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Analytical and Preparative Methods for Purification of Antibodies

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Dedicated to the memory of Professor Vera Johanides

Summary

Monoclonal and polyclonal antibodies play a key role in medicine as well as in analytical biotechnology. After being used predominantly for analytical purposes over the last 20 years, the number of therapeutical applications is growing fast. This review describes methods for purification of antibodies on an analytical scale in the laboratory, as well as on a preparative scale in industrial production. The classical methods of precipitation and the modern chromatographic methods are described. Methods of precipitation are still widely used in the production of immunoglobulins G (IgG) from human plasma. The chromatographic methods, often in combination with precipitation for the sake of initial purification, are the almost exclusively used methods in the production of monoclonal antibodies. The chromatographic methods are sub-divided into non-affinity and affinity modes. The most widely used methods are presented and discussed.

Key words: monoclonal and polyclonal antibodies, IgG, IgM, purification, chromatographic methods

Introduction

Since the introduction of the hybridoma technology more than 20 years ago by Milstein and Koehler (*1*) the monoclonal antibody has revolutionized biotechnology and biomedicine. As diagnostic agents in many diseases, especially in neoplastic diseases, monoclonal antibodies have been proven to be extremely useful. Recent development in cancer therapy has resulted in FDA approvals of several monoclonal antibodies as the new class of biotherapeutic cancer drugs. Currently, therapeutic monoclonal antibodies represent more than 30 % of the biotechnology products in clinical development in the US. Apart from the therapeutical monoclonal antibodies, the importance of polyclonal immunoglobulins obtained from human plasma has grown over the last 10 years. Plasma-derived human IgG are increasingly used for treatment of genetic and acquired immune deficiencies as well as several auto immune diseases (*2*). The need to develop effective, economical and rapid purification methods of monoclonal and polyconal antibodies from a variety of biological fluids becomes imperative for *in vitro* or *in vivo* application.

Antibody purification can be divided into two main groups: precipitation methods and chromatographic methods. The latter is grouped further according to the operational characteristics into non-affinity and affinity chromatography. Depending on the application of the

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antibody, purity and recovery will likely determine the strategy of purification. In this chapter, overview of common chromatographic methods for antibody purification will be described. In general, multiple step purification and combination of methods are required to obtain sufficient purity of antibodies. Since every antibody is unique and each has a distinctive distribution of hydrophobic, positive and negative charge characteristics, modification and optimization purification procedures might be necessary (*3*).

Antibodies are globulin proteins (immunoglobulins) that react specifically with the antigen that stimulated their production. Based on the electrophoretic migration rate, there are three types of globulins: alpha, beta and gamma. Antibodies are gamma globulins. There are five classes of antibodies: IgG, IgM, IgA, IgD and IgE. Within these classes, subclasses also exist. IgG and IgM are antibody classes involved in the primary and secondary immune response that can be found in the sera in relatively high concentrations. Only the purification of these two classes will be discussed in detail.

Immunoglobulin Structure

Immunoglobulins are glycoproteins made up of light (L) and heavy (H) polypeptide chains as based on their respective relative molecular mass. Light chains have a relative molecular mass of ca. 25 kDa, whereas heavy chains have a relative molecular mass of 50–70 kDa. The simplest molecule has a Y shape and consists of 4 polypeptide chains: 2 H and 2 L (Fig. 1). The 4 chains are linked by disulfide bonds. An individual antibody molecule always consists of identical H chains and identical L chains.

L and H chains are subdivided into variable and constant regions. The regions are composed of 3-dimensionally folded, repeating segments called domains. An L chain consists of one variable (VL) and one constant (CL) domain. Most H chains consist of one variable (VH) and 3 constant (CH) domains. Each domain is approximately 110 amino acids long. The variable regions are responsible for antigen binding, whereas the constant regions are responsible for various biologic functions, *e.g.* complement activation and binding to cell surface receptors. The remarkable specificity of antibodies is due to the variable antigen binding regions (*4*).

Because IgG has 2 identical antigen-binding sites, it is said to be divalent. There are 4 subclasses, IgG1-IgG4, based on antigenic differences in the H chains and on the number and location of disulfide bonds. IgM is the main immunoglobulin produced early in the primary response (*5*). In serum, it is a pentamer composed of 5 H2L2 units with an addition of one molecule of J (joining) chain. The pentamer with M_r of 900 kDa has a total of 10 antigen binding sites (Fig. 2).

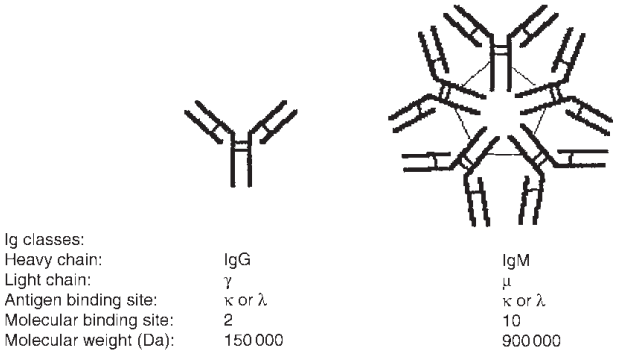


Fig. 2. IgM is a pentamer composed of five IgG-like molecules and coupled by J (joining) chain; it has a total of 10 antigen binding sites and M_r of 900 000 Daltons

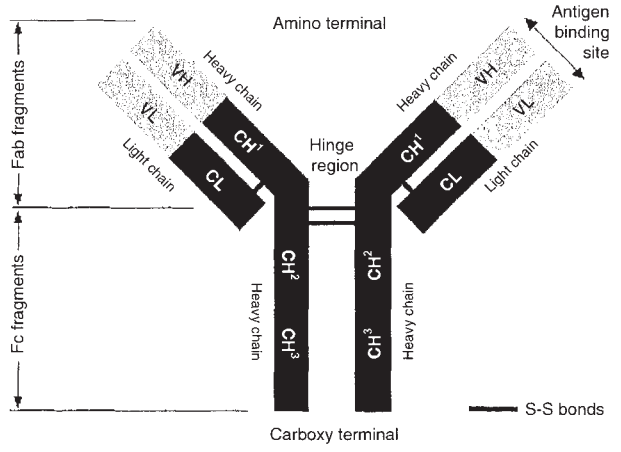


Fig. 1. Structure of an antibody molecule; the Y-shaper IgG consists of two light chains and two heavy chains. Each heavy chain consists of a variable region and a constant region that is divided into three domains: CH^1, CH^2, CH^3. The CH^2 domain contains the complement binding site and the CH^3 domain is the attachment site of IgG to receptors on neutrophils and macrophages. The antigen binding site is formed by the variable regions of both the light and heavy chains

Limited proteolytic cleavage with enzymes such as papain, pepsin and ficin produces well-defined fragments of antibody molecules, containing different combinations of domains. Two antigen-binding fragments (Fab) with ca. M_r of 50 kDa and the crystallizable fraction Fc are produced by the digestion of IgG with papain (*6,7*). Pepsin or ficin digestion of IgG results in a dimer, $F(ab')_2$, with M_r of ca. 110 kDa (*8,9*). The Fc fragments are fully or partially digested under these conditions (Fig. 3).

Fragmentation of IgM can be obtained by similar enzymes with less well-defined fragments than is the case for IgG molecules (*10,11*). Trypsin and pepsin, as

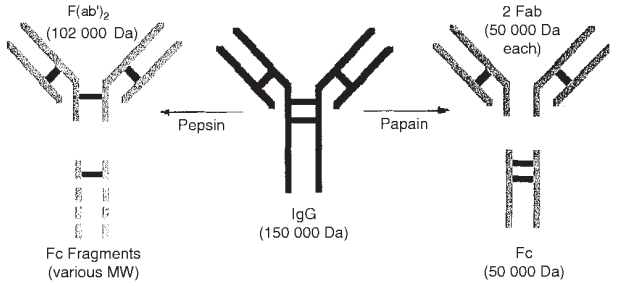


Fig. 3. Proteolytic cleavage of antibodies

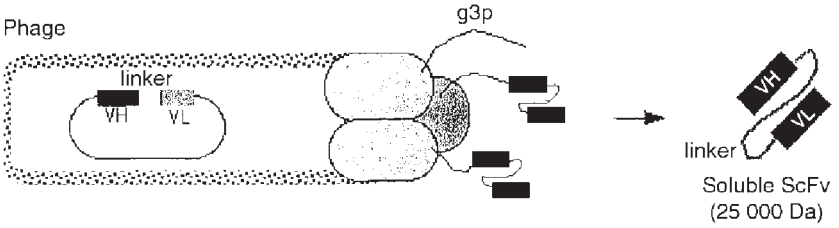


Fig. 4. single chain variable fragment (ScFv); the variable regions of the antibody heavy (VH) and light (VL) chain genes are joined by a flexible linker and cloned in recombinant phage. The phage display fully functional antibody fragments on their surface. Following affinity selection, positive recombinant phage can be used to infect *E. coli* to produce soluble ScFv

well as 2-mercaptoethylamine-HCl (2-MEA-HCl), generate a variety of IgM fragments. Recent developments in recombinant phage technology enable researchers to design and produce a repertoire of single-chain variable fragment genes in which the the variable regions of the antibody heavy (VH) and light (VL) chain genes are joined by a flexible linker (*12,13*). The soluble phage-displayed recombinant antibody fragments are called ScFv and are functional and useful as immunological reagents (Fig. 4). One of many advantages of this technology is that a so called peptide tag such as poly-histidine located at the carboxyl terminus of ScFv recombinant antibodies can be integrated and expressed. Peptide tags on the ScFv molecule facilitate an easy affinity purification strategy by anti-peptide tag antibody affinity chromatography or by immobilized metal-ion affinity chromatography (*14,15*).

Polyclonal and Monoclonal Antibodies

There are two different approaches to generating antibodies: development of polyclonal and monoclonal methods. Polyclonal antibodies are often raised by hyperimmunizing animals with antigens. The hyperimmune animal serum contains a mixed population of antibodies against an entire range of epitopes or determinants present on the antigens. Hyperimmune sera often have higher levels of IgG – up to 10 % of the total IgG content is specific antibody. The use of these mixed populations of antibodies sometimes creates problems in immunochemical techniques. Therefore, the preparation of homogenous antibodies with a defined specificity is desirable.

One of the most important scientific advances of this century is the hybridoma technology introduced by Milstein and Koehler in 1975 (*1*). This technique allows the growth of clonal populations of cells secreting antibodies with a defined specificity, *e.g.* reactive against one single epitope. These immunoglobulins, which are known as monoclonal antibodies, are identical since they are produced by descendants of one hybridoma cell. The monoclonal antibodies offer many advantages due to their specificity of binding, homogeneity and their ability to be produced in unlimited quantities. The monoclonal antibody can be produced *in vitro* (cell culture, bioreactors) or *in vivo* as ascites fluid in mice or rats. Although monoclonal antibodies produced by hybridomas are homogenous in nature, a variable post-translational glycosylation is possible that can cause differences in isoelectric point (pI).

Chimeric or humanized monoclonal antibodies consisting of mouse variable regions and human constant regions are being constructed for use in treating a broad range of human diseases (*16,17*). The advantages of the chimeric antibodies include complement activation and less immunogenicity for subsequent administration (*18*).

General Considerations

The most common sources of specific antibodies are plasma, serum, ascites fluid and cell culture supernatant. The major contaminants present are albumin, transferrin and non-specific endogenous antibodies, which are either produced by the host animal or added as supplements to the media (fetal calf serum or horse serum). Unlike other contaminants, endogenous antibodies will be more difficult to separate from the specific antibodies. Affinity purification with immobilized reactive antigens can result in a highly purified specific antibody preparation if a sufficient amount of reactive antigen is available. If the antibody-producing hybridoma cells could be grown in the culture or bioreactors in serum free media, the purification of the antibody would be much simpler. Chromatographic separation of IgG and IgM based on the difference in size, charge, hydrophobicity and affinity or combination of several separation modes will be described in the remainder of this chapter.

Since biological fluids and crude cell supernatant or feedstreams are the starting materials of the antibody to be purified, precautions should be taken to prevent column fouling due to floating fats and other lipids, cell debris and mucinous impurities. Filtering raw samples through 0.2 μm membranes prevents fouling by gross cell debris. Since most foulants are negatively charged and most antibodies are strongly electropositive, foulant removal can be achieved by anion exchange at physiological pH and ionic strength. The simplest removal can be obtained by adding microgranular cellulosic anion exchange media directly to the raw material. A simple clarification agent and non-ionic lipid absorbent can also be used to bind impurities effectively. Another approach is to use an in-line pre-filter macroporous based anion exchanger as a guard column. Column cleaning as suggested by the packing material manufacturer should be routinely performed to keep the column performance intact and avoid microbial contamination (*19*).

Precipitation Methods

Fractionation of antibodies by precipitation using ethanol has been in practice for many years. In plasma fractionation, precipitation is still the method of choice. In the case of the Cohn fractionation, immunoglobulins are precipitated in the so-called precipitate II+III with 25 % ethanol, at pH=6.8 and the temperature of –5 °C (*20*). As a safeguard against infections, viruses are inactivated with the solvent/detergent method and treatment at low pH and by pasteurization (*21,22*). The protein solution obtained in this way contains human immunoglobulin G of all four subclasses and is increasingly used in therapy. By the time, monoclonal antibody emerged, various precipitation mechanisms were already in use with polyclonal antibodies. All precipitation methods alter the properties of the solvent that cause selective precipitation of antibodies using salts such as ammonium sulfate or organic polymers such as polyethylene glycol (PEG). Alternatively, precipitation of the contaminants can be achieved by reducing the solubility of contaminant proteins using organic complexants such as short chain fatty acids or organic bases. In addition to their unique selectivities, each method offers a different mix of practical advantages and limitations. In general, poor reproducibility of precipitation processes and offset between purity and recovery curves are characteristic for precipitation methods in purifying antibodies. Nevertheless, precipitation methods make an effective partnership with chromatographic methods. Salt precipitation followed by Ion Exchange HPLC is the classical IgG purification method. In IgM purification, euglobulin precipitation followed by Size-Exclusion HPLC or Hydroxyapatite-HPLC offers an effective purification method (*23*). Precipitation by electrolyte depletion is traditionally referred to as euglobulin precipitation (*24*). The major class of antibodies precipitating in water are IgM and IgG3 (*25*).

Non-Affinity Chromatographic Methods

Non-affinity chromatographic methods of purifying antibodies from a protein mixture are based on differences in size, charge, hydrophobicity and interaction with specific media such as hydroxyapatite.

Size exclusion chromatography

Size exclusion chromatography (SEC) has been popular for laboratory scale purification of antibodies due to its simplicity. Since the separation is determined predominantly by the gel matrix, method development is minimal. Due to the large size of the molecule, SEC is useful in purifying antibodies of the IgM isotype. The major limitations of SEC that restrict the wide application of this method for antibody purification, especially for commercial use, are low resolution, low capacity and its general slowness. Since SEC only allows a limited volume of sample to be injected into the column, ascites fluid with high IgM concentration is more suitable for this method in contrast to cell culture supernantant. Due to the gentle separation conditions, antibodies separated by this method typically retain their biological activity in contrast to those produced by other methods that require extreme exposure to harsh pH or high concentration of salts or chaotropical agents. A reasonable purity of 50–80 % for IgG and 80–90 % for IgM antibodies are typically expected from SEC. However, the separation can not discriminate between the specific target monoclonal antibodies and non-specific antibody contaminants of the same class. Major contaminating proteins in IgG separations are complements C3 and C4, haptoglobulin, β1-haptoglobulin, ceruloplasmin and inter-α-trypsin inhibitor. Typical contaminants found in IgM fractions are α-2-macroglobulin and lipoproteins (*26*).

It is important that the antibody be fully soluble under the buffer conditions. A buffer of moderate salt concentration is preferable since IgGs and especially IgMs are poorly soluble at a low ionic strength buffer. High concentrations of salts or organic additives such as polyethylene glycol in the running buffer may also alter SEC separation performance. Like salt, glycine has a solubilizing effect on antibodies, especially IgM (*27*), and it is a recommended additive in the running buffer for SEC.

It is highly desirable to choose an inert SEC media to avoid charge interactions, hydrophobic interactions, hydrogen bonding and affinity interactions between antibody molecules and the media that can cause elution interference for antibodies (*28,29*). Agarose-based media often bear residual sulfates, while dextrans carry residual carboxyls and polyacrylamides carry primary amino groups that potentially can cause charge interactions. These interactions can be reduced by adding 0.2–0.5 M sodium chloride to the buffer (*30*). Most of SEC media are rich in hydroxy groups as potential hydrogen donors which contribute to non-specific binding of antibodies. The addition of urea or sucrose as hydrogen donors/acceptors in the buffer can reduce these effects (*31*).

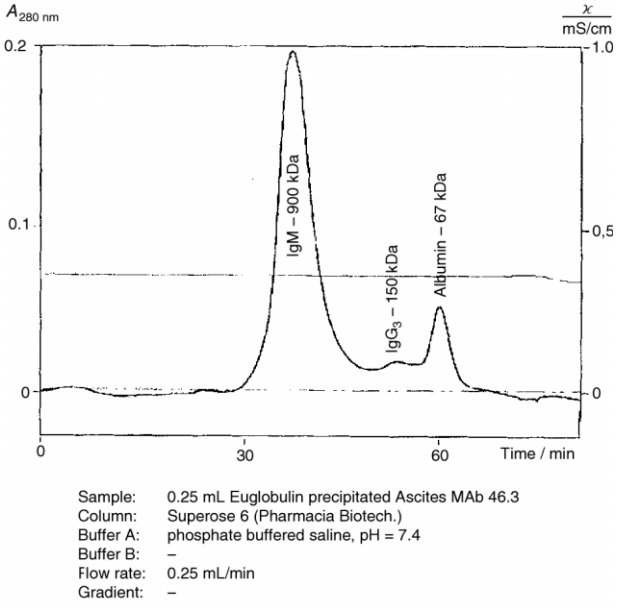


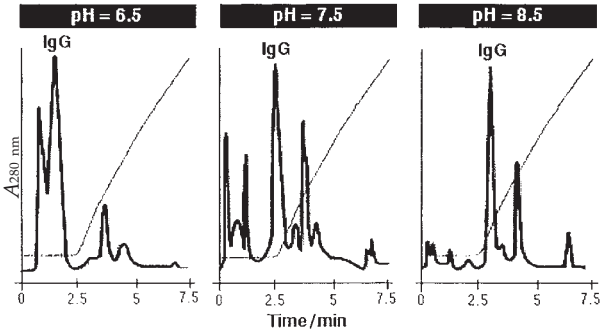
Fig. 5. Purification of IgM by Size Exclusion Chromatography following Euglobulin precipitation; monoclonal antibody of IgM isotype (MAb 46.3) was purified from ascites by euglobulin precipitation. Precipitated IgM was contaminated by IgG3 and albumin which were separated further by Size Exclusion Chromatography on a Superose-6 column

The most powerful use of SEC is in combination with other chromatographic steps following precipitation, such as hydrophobic interaction or ion exchange HPLC. An example of the purification of a monoclonal antibody MAb 46.3 (IgM isotype) by SEC following euglobulin precipitation is shown in Fig. 5.

Ion-exchange chromatography

Ion exchange chromatography (IEC) has been a platform for antibody purification for many years (*32*). IEC separates proteins based on differences in the surface charge of the molecules. Because antibody molecules have a more basic isoelectric point than the majority of other serum or contaminating proteins, IEC is useful in purifying antibodies regardless of isotype (*33–35*). The general strategy is to keep the pH below the isoelectric point for antibodies so that they will not bind to an anion exchanger such as DEAE modified resin or, alternatively, to raise the pH above the pI where the antibodies will bind to the DEAE-groups. The opposite strategy works for cation exchangers. The bound antibodies are commonly eluted with a salt or pH gradient. Under these conditions, most eluted antibodies are expected to maintain their biological activity (*36*).

Due to the fact that every antibody is unique and can vary in its pI, binding to an IEC resin needs to be explored and determined experimentally on an individual basis. The contaminant spectrum with which an antibody is associated on a cation exchanger is usually different from the spectrum on an anion exchanger. Even on the same exchanger, altering the pH may significantly change the profile of contaminants co-eluting with the antibody (*37*). Therefore, it is recommended that the appropiate pH for optimal separation be determined. Effects on the retention of IgG on an anion exchange resin (Poros 20 HQ) under various pH conditions is shown in Fig. 6.



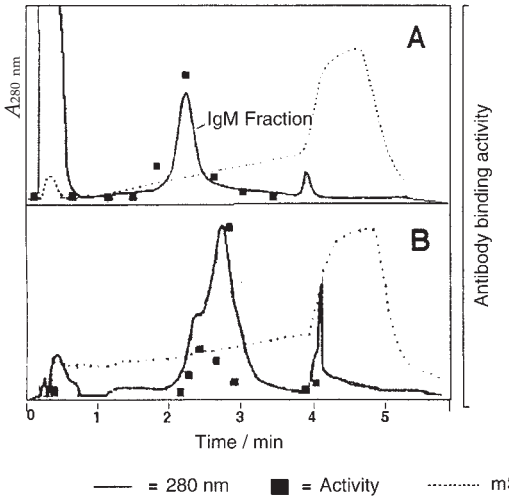
Sample: 50 μL mouse asciles
Column: POROS 20 HQ anion exchange 4.6 x 100 (PerSeptive Biosystems)
Buffer A: 20 mM Tris/Bis Tris
Buffer B: 20 mM Tris/Bis Tris + 0.5 M NaCl
Flow rate: 8 mL/min
Gradient: 0-100 % B in 20 column volumes

Fig. 6. pH Map on an anion-exchange resin; the appropriate pH for optimal separation of IgG from crude ascites on POROS HQ anion exchange resin was determined by pH mapping experiments. High purity and yield of IgG was achieved by pH = 8.5

Likewise, the elution conditions of IEC can be optimized. Elution can be conducted in either step or linear gradient formats. In general, a linear gradient offers better reproducibility with a disadvantage of dilution effects.

IEC resins are available in two charge modes (anion and cation exchangers) with several ionic forms (strong and weak exchangers). It makes this technique broadly applicable in providing different selectivities ideal for initial capture or intermediate purification of antibodies. Moreover, IEC is capable of separating a target monoclonal antibody from a host or media supplement-derived polyclonal antibody.

Although it is not impossible to obtain a single step purification of antibody by IEC, such an effect requires a



Sample: 2 mL crude murine ascites of MAb 46.3 (IgM)
Column: POROS 20 HS cation exchange (4.6 x 100)
Buffer A: 50 mM sodium phosphate, pH = 7.2
Buffer: Buffer A + 1.0 M NaCl, pH = 7.2
Flow rate: 8.5 mL/min
Gradient: 0-35 % B linear gradient in 20 CV

Sample: 2 mL of IgM fraction from A
Column: POROS 20 HQ anion exchange (4.6 x 100)
Buffer A: 30 mM sodium phosphate, pH = 7.2
Buffer B: Buffer A + 1.0 M NaCl, pH = 7.2
Flow rate: 10 mL/min
Gradient: 15 to 20 % B step gradient held for 2 CV;
20-35 % B linear gradient in 15 CV

Fig. 7. IgM purification by a combination of cation and anion exchange chromatography; panel A represents the separation of crude ascites of monoclonal antibody IgM 46.3 (diluted 1:5 in PBS) on a cation exchange column. The IgM peak was pooled and a 2 mL aliquot of this pool was injected onto the anion exchange column. The resulting chromatogram is shown in Panel B. In each chromatogram, the activity of collected fractions as measured by ELISA is shown. High purity of IgM was achieved by this combination method. The rapid separation with high flow rates minimized the risk of aggregation and helped preserve the biological activity of the labile IgM

rigorous and systematic optimization of separation (*38, 39*). Furthermore, high single step purity requires narrow peak cutting that can reduce recovery significantly. A combination of both anion and cation exchangers offers a powerful method for antibody purification, such as that of IgM (Fig. 7). Alternatively, a combination of IEC with a non-charge based separation method such as SEC, to purify IgM or with hydrophobic-interaction HPLC for IgG purification is common (*33,36,40*).

Recent development of new IEC media introduced by several manufacturers support a rapid separation of complex proteins including antibodies. Perfusive or convective media offer high speed processing that encourages more thorough optimization (pH, gradient and surface chemistry) and ultimately results in better purification products. The high flow rates might minimize the risk of aggregation and help preserve biological activity of labile antibody molecules, especially of the IgM subclass (*41*).

Hydroxyapatite chromatography

Hydroxyapatite chromatography (HAC) has recently gained a wider popularity as a purification tool for antibodies because of the recent development of uniform ceramic media that provide excellent physical strength, high flow properties and scale-up capabilities (*42*). Hydroxyapatite, which has the formula of $Ca_{10}(PO_4)_6(OH)_2$, acts both as the ligand and the matrix. The interactions of antibodies with hydroxyapatite are complex and mostly based on attraction of the amino and carboxyl groups of the molecules to the phosphate and calcium sites of hydroxyapatite.

The samples are usually applied in 5–50 mM phosphate buffer at pH=6.8 and the antibodies are eluted from the hydroxyapatite by a gradient elution of phosphate buffer up to 0.5 M at pH=6.8. Solubility problems can occur for many IgM species and some IgG molecules under the low phosphate loading conditions. Aggregation that can lead to peak broadening and smearing can reduce the recovery significantly. Addition of 0.1 M NaCl to the loading buffer improves the solubility, especially for IgMs, without affecting the binding efficiency of the antibodies (*43*). Moreover, the addition of NaCl to the loading and elution buffers improves binding selectivity which can result in a higher purity and yield of antibodies (Fig. 8).

HAC is useful for fractionating antibodies regardles of species, class or subclass. Purity up to 90 % from serum or ascites in a single step is possible. Low levels of albumin, transferrin and β-1-haptoglobulin are found as contaminants (*43–44*). A combination of HAC with SEC is especially effective for purifying IgM.

It has been reported that monoclonal antibodies are better separated at a lower pH than the conventional elution using pH=6.8 (*45*). However precautions should be taken since the crystalline structure of hydroxyapatite degrades rapidly below pH=5. Matrix degradation can also occur due to acid secretion by microbial contaminations following poor cleaning and sanitization (*43*). Loss of separation performance, capacity, reproducibility and short column lifetimes as low as 24 hours can only be prevented by rigorous cleaning and appropriate storage of the column, *e.g.* in 60 % methanol (*43*).

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) separation is based on adsorption of biomolecules to hydrophobic groups such as phenyl or butyl which are covalently bound on the hydrophilic HIC support. Since antibodies have a stronger hydrophobicity than the ma-

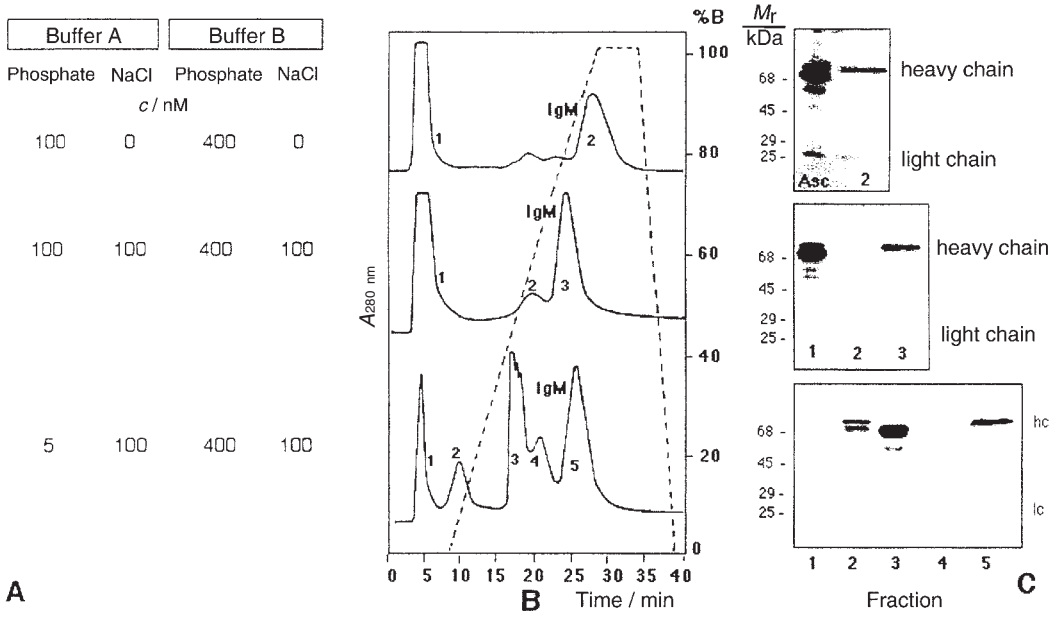


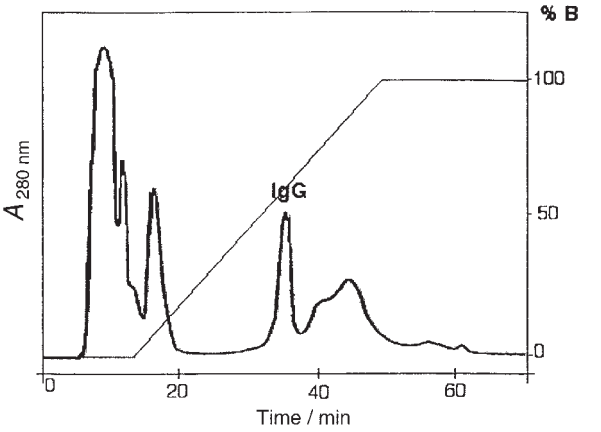
Fig. 8. Separation of mouse IgM from crude ascites by Hydroxyapatite Chromatography under various buffer conditions (A); the effect of solubilizing sodium chloride in the buffer is demonstrated by a sharper IgM peak (B). The SDS-PAGE analyses of the fractions are shown in C. Starting material (mouse ascites) is shown in lane 1 on the top panel (Asc). Mass recovery of IgM can be improved from 75 to >90 % by a simple inclusion of NaCl in the buffer

jority of their contaminants (*46*), HIC is an effective technique for purification. The adsorption of antibodies to the HIC matrix is enhanced by the presence of high salt concentration (*47*), the choice of which has effects in binding selectivity. Salt concentration should be optimized individually for each antibody depending on its hydrophobicity. Bound antibodies are eluted by decreasing the ionic strength and/or addition of hydrophobic competitors, such as organic solvents, in linear gradient modes.

Due to denaturing risks from a combination of strong organic solvents with excessivelly hydrophobic ligands, a careful media selection should be taken to obtain high recovery of bioactive antibodies. Generally, a strong hydrophobic media for antibody separation is not recommended. Phenyl ligands have been sucessfully reported for monoclonal antibody purification since the required salt concentration is often beneath the level at which antibody precipitation begins to occur (*48*). Strong organic solvents in the mobile phase may improve recovery, however, they also increase the probability of antibody denaturation. An addition of 25–50 % ethylene glycol in the elution buffer is generally sufficient for obtaining high purity and recovery of antibodies.

Similarly to other techniques, HIC requires routine method development for optimal sample application conditions. A screening of conditions of mobile phase pH that allows the most favorable binding selectivity of the antibody should be evaluated. The hydration of residues within the hydrophobic sites on antibody molecules is known to be pH-dependent and consequently pH has a significant impact on selectivity (*49*). Buffers containing potassium or sodium phosphate with ammonium sulfate at pH=8.5, 7.0 and 5.5 should be screened for optimal selectivity.

In general, HIC is very useful in purifying antibodies of the classes and subclasses from all species. It is especially attractive since it easily removes the contaminants that are most difficult for the other HPLC techniques such as SEC, IEC and HAC. Because albumin and transferrin are usually unretained under antibody binding conditions, HIC is often a suitable first purification step. Furthermore, HIC partially removes up to 50–75 % of non-specific antibodies present in the starting material. In combination with IEC, complete removal of the non-specific antibodies can be achieved. Fig. 9 illustrates the separation of IgG from ascites fluid by HIC.



Sample: Cell culture supematant of murine hybridoma
Column: Merck Fractogel EMD Propyl 650 (S)
Buffer A; 20 mM sodium phosphate + 1.0 M ammonium sulfate, pH = 7.0
Buffer B: 20 mM sodium phosphate, pH = 7.0
Flow rate: 1 mL / min
Gradient: 0-100 % B in 20 column volumes

Fig. 9. Separation of a monoclonal antibody by hydrophobic interaction chromatography; IgG is eluted during the decreasing gradient at a salt concentration of about 500 mM ammonium sulfate

Affinity-based Separation

Protein A affinity chromatography

After the introduction of Protein A for the affinity purification of IgG in 1972 by Hjelm (*50*) and Kronvall (*51*), this method for antibody purification became so popular that it currently ranks with ion-exchange chromatography.

Protein A (Cowan strain I) is a wall component of *Staphylococcus aureus* that binds the Fc portion of IgG specifically. Variations in Protein A affinity for IgGs from different species and subclasses are well known (*52*). The most significant feature of Protein A affinity chromatography is its simplicity – it can be performed quickly and easily with or without sophisticated instrumentation. Most mammalian and mouse/rat IgGs bind well to Protein A, achieving efficient purification under physiological condition (*53,54*). Bound antibodies most commonly are eluted by low pH buffer, such as 0.1 M glycine-HCl, pH=3.0 or 0.1 M citric acid, pH=2.4 using a step gradient. Neutralization of the solution immediately after the elution preserves the biological activity of the purified antibodies.

Protein A affinity chromatography is capable of providing total IgG purity as high as 95 % in a single step, however, non-specific antibodies from the host or serum supplements, which also bind to Protein A, co-elute with the target IgG and contaminate the preparation. The contamination by non-specific antibodies can be reduced with linear gradient pH elution, however it cannot be eliminated completely (Fig. 10). An additional ion-exchange chromatography step can be utilized to remove the non-specific Ig. Antibody aggregates bind to Protein A with higher affinity than monomeric antibody, therefore, linear gradient pH elution can partly resolve antibody aggregates from the monomeric antibody (Fig. 10). α_2-macroglobulin and kinogen are also known to bind to Protein A and might be found as contaminants in the antibody preparation (*55*).

The most widely recognized concern with Protein A purification is antibody denaturation that can manifest as aggregation, fragmentation and loss of biological activity due to harsh elution conditions (*56,57*). Some antibodies undergo permanent conformational alterations under extremely low pH (*58*). In such cases, minimum elution conditions should be determined. Elution above pH=4.5 rarely has effects in antibody denaturation. Elution with chaotropic salts such as urea, sodium isothiocyanate and potassium iodide are also possible (*54*). Another known problem with Protein A is leaching from the column. This is potentially a concern for *in vivo* anti-

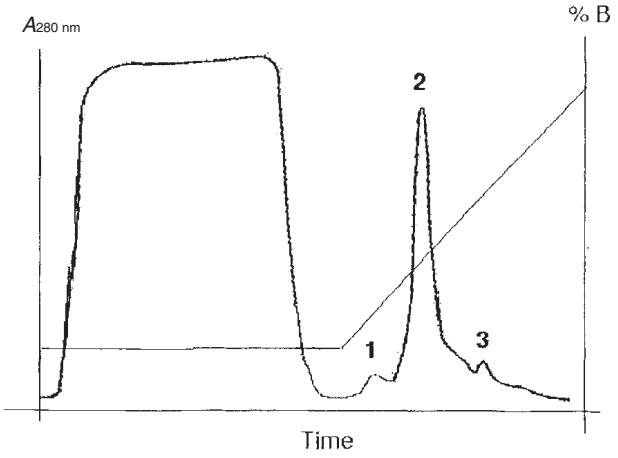


Fig. 10. Separation of IgG by linear pH gradient elution; cell culture supernatant of murine hybridoma (IgG2a) was fractionated on a Protein-A Sepharose column. Running buffer: Phosphate buffered saline, pH = 7.4. Bound proteins were eluted by 10 column volumes of a linear gradient to 0.1 M citric acid, pH = 2.2. Bovine IgG (Peak 1) and IgG aggregates (Peak 3) were partially separated from the main elution peak of murine IgG2a (Peak 2)

body use, since Protein A is a potent immunomodulator and has proven toxic in clinical trials (*59,60*). Recombinant Protein A (r-Protein A), a genetically engineered form of Protein A that is produced in a non-pathogenic form of *Staphylococcus aureus*, is now currently available. It is derived from secreted extracellular variants that lack the cell-wall associated region (*61*). Compared with Cowan strain I, there are minor differences in the amino acid sequences of the IgG binding domains, however, the antibody binding property is indistinguishable. The main advantage of r-Protein A is that toxic contaminants from a pathological bacterial source can be eliminated.

Protein G affinity chromatography

As an alternative to Protein A, Protein G, which is derived from group C and G streptococci, offers a similar application for purification of antibodies (*62*). There are some advantages in the binding selectivity of mouse and rat IgG for Protein G over Protein A (*52*). Wild type Protein G contains albumin-binding domains as well as IgG-Fc and Fab binding domains (*63*). Currently, recombinant Protein G without an albumin binding property is available. Purification performance varies significantly among commercial Protein G products (*64*) and binding capacities may vary by a factor of 2–3. This partly reflects variations in ligand density, spacer characteristics and the variations in Protein G itself.

Although Protein G offers some advantages over Protein A, the concerns associated with the use of Protein A such as non-specific antibody contamination and ligand leaching are also shared with Protein G. Moreover, Protein G is more expensive than Protein A. Alternatively, to obtain a broader range of antibody binding, a hybrid of recombinant Protein A/G may be useful (*65*).

Protein L

Another microbial protein that binds mammalian immunoglobulin has been recently made commercially available. The protein is originally derived from the cell wall of bacteria *Peptostreptococcus magnus* and is termed Protein L (*66*). The protein has the unique ability to bind immunoglobulins through the κ light chains without interfering with the antigen-binding site and allows a wider range of antibody classes and subclasses than Protein A or Protein G (*67*). Protein L binds IgM most strongly, apparently as a result of the pentameric nature of IgM which allow five times as many binding sites (*66*). Furthermore, Protein L is useful for purifying single chain variable fragments (ScFv) and Fab fragments of immunoglobulins (*67*).

Biomimetic ligand affinity chromatography

Recent developments in techniques such as rational design and combinatorial chemistry have allowed generation of a vast array of new synthetic ligand structures that might be useful for purification of biomolecules including antibodies. These techniques have been successfuly used to identify low molecular weight synthetic molecules that mimic the immunglobulin binding activity of native biological macromolecules such as *Staphyloccoccus aureus* Protein A (*68*). This biomimetic ligand has been reported to bind IgG competitively with Protein A in solution and when immobilized on agarose beads, it displays an affinity constant of 10^5–10^6 M^{-1} (*69–71*). The immobilized ligand (Mimetic A, ProMetic BioSciences, Inc.) has been used on affinity adsorbents to purify polyclonal IgG from diverse sources effectively. The combination of fast and effective affinity chromatographic techniques and the synthetic nature of biomimetic ligands offer many advantages over conventional antibody purification methods, especially for downstream processing of *in vivo* products. A mimetic ligand might suspend all concerns pertaining to biological origin and toxic contaminants from a pathological bacterial source (*72*). In addition, the robustness of the ligand enables harsh cleaning, sterilization and depyrogenation which ensures long operational lifetimes. The high binding selectivity of the mimetic ligand to human IgG might be useful to purify the fast growing products of therapeutic human IgGs or chimeras with human Fc regions. Fig. 11 shows the isolation of polyclonal human immunoglobulin G from plasma, using such a combinatorial ligand. It was shown in this experiment that purity of the isolated IgG was 92.5 %. Sanitation of the affinity resin was performed with 1 M NaOH. Difficulties arose with recovery. Whereas in experiments on a small scale recovery was almost 100 %, losses appeared as a consequence of up-scaling with recovery dropping to only 60.5 (*74*). All combinatorial ligands shown here were differently modified triazins that had been developed by molecular modelling, mimicking immunoglobulin binding activity of protein A (*69*).

In parallel experiments the group of Fassina *et al.* (*73–75*) has developed different combinatorial ligands, which are also able to bind immunoglobulins of other classes.

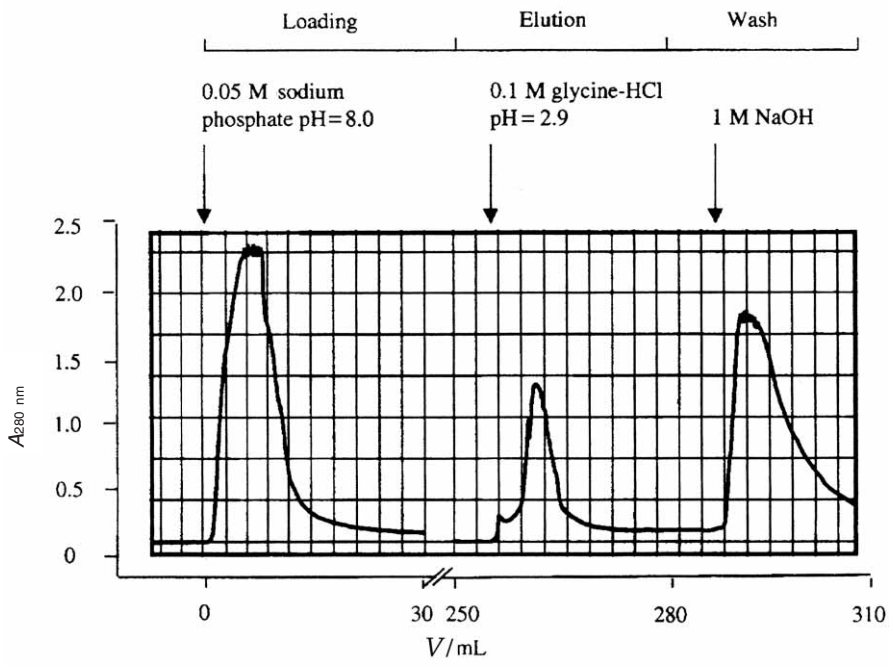


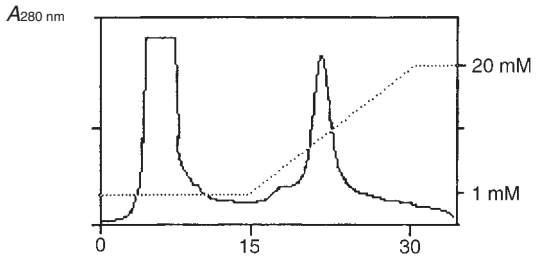
Fig. 11. Chromatography of human plasma on an immobilized combinatorial adsorbent (22/8); A column (7 × 1.2 cm) of immobilized 22/8 (5 mL 90 μmol/g moist weight gel) was equilibrated with sodium phosphate buffer (0.05 M, pH = 8.0) at 20 °C. Four-fold diluted human plasma (12.5 mL) was applied and the column washed with sodium phosphate buffer (0.05 M, pH = 8.0) until the absorbance at 280 nm was < 0.002, whence bound proteins were eluted at a flow rate of 0.6 mL/min with glycine-HCl buffer (0.1 , pH = 2.9), followed by a wash with NaOH (1 M)

Immobilized metal affinity chromatography

Immobilized Metal Affinity Chromatography (IMAC) has been used for protein purification since 1975 (*76*) but it has not been widely utilized for antibody purification, despite its extraordinary capabilities, until recently (*77,78*). IMAC is a powerful tehnique to purify IgG molecules due to the affinity binding of histidyl residues in the antibody molecules to a chelated nickel support. Mammalian IgGs have a highly conserved histidyl cluster at the junctures of the CH2 and CH3 domains (*59*). Under alkaline conditions, nickel-loaded iminodiacetic acid (Ni-IDA) captured IgG through the histidyl residues selectively and effectively (*77,78*). The binding specificity may be increased by the presence of 0.5 mM imidazole in the loading buffer (*79*). The bound antibodies are eluted by titrating the histidyl residues using low pH (*80*). Alternatively, a competitive elution with imidazole or its analogues, histidine and histamine, achieve similar results. A typical chromatogram is shown in Fig. 12.

IMAC is particularly suitable for the separation of biomolecules such as antibodies since it can be performed under very mild, non-denaturing conditions. Single step purification is often possible for antibodies produced in cell culture supernatants. However, monoclonal antibodies produced in ascites fluid often need an additional step to remove non-specific IgG and other contaminants. A combination of IMAC with hydrophobic interaction or ion-exchange chromatography would provide an excellent method for antibody purification (*78*).

The recent advances in the production of recombinant single-chain Fv antibody fragments (scFv) in bacteria permit rapid access to genetically engineered molecules with antigen-binding properties and clinically useful applications either for *in vivo* diagnosis or therapy. Metal chelate chromatography has been widely utilized in purifying expressed scFv from bacterial supernatant (*81,82*). A simple insertion of a hexa-histidine tail fused at the C-terminus can provide an affinity tag which selectively binds to metal ions immobilized on an iminodiacetic acid derivatized solid phase matrix. Immobilized Cu^{2+}, Ni^{2+} and Zn^{2+} ions have been shown to be effective in retaining the His-tagged antibody fragments with up to 90 % purity on elution (*82*).



Sample: 0.25 mL mouse ascites
Column: TSK gel chelate SPW (Toso Haas)
Metal ion: Ni^{2+} saturated
Buffer A: 1 mM imidazole in 20 mM HEPES – NaOH buffer, pH = 8 + 0.5 M NaCl
Buffer B: 20 mM imidazole in 20 mM HEPES – NaOH buffer, pH = 8 + 0.5 M NaCl
Flow rate: 0.8 mL / min
Gradient: 30 min linear gradient from 1 mM to 20 mM imidazole

Fig. 12. Purification of IgG by Immobilized Metal Affinity Chromatography (MAC); the low concentration of imidazole in the loading buffer (buffer A) should enhance the binding specificity and addition of 0.5 M NaCl in the buffers may improve the purity of the antibody. Elution can be achieved by reducing pH or by competitive elution with imidazole

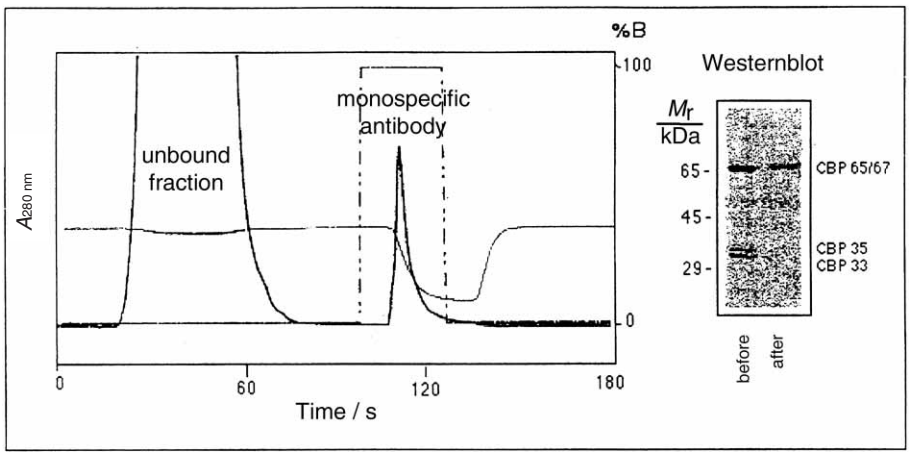


Fig. 13. Purification on monospecific rabbit antibody against Annexin VI; the rabbit polyclonal antibody against rat Annexin VI (CCBP 65/67) reacts against other annexins (CBP 35 and CBP 33). Monospecific polyclonal antibodies against Annexin VI were prepared by immobilized antigen affinity chromatography. Purified Annexin VI is immobilized on Epoxy activated CIM disk (BIA Separations). 1 mL antiserum is diluted 1:5 in PBS and applied to the disk (flow rate 8 mL/min). Bound antibodies were eluted by 0.1 M glycine-HCl, pH = 3.0 in a step gradient. The western blot analysis of EDTA extracts of Morris hepatoma rat plasma membranes with the antiserum before and after the affinity purification is shown

Immobilized antigen affinity chromatography

The classical immunoaffinity format utilizes antigens which are immobilized to chromatographic supports. This effective technique however is rarely used for purification of monoclonal antibodies primarily due to lack of sufficient amounts of antigen and labor intensive preparation of the purified antigens which are prohibitive to this approach. Other possible factors that might have negative impacts on the antibody binding are secondary steric modifications of the antigens induced by immobilization that might alter capture efficiency of the target antibody. Binding and elution may also alter the antigen binding site of the antibody. Moreover, leached antigens will interfere with the intended application of the antibody. Despite these limitations, this technique is attractive for purification of monospecific antibodies from a mixture of polyclonal antibodies (*83*). Fig. 13 demonstrates the purity of the monospecific antibody against calcium binding protein annexin 6 (CBP 65/67) separated by immobilized annexin 6 affinity porous disks.

Antigens that can be expressed as recombinant proteins and engineered to bear multiple histidyl residues at their N or C terminus would facilitate a simple immobilization procedure in preparing immunoaffinity absorbents on metal affinity resins. This technique has been used sucessfully in purification of antibody or antibody fragments (*84,85*). By taking advantage of biospecific interactions of antigen and antibody molecules, separation of bioactive forms of single-chain antibody fragments (scFv) from inactive scFv molecules can be achieved in a single step by using immobilized antigen affinity chromatography (*78*).

Conclusion and Outlook

Antibodies, especially monoclonal ones, play an important role in many research, diagnostic and therapeutic applications. HPLC has proven to be a powerful technique for the analysis, separation and purification of antibodies. A variety of chromatographic methods based on physico-chemical separation mechanisms, biological affinity and mixed mode mechanisms are available to achieve a succesful antibody purification. Each method and each combination of methods has its particular strengths and weaknesses. To define a broadly applicable strategy in purifying monoclonal antibodies is challenging due to the chemical diversity of antibodies. A succesful purification strategy needs to be developed systematically and tested individually for each antibody. Antibody purification for research and *in vitro* applications can usually be accomplished in a single step or two fractionation steps, while antibody purification for *in vivo* applications require additional steps due to the stringent purity standards. The powerful combination of affinity chromatography and synthetic biomimetic ligands that can selectively bind antibodies offers a promising approach to purification, especially of *in vivo* products.

Recent advances in high throughput HPLC media, such as perfusive and convective media, enable purification of antibodies in a much shorter time compared to the conventional media. Not only will this encourage a more systematic method development and optimization process that can lead to a better product, but it will likely also have a positive economical impact in downstream processing.

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Analitički i preparativni postupci za purifikaciju antitijela

Sažetak

Monoklonska i poliklonska protutijela imaju bitnu ulogu, kako u medicini, tako i u analitičkoj biotehnologiji. Primjena protutijela bila je u posljednjih dvadeset godina ograničena na analitiku i dijagnostiku. U novije vrijeme protutijela se sve više koriste i u terapeutske svrhe. U ovom su pregledu prikazane metode za izolaciju protutijela u laboratorijskom i u preparativnom razmjeru za njihovu industrijsku proizvodnju. Pri tome su opisane kako takozvane »klasične«, precipitacijske, tako i suvremene kromatografske metode. Precipitacijske metode nalaze još uvijek raznovrsnu primjenu u proizvodnji imunoglobulina G iz humane plazme. Kromatografske se metode često kombiniraju s precipitacijom koja omogućava prvi stupanj čišćenja željenoga protutijela, a najčešće se koriste za izolaciju monoklonskih protutijela. Dijelimo ih na afinitetne i neafinitetne kromatografske metode.