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Review article

Purification of antibodies by affinity chromatography ☆

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Abstract

This review focusses on affinity purification of immunoglobulins, a methodology which is a powerful tool to obtain pure and intact antibodies. Affinity techniques allow antibody purification both in a single step chromatographic procedure as well as in complex purification protocols depending on the intention to use the target antibody. The purification strategies for antibodies by interaction with affinity ligands such as antibodies and Fe receptors or low molecular weight compounds are described.

Keywords: Review; Purification; Antibodies; Immunoglobulins; Affinity chromatography

1. Introduction

For several years, there have been standard procedures for the purification of nucleic acids, whatever their source. Following them rigidly, most probably, is the key to success. This is not the case for protein purification. Each protein purification method is unique, and minor changes in upstream processes, such as the replacement of a broth component for the cultivation of cells, may make it necessary to change the method applied to isolate a specific target protein. However, the situation is not as bad as it may seem from these former remarks. There is at least a standard frame of reference that can be applied to most

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proteins in combinations, and many purification schemes resemble each other, even if proteins of different functions and structures are to be purified. It will always be necessary to remove particles from solutions and desirable to concentrate the proteins of interest. Proteolytic degradation and denaturation should be avoided. The purification of proteins belonging to the same class and therefore exhibiting close structural and functional relationships may require a very different methodology. In this respect, antibodies are a typical example. A combination of methods is applied to purify antibodies, the most common of which are: salting out, ion-exchange chromatography, and affinity chromatography. This review focuses on affinity methods and evaluates the power and limitations of this methodology.

2. Structure and sources of antibodies: the need for purifying them

There are numerous textbooks as well as reviews dedicated to the structure and function of antibodies, and the reader is referred to them for detailed information. However, a very short sketch is presented in the context of purification.

Antibodies or immunoglobulins consist of pairs of disulfide-linked heavy (H)- and light (L)-chain dimers. The heavy and light chains contain variable (V) and constant (C) domains. The variable domains of both H and L chains form the binding sites for interaction with the antigen. The constant domains of the H chain provide the antibody molecule with several biological functions, some of which are masked until the antigen binds to the binding site and causes a structural refolding. There are five different H chains (γ, α, μ, δ, ε) and two different light chains (κ and λ), determining the immunoglobulin classes IgG, IgA, IgM, IgG, IgD, and IgE. Within these classes, structural differences allow a distinction between subclasses and allotypes.

The smallest fragment of an antibody that is able to bind the corresponding antigen contains the variable domains of the H and L chains. It can be obtained by limited proteolytic degradation, which results in the formation of Fab or $F(ab)_2$ fragments (depending on the proteases and conditions used) and the Fc part, which consists of the constant region, respectively.[1] The methodology of molecular genetics now allows the construction of single-chain antibodies from their genes by fusing the V (L) and V (C) parts.

Each kind of antibody is produced by a special clone of plasma cells after their differentiation from B-lymphocytes. After stimulation by the antigen, these clones proliferate and produce considerable amounts of the specific antibody. The antibodies exhibit unique binding properties toward a wide range of natural and synthetic substances. These features have prompted remarkable efforts to obtain such antibodies in sufficient amounts.

An increasing number of modern diagnostic and therapeutic technologies are based on the interaction between antibodies with high affinity and their specific antigens. In vitro diagnostic procedures, like radioimmunoassay (RIA), immunoradiometric assay (IRMA), enzyme-linked immunosorbent assay (ELISA), or special blot techniques with radio-

[1] Note that the designation of this fragment resulted from the ease of its crystallization and not from its composition.

labeled antigens or antibodies linked to radioactive isotopes, enzymes, fluorescence markers, or even to other immunoglobulins allow the detection of minimal amounts of proteins, nucleic acids, and complexes of carbohydrates and lipids with a high degree of specificity in various media.

Often, purity of the immunoglobulins is critical because other substances in the source material may interfere with the detection process. For example, in microscopy, background staining due to nonspecific binding can interfere with or even vitiate the detection of the target antigen.

Therapeutically, antibodies have been used for a long time in vivo for passive immunization (neutralization) in case of anticipated or ongoing viral and bacterial infections, or contamination with biological or chemical toxins. In recent years, the high specificity of antibodies and the development of techniques for the production of specific antibodies by chemical and biological means, including molecular biology (e.g., monoclonal antibodies), have led to the introduction of treatment strategies where radiolabeled monoclonal antibodies reactive with tumor-associated antigens can be targeted to malignant cells and selectively deliver diagnostic or even cytotoxic isotopes in vivo.

Furthermore, antibodies are important tools for the purification of their antigens for research purposes as well as for their use as pharmaceuticals on an industrial scale. The specificity of the interactions of antibodies and their corresponding antigens also allows the functional manipulation of target antigens within a complex chemical environment, such as blood or cell extracts.

The sources from which antibodies may be isolated are nowadays not limited to the body fluids of immunized animals or human beings. Instead, cultures of engineered cells, like hybridoma, or bacterial cells permit the production of designed antibodies, having distinct properties with respect to their binding and effector functions, and now represent the main sources of commercially used antibodies. However, the variety of antibody species and the sources they derive from as well as their intended use may cause serious problems in selection of appropriate purification procedures. Often, the difficulties associated with the purification of antibodies have hindered their use. For instance, the withdrawal of a therapeutic antibody from the market for reasons associated with its purification has happened several times within the last few years. The purification of antibodies presents several problems: First, because the humoral immune system is incredibly diverse and able to produce an unlimited number of different immunoglobulins, every antibody is unique. This may cause an established purification procedure to fail when applied to a certain antibody. Second, antibody molecules are multifunctional. A purification protocol working well in preserving the antigen-binding properties of an antibody may cause, e.g., an altered interaction of the purified antibody with cell receptors. Third, antibodies have to be purified from very complex solutions: blood or blood fractions, milk, cell culture supernatants with varying ingredients, and extracts from cells of different origin (mammalian, bacterial, fungal, vegetable), etc. That is why often, if applicable, the purified antibodies are not used for laboratory or diagnostic purposes, but rather whole serum or cell culture supernatants. However, in many cases, purification is indispensable [1]. Aside from therapeutic applications, there is a need for using purified antibodies for several reasons. Antibodies are often used in a labeled form. To have the label, whether a protein (e.g., an indicator enzyme) or a low-molecular-weight substance

(e.g., a fluorophore), couple reproducibly, contaminating substances, i.e., other proteins, must be removed. A similar situation is encountered when antibodies must undergo proteolytic fragmentation to remove parts of the immunoglobulin in order to obtain Fab fragments. Purified antibodies are needed when a calibration of the protein content of an antibody solution is necessary to assure stoichiometric equivalence of the antibodies with their ligands.

3. Affinity purification: the method of choice for antibodies

The choice of appropriate methodology for the purification of antibodies, as for every protein, always depends on the yield and purity of the antibodies as well as on the overall costs of the procedure. The effectiveness of affinity purifications relies on the ability of the antibody to recognize specifically an affinity adsorbent, which consists of an immobilized ligand, sometimes a spacer, and the matrix to which the ligand is attached. Several matrices can be used with the same ligand, but they will perform differently. Often, detailed studies are available (e.g., Fahrner [2] studied several commercial protein-A-affinity chromatographic sorbents) which may help in deciding which method fits best in one's separation problem. Occasionally, a method recommended for the purification of a member of a antibody class, which should work, does not. For instance, a monoclonal IgG antibody cannot be purified by either Protein A- or Protein G-agarose chromatography. Sometimes, such antibodies do not bind at all or insufficiently for use in chromatography. Very often, the elution conditions are too harsh, and irreversible inactivation of the antibody occurs. A series of buffers for binding and elution with different pH, salt content, etc. may be tried, or other affinity adsorbents may be used instead.

With respect to the specificity of an antibody, it is worth mentioning that trying a monoclonal antibody involves the isolation of a single antibody that is not contaminated by other antibodies.

The purification of immunoglobulins by affinity techniques takes advantage of their domain structure. Two general strategies can be deduced from their architecture. The first one takes advantage of the specificity of antigen binding; the second one targets the constant part of antibodies, the Fc fragment. While there is little difference in the use of the antigen for purification, there are very different ligands targeting the Fc part.

3.1. Affinity purification using antigen

Because there is specific antigen recognition by antibodies, affinity purification with immobilized antigen as affinity ligand is widely used. This method is of advantage if a specific antibody must be obtained from a mixture of immunoglobulins with different specificities. The purification of monospecific antibodies from sera of immunized animals is a typical example of such a challenge. The often-occurring cross-reactivity of antibodies sometimes allows affinity purification with ligands different from the antigen used for immunization. Such an approach is valuable if, e.g., the antigen is expensive, hard to handle, or otherwise restricted in its use. The method of using immobilized antigen has also been used for the selection of antibodies with desired affinities [3]. In principle, the

same conditions for binding, washing, and elution are used for the isolation of antigens with immobilized antibodies. This method is the widely used immunoaffinity chromatography, e.g., the isolation of antibodies with anti-antibodies [4]. It is applied where antibodies of low affinity [3] are better suited, for one can choose milder conditions for the elution of bound antigen.

3.2. Affinity purification with anti-antibodies

The constant domains of both the H and L chains of antibodies are potential targets for the affinity purification of antibodies by using immobilized anti-antibodies. Especially for the isolation of rat monoclonal antibodies, which very often resist their purification by Protein A and even Protein G, anti-rat-IgG light-chain antibodies, coupled to agarose, can be used [1]. Normally, binding can be achieved at pH 8, while elution occurs at lower pH. Care should be taken to avoid long periods of exposure of antibodies eluted by acidification because this may often damage the antibody. Neutralization by adding concentrated buffers, followed by rebuffering through dialysis or size-exclusion chromatography should be performed as soon as possible after elution to prevent inactivation.

Since the anti-antibody ligands are proteins, care must be taken to avoid contamination of the antibody by traces of the ligand or proteolytic degradation products that may be solubilized during chromatography, especially during elution [5].

3.3. Bacterial Fc receptors

Numerous cells and viruses have proteins on their surfaces which bind to the Fc part of immunoglobulins. Because of this interaction, such proteins are designated as Fc receptors. In bacteria, these immunoglobulin-binding proteins are considered to be virulence factors, which enable pathogens to evade the host's immune response [6,7]. As a result of the initially clinically oriented research, bacterial Fc receptors were intensively characterized and became important tools for the affinity purification of immunoglobulins. Several bacterial Fc receptors have been described, classified, and their genes cloned. The best-studied in this group are Protein A from *Staphylococcus aureus* and Protein G from *Streptococcus* spp., which bind IgG from different sources [8,9]. Although they share no sequence homologies, they (and other bacterial Fc receptors) bind to the same part of IgG constant regions, indicating a convergent evolution of such proteins. Proteins A and G are used as ligands in many affinity applications. Most probably, protein-A-affinity chromatography is the most common method for antibody purification. That is because Protein A has some important virtues for application as an affinity ligand. It is well-characterized [10,11] (even its crystal structure has been solved [12]) and it is obtainable in large quantities from recombinant bacteria. Its molar dissociation constant is at about 10^{-7}, and this reflects its high affinity for the Fc region of immunoglobulins. Protein A is stable over a wide range of pH (pH 2–11) and is able to refold after treatment with denaturating solutions of urea and guanidinium salts. Because it is remarkably resistant to 0.5 M NaOH, clean up of Protein A can be accomplished with only a minor decrease in functional capacity and no adverse effect on leakage of Protein A into the eluate [13]. Protein A sorbents allow several cycles of binding and eluting antibodies which improves their operating efficiency. Furthermore, because

Protein A contains four binding sites, two of which are able to interact with the Fc part of immunoglobulins at the same time, coupling of Protein A to supports is easy. Whatever the chemical nature of the coupling procedure may be, the resulting Protein A conjugate is effective in binding antibodies. Protein A sorbents are available from several commercial suppliers in the form of Protein A coupled to different matrices. On the laboratory scale, agarose-based Protein A adsorbents are by far the most popular. Prepacked columns of different sizes as well as spin columns and even complete purification kits, containing the adsorbent together with the buffer systems required for binding, washing, and elution, are also available.

For large-scale purification of antibodies with Protein A, conventional affinity chromatography has some limitations. Agarose beads are relatively large and therefore exhibit slow intraparticle diffusion. Furthermore, cell debris must be removed from cell culture fluids prior to loading the affinity column. Attempts to circumvent such problems point in the direction of membrane systems or expanded beds for chromatography [14,15].

Different antibodies may be purified by means of Protein A chromatography (Table 1). Polyclonal and monoclonal IgG from human and most laboratory animals (mouse, rabbit, guinea pig) bind well, with the exception of monoclonal IgG_3 and mouse IgG_1. [16,17]. Varying the binding buffer composition may help to achieve binding of such antibodies. For nonbinding antibodies from these species as well as for immunoglobulins from rat,

Table 1
Binding of immunoglobulins to Protein A

Immunoglobulin	Binding
Human IgG_1	s
Human IgG_2	s
Human IgG_3	w
Human IgG_4	s
Human IgM	w
Human IgA	w
Human IgD	n.b.
Mouse IgG_1	w
Mouse IgG_{2a}	s
Mouse IgG_{2b}	s
Mouse IgG_3	s
Mouse IgM	n.b.
Horse IgM	n.b.
Horse IgG	w
Rabbit IgG	s
Goat IgG	w
Guinea pig	s
Sheep IgG	w
Dog IgG	s
Pig IgG	s
Cow IgG	w
Rat IgG	w
Chicken IgG	n.b.

The data were compiled from different internet sources.
S: strong binding; w: weak binding; n.b.: no binding.

goat, and chicken, Protein G is used instead. Protein G exhibits binding sites not only for antibodies but for albumin and α2-macroglobulin, which would contaminate antibodies after their Protein-G-based affinity purification. However, Protein G is now available in genetically modified form, with the additional binding sites removed.

A limitation in using bacterial Fc receptors for purifying antibodies is the elution at low pH, which can alter the conformation of immunoglobulins [18]. Therefore, attempts have been made to modify the structure of Protein A in order to obtain affinity ligands that allow milder conditions for elution. One of these attempts is the genetic manipulation of so-called Protein Z, which is an IgG-binding-domain derivative, based on the B domain of Protein A [19,20]. The affinity for immunoglobulins could be lowered and antibodies could be eluted at pH above 4.

3.4. Protein-A-mimetic ligands

Although Proteins A and G are obtained from genetically modified bacteria, their isolation is still complex and expensive. Furthermore, if antibodies to be purified by means of bacterial Fc receptors are used as therapeutics, time-consuming analyses are required to ensure the absence of potentially hazardous contaminants, such as pyrogens and viruses. It is well known that Protein A leakiness from columns causes contaminations that will interfere with analysis of the purified antibody [21,22]. Therefore, considerable efforts have concentrated on the synthesis of low-molecular-weight substances, able to bind immunoglobulins, like Protein A (Table 2). The screening of peptide libraries has identified several low-molecular-weight peptides that fulfilled such requirements [24,25]. Their performance as ligands in affinity chromatography has been demonstrated to match closely that of Protein A, they may even have broader selectivity. The multimeric peptide TG19318, e.g. [26], binds IgG from human, rabbit, rat, mouse, goat, and horse sera as well as rat and mouse monoclonal antibodies from cell supernatants and ascites fluids. Furthermore, this peptide was found to be nontoxic in mice up to administered 2 g/kg. Sodium bicarbonate was effective in eluting bound immunoglobulins at pH 9, as was acidification. Coupled to a rigid ceramic composite support [27], the peptide ligand is remarkably stable toward proteolysis. While Protein A, immobilized on a similar matrix, lost about 75% of its initial antibody-binding capacity, the Protein-A-mimetic adsorbent was stable in the presence of chymotrypsin and lost only about 25% binding capacity upon incubation with trypsin. At least in one case, TG19318, immobilized on hydroxysuccinimide-activated agarose even bound to a mouse monoclonal IgE from crude ascites fluid [29]. Although it must still be demonstrated that a ligand, such as TG19318, is generally useful in affinity chromatography for the isolation of IgE, this result is promising for antibodies of the IgE class, if they need to be purified by nonaffinity techniques or by using anti-IgE antibodies, for neither Protein A nor Protein G can bind to monoclonal IgE [30]. Sorbents which bind to IgG could be used in a two-step procedure, with, first, Protein A adsorption to remove IgG, followed by IgE adsorption and elution [31]. The purification of IgM was also demonstrated with the use of TG19318 as affinity ligand [32].

The group of Lowe [34,35] followed a different approach in order to find Protein-A-mimetic ligands. Knowing of the potential of reactive dyes as ligands for pseudoaffinity chromatography [36], they designed non-peptidyl low-molecular-weight synthetic mole-

Table 2
Protein-A-mimetic ligands

Ligand/matrix	Immunoglobulins purified	Conditions of affinity chromatography	K_a [M^{-1}]	References
Tetramer of the IgG binding domains A and B of Protein A/Sepharose 4B	Binds IgGs from various species. The capacities are highest with porcine, rabbit, human and guinea pig, intermediate with bovine, horse and sheep, and lowest with mouse, goat, rat and chicken sera.	*Capacity:* 23.5 mg human IgG/g moist weight gel *Binding*: 100 mM phosphate, pH 7.0 *Elution*: 300 mM KCl–HCl, pH 2.3	–	[23]
TG19318 (multimeric peptide)/ ceramic support	IgG, IgM, IgE, IgY	*Capacity*: 25 mg IgG, 2–8 mg IgM, ~ 5 mg IgE, and 65 mg IgY/ml gel, respectively *Binding*: 50 mM buffer, pH 6.5–7.0 *Elution*: 100 mM Hac or 100 mM sodiumbicarbonate, pH 9.0 *Recovery*: 85–95% *Purity*: 90–95%	3.3×10^7	[24–33]
2-(3-aminophenol)-6-(4-amino-1-naphthol)-4-chloro-*s*-triazine (22/8)/agarose beads (the ligand is stable in 1 M NaOH for at least 7 days)	Human IgG from plasma Murine IgG from ascites Bovine IgG removal from fetal calf serum	*Capacity*: 51.9 mg human IgG/g moist weight gel *Binding*: 50 mM phosphate, pH 8.0 *Elution*: 100 mM glycine–HCl, pH 2.9 *Recovery*: 60% *Purity*: 92.5%	1.4×10^5	[34–36]
FastMabs A® (low molecular weight ligand)/agarose	IgG1, IgG2a, IgG2b, IgM from mouse IgG2b, IgM from rat	*Capacity*: 50–70 mg mouse IgG/ml gel *Binding*: acetate buffer, pH 5.1 substituted with 0.5–1.5 mg/ml sodium lauroyl sarcosine *Elution*: 50 mM phosphate buffer, pH 7.0 *Recovery*: 90–98% *Purity*: 84–99% (35% for rat IgM)	–	[37]

cules that mimic Protein A. Following X-ray crystallographic structure analysis for identifying the specific recognition and complexation sites between the B domain of Protein A and the Fc part of IgG, they synthesized a series of biomimetic low-molecular-weight molecules and found one that binds IgG competitively with Protein A by mimicking a key dipeptide motif of the B domain. The interaction was found strong enough (molar dissociation constant of 10^{-6}). An adsorbent with the immobilized ligand was used to purify IgG from human plasma and murine IgG from ascites fluid, and to remove bovine IgG from fetal calf serum. Such synthetic affinity ligands are inexpensive, completely defined and, if nontoxic, ideal substitutes for protein ligands. Adsorbents are readily cleanable and even sterilizable, which is another advantage because reusing the adsorbent becomes safe.

3.5. Histidine-ligand-affinity chromatography

Under certain circumstances, natural antibodies exhibit catalytic functions within their variable region. Such enzymatic activities, in particular peptide- and DNA-degrading activities, are of particular relevance in some autoimmune disorders, like rheumatoid arthritis or lupus [38,39]. Vijayalakshmi [18,40] has pointed out that a detailed study of the structural basis of the catalytic functions of antibodies requires efficient and specific purification methods that not only ensure good recovery, but also the conformational integrity of the purified immunoglobulins. One must ensure especially that enzymes present in the original biological fluid (serum) are not contaminating the final antibody preparation. Using histidine-ligand-affinity chromatography, the purification of intact antibodies with peptidase activities was successful, with a recovery of high-purity IgG directly from the sera of autoimmune disease patients. The preservation of catalytic activities of the antibodies was attributed to the mild elution conditions that were applicable. The addition of sodium chloride to the binding buffer was sufficient for elution, and this is considered a major advantage of this type of affinity chromatography.

3.6. Metal-affinity chromatography

Some immunoglobulins show an innate affinity for metal due to a histidine-rich sequence. For instance, murine IgG_1 has been shown to contain a metal-affinity site near the carboxy terminus of the H chain. On this basis, metal-affinity purification techniques have been developed (Table 3) [41]. Antibody, bound to the metal-affinity column, was eluted by decreasing the pH, and IgG_1 could be eluted at about pH 6. The purity of the immunoglobulins did not attain that of Protein A purification: Murine IgG_1 is recovered in only approximately 60% purity, with transferrin as a major contaminant. The humanized murine IgG_1 isolated from cell culture supernatant was, however, 90% pure and did not contain any light chains or contaminating albumin [42]. In combination with ion-exchange chromatography, metal-affinity chromatography is an alternative to Proteins A/G when gentle elution is essential. Recoveries of antibody were greater than 90%. In this paper [42], the authors speculated that the histidine-rich sequence responsible for metal binding could be genetically engineered, resulting in a metal-binding site of even higher affinity for potential application in antibody imaging and therapeutics. His-tagging of recombinant proteins at their amino or carboxy termini is now a widely used method to ease the

Table 3
Metal chelate affinity chromatography of immunoglobulins

Chelating ligand	Chelated metal ion	Immunoglobulin purified	Purity (%)	References
Imidodiacetic acid (IDA)	Ni^{2+}	Humanized murine IgG_1	90	[42]
	Ni^{2+}	Murine IgG_1	60	[42]
	Ni^{2+}	Fc fragment	~ 80	[43]
Tris(2-aminoethyl)amine (TREN)	Cu^{2+}	Goat IgG	>95	[46]
Tris(carboxymethyl)ethylenediamine (TED)	Fe^{3+}	Human IgG	n.d.	[37]
Nitrilotriacetic acid (NTA)	Ni^{2+}	single-chain antibody fragment	n.d.	[47]
	Ni^{2+}	Ig-like domain of human G-CSF	80	[48]
	Ni^{2+}	murine anti-dsDNA autoantibody	80	[49]
8-Hydroxyquinoline (8-HQ)	Yb^{3+}, Al^{3+}, Fe^{3+}	Human IgG	70–80	[50]

n.d.: no data available.

downstream purification. It is also applied to antibodies. Especially, if single-chain antibodies or fusion proteins, consisting of antibody fragments and indicator proteins, must be purified, metal-affinity chromatography is often used, for such constructs lack the target of Fc receptors, the Fc region. Examples are the purification of tumor-specific antibodies, expressed in tobacco plants [43], a green, fluorescent single-chain Fv fusion protein [44], and a single-chain fragment derived from a human monoclonal IgM [45].

Metal-affinity chromatography with copper ions was also used for the rapid, single-step purification of immunoglobulins from goat serum [46]. Adsorption was achieved at pH 7, and subsequently, the immunoglobulin fraction was recovered, with a decreasing linear pH gradient, at about pH 5.5. Immunoglobulin purity could be improved to 95%, if the adsorption was performed at pH 6.0. The capacity of the gel was dependent on the flow-rate during the adsorption step. With 15–17 mg immunoglobulin bound per ml of gel, the capacity of the commercial adsorbent used was in the range of Protein A/G sorbents.

3.7. Thiophilic interaction

Originally developed by Porath and co-workers in 1985 [51–55], this method still lacks wide acceptance. SH-containing ligands, originally mercaptoethanol, are immobilized on divinylsulfone-activated matrices to form so-called thiophilic adsorbents or T-gels, which exhibit salt-promoted selective adsorption of proteins by interacting with as yet poorly identified thiophilic regions of such proteins. These gels are particular selective for immunoglobulins. Polyclonal as well as monoclonal antibodies have been successfully purified using T-gels [53] and binding is almost independent of the antibody source and class. Both aliphatic as well as π-electron-rich aromatic and heteroaromatic ligands have been studied. All of these adsorbents bind immunoglobulins at high concentrations of water-structure forming salts. Later on, it was observed that adsorbents, derived from coupling heterocyclic ligands to epoxy-activated matrices, bind immunoglobulins with similar selectivity. Desorption of the proteins bound is easily achieved by omitting the salt from the buffer. The main advantages of using thiophilic chromatography instead of

Protein-A- or Protein-G-based techniques are these mild conditions for elution at neutral pH, the opportunity to autoclave thiophilic gels, and the low cost of these adsorbents. However, the binding of antibodies to thiophilic adsorbents is far less specific than affinity purification with bacterial Fc receptors as ligands. This may, however, turn into an advantage, for thiophilic adsorption chromatography is also applicable to the purification of antibody fragments. Fiedler and Skerra [56] have purified a recombinant Fab antibody lacking the Fc part of immunoglobulins by means of T-gel chromatography, while others have separated papain-digested immunoglobulins and their fragments, and were also able to separate them from the protease used in fragmentation [57,58].

The elution conditions in thiophilic chromatography may be simplified in order to combine the purification step with the rebuffering necessary for chemical derivatization of the antibody protein. This has been demonstrated, e.g., by Lutomski et al. [57] who eluted IgG, bound to the T-gel, directly with a buffer suitable for biotinylation, thereby omitting an additional buffer change.

In some respect, thiophilic chromatography resembles hydrophobic interaction chromatography and is, therefore, not a real affinity method. However, comparing the behavior of various serum proteins to a T-gel and to Phenyl-Sepharose, the binding and elution pattern turned out to be different. Furthermore, proteins that adsorb well to hydrophobic adsorbents, such as albumin, are not bound at all to thiophilic gels. The finding of Scoble and Scopes [59], who demonstrated in a systematic study that the treatment of agarose matrices with divinylsulfone alone is sufficient to obtain adsorbents with the performance of thiophilic gels, is another puzzling observation which demonstrates that thiophilic interaction must be considered as an exceptional affinity interaction.

Another disadvantage of thiophilic chromatography besides its lack of specificity is the need to handle the antibodies at high salt concentrations. The need for high salt concentrations in the binding buffer is inconvenient for the samples from which the immunoglobulins are to be purified must be adjusted by either adding the salt to the appropriate concentrations, or by changing the buffer using procedures such as dialysis which are often time consuming. However, Scholz et al. [60,61] synthesized thiophilic adsorbents by coupling derivatives of mercaptopyridine and quinazoline to divinylsulfone-activated agarose, which are able to bind immunoglobulins in a salt-independent manner at neutral pH. Whether this type of chromatography which exhibits the same binding specificity for proteins from human and animal sera as T-gel chromatography at high concentrations of lyotropic salts is an example of hydrophobic charge induction chromatography which was introduced by Burton and Harding [62] remains to be verified. It is noteworthy, however, that recently, this type of adsorbent became commercially available for the purification of all classes of human IgG as well as for monoclonal immunoglobulins from mice.

3.8. Lectin-affinity chromatography

Immunoglobulins are glycoproteins and can therefore be purified using affinity chromatography based on immobilized lectin adsorbents [63]. An example is the binding of dog serum immunoglobulins G, A, M, and E to concanavalin A [64]. All of the IgE and IgM, but only 60% of IgG and 58% of IgA, bound to the Con A-Sepharose. By means of a sequential

elution with different buffers, a separation of antibodies occurred: IgE could be eluted by mannose, methylglucose, and methylmannoside. IgG was eluted by glucose, mannose, methylglucose, and methylmannoside. IgA and IgM were eluted by methylmannoside only.

Especially for the isolation of human IgA, lectin-affinity chromatography is of importance. Human IgA possesses a series of O-glycans which are closely located within the hinge region of the molecule. Purification of IgA is sometimes necessary for a detailed analysis of its glycosylation and the harsh conditions for the elution of IgA as, for instance, from anti-IgA columns, can have profound effects upon its O-glycans. The most commonly alternative is affinity purification on jacalin-agarose [65–67]. Jacalin-agarose as well as other jacalin-adsorbents are readily available from commercial suppliers. All binding, washing, and elution steps during the lectin-affinity chromatography can be performed at neutral pH, and elution is achieved with galactose or mellobiose.

3.9. Purification of recombinant antibodies using affinity tags

Recombinant proteins can be fused on the gene level to peptide sequences which allow the simple purification of the fusion proteins by affinity techniques [68]. Such peptides are called affinity tags and several of them are available on cloning vehicles. The implementation of such systems has also been demonstrated for antibody purification [49,69,70].

4. Perspectives

Purification methods for immunoglobulins have a long history of highly qualified attempts to obtain them in an active and pure manner. Until now, Protein-A-chromatography is the purification method of choice for a large scale of antibodies, but the harsh conditions necessary to elute these sometimes sensitive proteins have led to the development of alternative purification procedures. One of the most fascinating developments of the recent years are protein mimetic ligands, but also thiophilic and hydrophobic charge induction chromatography are promising. These new developments introduced in large-scale production of antibodies will allow a wide application of pure and intact antibodies as diagnostic tools and pharmaceutics to improve and retain humans health.

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