus infection [5]. Monoclonal IgA may be produced by hybridoma technology in serum-free media, devoid of IgG and IgM. Purification of this class of immunoglobulins can be accomplished by classical chromatographic approaches with an acceptable degree of purity, but several steps, such as ammonium sulfate precipitation, ion-exchange and size-exclusion chromatography, are usually required [6,7]. The lectin jacalin, isolated from jackfruit seeds [8], binds to IgA and can be used conveniently for the affinity purification of IgA from colostrum or serum [9]. However, several aspects limit the use of this lectin for large-scale purification of monoclonal IgA from cell culture supernatants. First, jacalin is a biologically active lectin, being a potent T cell mitogen and a strong B cell polyclonal activator [10], thus, requiring careful control for ligand leakage in purified preparation. Second, jacalin binds to the carbohydrate moiety of IgA, and D-galactose is required to elute IgA from affinity columns. This is costly and impractical for large-scale operations.

Monoclonal antibodies of the E class have also been developed for therapeutic applications [11], but large amounts of highly pure material are required to extend the clinical investigations. In these cases, IgE are produced by hybridoma technology in media with low IgG and IgM content.

Purification of monoclonal and polyclonal IgE requires the use of conventional, often complex and laborious fractionation procedures, since specific and cost-effective ligands for this class of immunoglobulin usable for affinity chromatography applications are not yet available.

Antibodies of the E class are purified mainly by immunoaffinity chromatography, using anti-IgE antibodies immobilized on solid supports [12,13]. Even if selective enough for research application, scaling up immunochromatography for preparative applications is very expensive and not easily accomplished. Other approaches to IgE purification include classical chromatographic procedures based on combinations of different sequential procedures, such as salting out, affinity chromatography on lysine–Sephrose, ion-exchange and size-exclusion chromatography and immunoaffinity chromatography to remove interfering proteins [14]. Studies carried out with immobilized Protein A show that this protein, known to recognize the immunoglobulin Fc region, does not bind to monoclonal IgE, but binds only 12–14% of serum polyclonal IgE. Protein G binds to neither polyclonal nor monoclonal IgE.

IgM are also finding application in the diagnosis and treatment of certain important diseases, such as cancer [15]. Conventional approaches for the purification of IgM have been based on the combination of different fractionation techniques, such as precipitation, size-exclusion chromatography and ion exchange chromatography or electrophoresis. Proteins A, G and A/G, widely used for the affinity purification of antibodies from sera or cell culture supernatants even at the industrial scale, do not recognize immunoglobulins of the M class well and are not used to capture and purify IgM from crude sources. Alternative ligands for the affinity purification of IgM include the mannan-binding protein (MBP) which, after immobilization on solid supports, provides an affinity medium useful for IgM isolation based on a temperature-dependent interaction of the ligand with the immunoglobulin [16]. The use of immobilized MBP for the purification of IgM is based on adsorption in the presence of calcium at a temperature of 4 °C, and the temperature-dependent elution of adsorbed immunoglobulins in the presence of ethylenediaminetetraacetic acid (EDTA). This ligand shows low binding affinity for IgG, but binds to bovine and human IgM with lesser affinity than murine IgM. However, in addition to the complexity of MBP isolation, functional binding capacities of MBP columns are limited to 1 or 2 mg of IgM per ml of support. All these procedures are time-consuming and labor-intensive, may alter IgM functionality and are not compatible with industrial scale-up.

The growing application of monoclonal and polyclonal antibodies in immunotherapy and immunodiagnosics, and the strict standards for animal care, have led to a renewed interest in the use of chickens for antibody production, as opposed to mammals. Egg yolk represents an economical source of polyclonal antibodies [17,18], since their content of immunoglobulins (IgY) is similar or higher than in the serum of chickens, rabbits or humans, with levels ranging from 15 to 25 mg IgY per ml of yolk in the case of the hyperimmunized hen [19,20]. Production of immunoglobulins in chicken egg yolk

offers several advantages over mammalian antibodies. The bleeding step, which causes marked distress to the animals, is eliminated and the number of animals used may be considerably reduced. For highly immunogenic antigens, one chicken may produce 120 rabbit-equivalents of IgY over the period of 1 year, and at least 15 equivalents of antibodies, even in the case of less-effective antigens [21]. Therapeutic and diagnostic potentialities of chicken antibodies are strictly related to the phylogenetic differences between mammals and birds, which are greater than between mammalian species. Immunoglobulins produced in chickens against mammalian antigens are, therefore, directed against more antigenic epitopes, especially for highly conserved proteins, such as hormones [22,23]. In addition, Y immunoglobulins do not cross-react with mammalian IgG and do not bind to rheumatoid factors and bacterial or mammalian Fc receptors, thus, reducing nonspecific binding and minimizing interference in therapy or immunological assays involving mammalian sera [24,25]. Despite the advantages related to the production and use of chicken antibodies, only less than 2% of the total number of polyclonal antibodies produced worldwide and commercially available are raised in chicken. This low percentage is strictly related to the difficulties in isolating IgY from egg yolk, which is essentially composed of a considerable lipid fraction, dispersed in a water-soluble fraction containing the immunoglobulins, and to the failure of Protein A or G to bind to IgY [26]. Many methods have been described for the extraction and purification of IgY from egg yolk, based on a combination of several steps, including salt precipitation, alcohol precipitation, water dilution, ultrafiltration, size-exclusion chromatography, ion-exchange chromatography and dialysis [27–33]. All of these procedures are time-consuming and labor-intensive, may alter Ig functionality and cannot easily be scaled up for industrial applications.

2. Novel ligands for antibody purification deduced by screening combinatorial libraries and molecular modeling

Proteins A and G are very effective in the large-scale purification of monoclonal IgG, since support capacity can reach up to 30–40 mg IgG/ml of affinity sorbent under selected circumstances. Many monoclonals currently on the market for therapy or imaging are purified on these proteins. However, these ligands suffer from several drawbacks, including the high cost, their biological origin, the requirement for accurate analytical test to assure the absence of contaminants, the low stability toward sanitizing agents and, finally, a selectivity restricted to IgG.

The increasing number of monoclonals being developed for therapy, the role of the purification process in assuring the quality, consistency and safety of the products, and, finally, the emerging importance of other classes of immunoglobulins, prompted research activities focused on the identification of novel synthetic ligands with improved properties. Compared to Proteins A and G, optimal synthetic ligands should display not only reduced production costs at large-scale, but also increased resistance to chemical and biological actions, reduction in the amount of contaminants of biological nature, high capacity for large-scale purification and extended selectivity for other classes of

immunoglobulins not recognized by Proteins A or G. In addition, ideal ligands should display reduced toxicity and minimal leakage from affinity sorbents.

In the past, several synthetic ligands have been proposed as a replacement of Protein A in the affinity purification of antibodies, including thiophilic [34] and histidyl ligands [35]. However, lack of selectivity for antibodies has limited the widespread use of these ligands, which require moreover buffers not always compatible with the retention of antibody activity. Other synthetic supports for antibody purification such as Avidin [36] are not completely characterized from a structural point of view and, thus, preclude the detailed leakage and toxicity evaluation required by regulatory agencies. Recent investigations, based on the utilization of combinatorial technologies or computer modeling, have provided novel compounds with very promising characteristics and the potential of becoming effective ligands for antibody purification, even at the large-scale.

2.1. PAM (Protein A mimetic, TG19318)

In our laboratory, the search for a synthetic ligand able to bind to immunoglobulins and usable for affinity-chromatographic applications, has been undertaken through the synthesis and screening of combinatorial peptide libraries [37]. Peptide libraries are collections of peptide molecules, encompassing all the possible sequences for a given peptide length, which are synthesized and screened for activity simultaneously [38,39]. Previous studies have shown that peptide ligand multimerization enhances retention of recognition properties after immobilization on solid supports for the preparation of affinity columns [40–42]. Based on these considerations, a tetrameric peptide library has been designed, where four identical peptide chains are assembled, starting from a tetradentate lysine core, similar to that used for the production of multimeric antigenic peptides [43]. The multimeric library, composed of 5832 randomized tripeptide tetramers, has been produced by solid-phase peptide synthesis, following a simple manual procedure [44]. Screening of the activity of the multimeric library in terms of antibody recognition has been carried out by measuring the ability to interfere with the interaction between Protein A and biotinylated immunoglobulins, monitored on a solid phase by ELISA. The first screening cycle identified the sublibrary with arginine at the N-terminus as the most active sublibrary (60% inhibition). Subsequently, a second multimeric sublibrary was synthesized, with arginine at the N-terminus, the amino acid in Position 2 defined, and the third unknown. This second sublibrary was screened in the same assay at the same concentrations, and the best inhibitory activity was found in the sublibrary with threonine in the second position. A third sublibrary, composed of 18 tripeptide tetramers of the general formula $(\text{Arg-Thr-X})_4\text{-K}_2\text{-K-G}$, allowed the final identification of the most active multimer as $(\text{Arg-Thr-Tyr})_4\text{-K}_2\text{-K-G}$ [PAM, Protein A mimetic, TG19318]. This compound can be synthesized by solution or solid-phase methods in high yield and at limited costs. Its affinity constant for IgG, as determined by optical biosensor determinations, is close to $0.3 \mu\text{M}$. The tetrameric ligand TG19318 can be easily immobilized on preactivated solid supports, since the presence of the symmetric central core and the four peptide chains departing from it lead to an oriented immobilization where all the chains are not covalently linked and where the resin-bound chains act as self-built spacers to optimize interaction. All the supports tested so far

maintained the ligand recognition properties for immunoglobulins (CH-sepharose 4B™, Ultralink™, Eupergit™) although with different functional capacities, ranging from 10 to 25 mg IgG/ml of support [45,46]. Affinity columns with immobilized TG 19318 are not affected by the presence of denaturants, detergents or other sanitation reagents commonly used for pyrogen removal and are stable in repeated use. Adsorption and desorption of immunoglobulins can be accomplished with the eluents used for Protein A columns. Sterilization by autoclaving leads to a 10–30% capacity reduction, but this effect is presumably due to support instability under stringent temperature and pressure conditions rather than ligand instability [47]. The ligand is not immunogenic, even after conjugation to carrier molecules, such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) (unpublished observations). Experiments on mice indicate that TG19320 is nontoxic when administered per os (no toxicity for doses up to 2000 mg/kg) while for intravenous administration the dose leading to death in 50% of cases (LD₅₀) is around 150 mg/kg [45]. Preliminary experiments suggest that the ligand is more stable to proteolytic digestion when coupled to solid supports, and the enzymatic activity normally found in crude feedstock derived from cell culture supernatants do not lead to noticeable losses of capacity. A variant of this ligand, TG19320, where the amino acids are in the D configuration, maintains binding properties for IgG, is much more stable to proteolytic degradation, and is currently under evaluation for chromatographic applications [48]. Adsorption of antibodies on TG19318 affinity columns occurs with neutral buffers at low ionic strength, conditions fully compatible with the use of crude feedstock derived from cell culture supernatants. Elution of adsorbed immunoglobulins can be achieved simply by changing the buffer to acid or weakly alkaline conditions, with acetic acid at pH 3.0 or sodium bicarbonate at pH 9.0. Increasing the ionic strength of the dissociation buffer favors a more efficient elution of adsorbed antibodies.

The interaction affinity is strong enough to allow purification of antibodies directly from diluted supernatants, where the immunoglobulin concentration is very low, from 10 to 50 µg/ml. The main contaminant, albumin, is always efficiently removed in the purification step with any type of support tested for TG19318 immobilization. The ligand has a much broader specificity than Protein A, since not only will it purify IgG from different sources, but also IgY from egg yolk [47], IgM from ascitic fluid, sera, or cell culture supernatants [49], IgA from cell culture supernatants [50] and IgE from cell culture supernatants [51]. Preliminary investigations suggest that binding affinity is greater for IgM than for the other Ig classes, and under selected conditions isolation of IgM from IgG-containing samples can be achieved.

2.2. Peptide H

Short, linear peptides derived from the screening of combinatorial libraries, often do not display enough structural rigidity to provide recognition surfaces sufficiently selective for biotechnological or pharmaceutical applications. Cyclic peptides, on the other hand, show increased resistance to enzymatic degradation and constrained flexibility compared with the linear form. By screening dimeric tripeptide libraries (DTL), produced by starting from a bifunctional lysine residue at the C-terminus, and structurally

constrained by the presence of a disulfide bond formed by two cysteine residues at the N-terminus [52], a ligand for mouse IgG purification has been identified [53].

The screening assay has been performed by immobilizing the library, as a set of sublibraries on microtiter plates for ELISA determination by noncovalent adsorption and then treating the microtiter plate wells with a fixed concentration of mouse monoclonal antibody. The presence of bound antibody was detected by a subsequent treatment with a goat anti-mouse IgG, labeled with peroxidase, followed by chromogenic reaction with ABTS. This screening strategy led to the identification of Peptide H, a cyclic dimeric peptide of formula (C–F–H–H)₂KG, where the two cysteine residues at the N-terminus are covalently linked by a disulfide bridge. When tested in affinity chromatography experiments, this ligand proved useful for mouse and rat IgG purification, but less selective for human and rabbit IgG (unpublished observation). The capacity for mouse IgG was close to 1 mg IgG/ml of derivatized support (CH-sepharose 4B).

2.3. *APa (artificial Protein A)*

Nonpeptidic ligands for IgG have been developed from the X-ray crystallographic structure of the complex between the B domain of Protein A and the Fc fragment of IgG and the specific recognition and complexation elements. By using computer-aided molecular modeling, a series of biomimetic molecules around the Protein A dipeptide Phe132–Tyr133, identified as responsible for IgG binding, was designed [54]. One of these ligands, termed ApA (artificial Protein A), was found to bind to IgG in a competitive manner with an affinity constant of 1–10 μ M, as deduced by competitive ELISA experiments. Immobilized ApA proved to be useful for the purification of IgG from human plasma and ascitic fluid, with a purity of eluted IgG close to 98%. Both pH and ionic strength have a direct effect on binding, which is stronger near pH 7 and weaker at extremes of pH. Contrary to TG19318, interaction between ApA and IgG was enhanced at higher salt concentration, probably because binding is stabilized predominantly through hydrophobic interactions. After immobilization on solid adsorbents such as agarose, ApA did not show alteration of binding capacity following treatment with 1 M NaOH for 8 days. As in the case of TG19318, ApA proved useful also for the purification of IgM and IgA. The derivatized support capacity for IgG ranged from 5 to 12 mg IgG/ml of support, depending on the IgG isotype.

3. Conclusions

Among the classical techniques for protein purification, affinity chromatography deserves particular attention in the downstream processing of antibodies, since it allows a several-fold purification in a single step, usually with high recovery, and is compatible with the use of large amounts of very dilute preparations, as is often the case with monoclonal antibodies produced by hybridoma technology. The availability of synthetic ligands for immunoglobulin purification deduced by screening combinatorial libraries screening or computer modeling offers several advantages over the use of conventional ligands, such as Protein A. First, the possibility of facilitating the purification of classes

Table 1

Capacities (mg Ig/ml support) of different ligands for various immunoglobulin classes

	Protein A	Anti-IgE antibodies	Jacalin	Mannan binding protein	PAM	ApA	Peptide H
IgG	20–50 ^a	N.B. ^b	N.B. ^b	N.B. ^b	10–25 ^a	5–12 ^c	1 ^d
IgM	N.B. ^b	N.B. ^b	N.B. ^b	0.85	10 ^e	19.6	N.D. ^f
IgA	N.B. ^b	N.B. ^b	3.0–4.5 ^a	N.B. ^b	10 ^g	24	N.D. ^f
IgE	N.B. ^b	0.5	N.B. ^b	N.B. ^b	10 ^g	N.D. ^f	N.D. ^f
IgY	N.B. ^b	N.B. ^b	N.B. ^b	N.B. ^b	30–60	N.D. ^f	N.D. ^f

^a Depending on the type of support.^b No binding or very weak binding.^c Depending on IgG isotype (in mg Ig/g wet gel: IgG₁ 12.3, IgG₂ 10, IgG₃ 4.8, IgG₄ 8.7).^d Only for rat and mouse IgG.^e Selective for IgM with buffers at high ionic strength.^f Not determined.^g For monoclonal antibodies.

of immunoglobulins other than IgG, thus expanding the characterization and use of these immunoglobulins, which have found limited applications only because their purification has been complex and time-consuming. Second, the stability toward cleaning-in-place procedures, an important attraction for large-scale purification. While the capacity of sorbents derivatized with PAM and ApA is very high for certain classes of immunoglobulins such as IgM, IgA, IgE and IgY, where classical ligands are not performing very well, for IgG Protein A and Protein G still represent the most effective ligands from this point of view (Table 1). More work is needed to improve the binding capacity of supports derivatized with these novel ligands through the improvement of supports and coupling chemistry to optimize stability, selectivity and capacity. This may require hard efforts, since the high capacity of commercially available Protein A affinity sorbents is the result of improvements started 20 years ago.

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