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Review

Isolation of antigens and antibodies by affinity chromatography

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Abstract

Antibody–antigen binding constants are commonly strong enough for an effective affinity purification of antibodies (by immobilized antigens) or antigens (by immobilized antibodies) to work out a straightforward purification method. A drawback is that antibodies are large protein molecules and subject to denaturation under conditions required for the elution from the complex. Structures of antigens can vary but usually antigens are also equally subject to similar problems. The lability of the components can sometimes make the procedure sophisticated, but usually in all cases it is possible to find a satisfactory approach. In certain cases, specific interactions of the Fc part of antibodies are more facile to exploit for their purification.
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1. Introduction

Firm and specific interactions between antigens and antibodies allow their versatile applications in (immuno)affinity chromatography by employing either immobilized antigens or antibodies [1,2]. The basic principle is straightforward, but the interacting molecules have features which can cause troubles while using this method.

Possibilities for exploitation of immobilized antibodies to catch free antigens in fluids were described as early as in 1924 by Engelgardt who suggested ‘‘the method of the fixed partner’’ [3]. It took a long time for materials and immobilization methods to be developed into a proper measure, and actually, it was in the beginning of the 1950s when Campbell et al. were able to isolate antibodies with antigens immobilized on azid-activated cellulose [4]. Still, it took several more years to expand the use of the method to different types of antigens and antibody fragments [5–9]. Discovery of monoclonal antibodies in the late 1970s accelerated in many ways the development of immunoaffinity chromatography. For example, when employing monoclonal antibodies, the procedure of prior purification of the immobilized partner became less critical [10,11]. Immunoaffinity chromatography has steadily become an invaluable tool in the life sciences concomitantly with the development of chromatographic materials and immobilization methods during the last 20–30 years. The ease of producing recombinant proteins, including recombinant antibodies, also provides additional convenience for using this method.

For many routine applications, such as immunodiagnostics, antibodies are isolated as an antibody class. Undoubtedly, however, the diagnostic methods could sometimes benefit from higher levels of purification. Highly selective methods of purification of antibodies and their Fab fragments are of special importance for obtaining catalytic antibodies (abzymes) [12–15], as well as for revealing autoimmune catalytic antibodies [16–19]. Pure antibodies are also necessary for biochips and related systems, a field which is growing rapidly [20–27].

Whenever a suitable antibody is available, it provides a convenient and effective way to purify the antigen. Unfortunately, such an antibody is not always available. Another complication is that the immobilization process may lead to partial inactivation of the immobilized antibodies. Elution of the antigen from the column under rather harsh conditions also results in a limited life-span of antibody columns. Therefore, antigens are less commonly purified than antibodies by immunoaffinity chromatography.

This review aims at describing different principles and possible problems encountered in immunoaffinity chromatography.

2. Isolation of antibodies with affinity chromatography

Antibodies can be isolated from sera of immunized animals (polyclonal antibodies) or from the ascites or culture supernatant (monoclonal antibodies). For some purposes these liquids (antisera) can be used without additional purification, or the γ-globulin fraction can be isolated by routine sodium sulfate precipitation. To isolate specific polyclonal antibodies from a serum, affinity chromatography on immobilized antigens is often used, while isolation of a certain immunoglobulin fraction is usually sufficient to purify monoclonal antibodies.

2.1. Purification of antibodies on immobilized proteins A, G, and L

The cell wall proteins of *Staphylococcus aureus* have been known to possess a high affinity towards the Fc fragments of different types of immunoglobulins (Igs) [28–37]. Immobilized proteins A, G, or L possessing affinity for types 1–4 of IgGs can be used for the isolation of monoclonal antibodies from

ascites or culture supernatant [38–44]. Protein A also specifically binds to IgM and IgE [31,45]. Protein L binds to antibodies carrying the κ-light chains [44], while protein G binds to IgG and IgM [43,46,47]. The cell wall proteins of *S. aureus* exhibit a unique specificity [30,31] but it can vary from one mammal to another. For example, protein A has affinity to human IgG3 and murine IgG1 [31]. Different interactions of protein A with different antibodies therefore allow their fractionation by class [36,37,41]. The Fc fragments can be separated from the Fab and $(Fab)_2$ fragments after the treatment of the antibodies with papain or pepsin [28]. The fragments of IgM involved in the interaction with protein A have been identified [37].

Chromatography on a proper immobilized *S. aureus* protein allows isolation of homogeneous monoclonal antibodies in one step. Naturally, these immobilized proteins do not usually effectively purify polyclonal antibodies, because they contain a large population of different immunoglobulins. Immobilized *S. aureus* proteins are widely used for analytical purposes, such as studying the antibody composition in sera [28,31–33,43].

2.2. Purification of antibodies on immobilized antigens

Purification of antibodies on immobilized antigens includes three stages: (1) adsorption of the antibodies on the immobilized antigen, (2) removing non-specifically adsorbed proteins by washing, and (3) elution of the antibodies. The standard procedure described in a number of articles allows isolation of homogeneous [by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)] antibodies directly from serum [48–50]. The serum containing antibodies is passed through a column with immobilized antigens equilibrated with a neutral buffer solution (usually 10 m*M* phosphate buffer containing 0.15 *M* NaCl, pH 7.6). The amount of antiserum to be applied to the column is determined empirically. The maximum load can be determined by upfront. It should be noted that an immobilized antigen molecule can bind more than one antibody in polyclonal sera, because there are several antigen determinants on the surface of the antigen molecule. In a standard procedure, the column is washed to remove non-specifically bound proteins with the same buffer. To achieve a more effective removal of the non-specific proteins, as well as the antibodies with a low affinity, the column is washed more stringently with a mild acid (pH 5.5) or mild basic (pH 8.5) buffer solution.

The antibodies are normally eluted by buffer at pH 2.0–2.2. Such a treatment results in liberation of the most part of the adsorbed antibodies into the solution. To retain the native conformation of the antibodies, pH of the solution should be adjusted to a neutral value with 1 *M* phosphate buffer, pH 7.0, immediately after elution. When changing the pH of the gel (washing or neutralizing) it should be recognized that the buffer capacity of the immobilized protein material can be rather high over the whole pH range and changing of the pH can consume quite large volumes of a weak buffer. Therefore, it is a good practice to follow the pH of the eluate.

The standard procedure can be modified to achieve more effective purification. The washing conditions for nonspecifically bound proteins may need to be studied more carefully and apply other pH values, salt solutions, or traces of organic additives. The optimal elution conditions for the antibodies should be studied to achieve a minimum of denatured product. The binding strength between antibody and antigen may need to be adjusted to an optimum in the preparation of the affinity sorbent. However, these studies can be laborious and much of the benefits of simplicity of the immunoaffinity chromatography is thereby lost.

2.3. Immobilization of antigens

A major effort for the affinity purification of an antibody is the preparation of an immunosorbent, i.e., antigens immobilized on an insoluble support. Basically, several supports and immobilization methods are useful. For a standard usage, a macroporous hydrophilic carrier with good flow properties is recommended. The immunosorbent works efficiently when the antigen determinants retain the native state and are accessible to the antibodies. Cross-linked Sepharose fulfils most of the features of an ideal carrier and therefore it is commonly used [51,52]. For a large-scale preparative purification, other alter-

natives may be considered for economical or other reasons.

Normally, a mild immobilization method is used to retain antigen in its natural conformation. Amino, carboxyl or thiol functions on a protein antigen surface are subjects for coupling to the support. Thiol groups are sometimes problematic if such a reaction can disrupt an essential structural intramolecular bond. In the case of non-protein antigen or small haptens, special chemistry may be needed.

The degree of immobilization of an antigen is an adjustable parameter in immunoaffinity chromatography affecting the binding capacity and binding strength. Systematic studies on such effects exist with protein antigens immobilized on CNBr-activated Sepharose. The degree of bound protein increases until the activation reaches approximately 100 mg CNBr/ml of gel. A further increase of activation has no effect on the amount of bound protein. Usually interactions of antibodies and antigens are so strong that very stringent conditions should to be used for elution resulting in inactivation of the protein being purified. Remarkably, it was found that if the activation degree of Sepharose increases from 70 to 150–200 mg CNBr/ml of the gel, antibodies can be eluted by milder conditions [51,53–55]. Presumably, the multipoint binding of an antigen molecule results in changes in its conformation that leads to weakening of the interactions between the antigen and the active site of the antibody. Unfortunately, commercial preparations of CNBr-activated Sepharose are of standard activation degree (about 70 mg CNBr per ml of the gel). Therefore, sometimes it is necessary to activate Sepharose independently. A useful device for CNBr activation of small gel batches reproducibly was described [56]. However, if the biological activity is considered to be important for the bioaffinity, but it is lost during this procedure, other milder methods, like immobilization through thiols can be used. Aldehyde functions can be introduced to agarose using relatively easy chemistry [57]. Proteins can be bound to aldehyde-agarose through Schiff base formation with or without borohydride reduction, while without the reduction, a part of the initially bound protein may slowly leak from the column [58]. Epoxy–activated Sepharose may be sometimes beneficial because of its low ionic character as compared to CNBr activated agarose.

Recently a method of immobilization was suggested allowing achievement of a very high antigen (alkaline phosphatase) concentration on the support—salicylhydroxamic acid-modified Sepharose [59]. Also, the nonstoichiometric polyelectrolyte complexes with attached antigens have been suggested for isolation of antibodies [60]. At the first step, an antigen–polycation conjugate was added to a solution containing antibodies. The conjugate was prepared by the covalent attachment of the antigen to polycation, poly(*N*-ethyl-4-vinylpyridinium) bromide containing 7% hydroxyethyl groups. After the antibody–antigen–polycation complex was formed, precipitation of the conjugate was induced by polyelectrolyte complex formation by the addition of polyanion, poly(methacrylic) acid and with a pH-shift from 7.3 to 6.5. The antibodies were eluted from the precipitate at pH 3.0.

2.4. Immobilization of oligomeric proteins for the preparation of immunosorbents

Purification of antibodies (especially the elution step) becomes complex while using immobilized oligomeric proteins as the antigens [61]. It is well known that the intersubunit interactions in oligomeric proteins are not firm and break down under rather mild conditions (changes of pH or the ionic strength of the medium, low or high temperatures). The K_d value for the oligomer–monomer equilibrial system is seldom lower than 10^{-7} M (usually it is about 1 μM), and the K_d value for the antigen–antibody complex is about 10^{-9}–10^{-11} M [61]. Therefore, in the case of immobilization of an oligomeric protein through a single subunit, the oligomeric protein will dissociate into subunits before the dissociation of the antibody–antigen complex takes place. Thus, oligomeric proteins immobilized through a single subunit cannot be used as the immunosorbents. Covalent binding of an oligomeric protein with subsequent linking of the subunits with the cross-linking agents is not always useful, because it may destroy the antigen determinants. The safest method is usually the immobilization of oligomeric proteins on supports containing a high concentration of active groups allowing multipoint binding through several subunits. Although enzymes may loose their activity under such conditions, they often retain the

ability to bind antibodies and this procedure may even facilitate the elution of the antibodies.

2.5. Elution of antibodies from an immunosorbent and approaches used to increase yield of antibodies

The fact that the interaction between antibodies and antigens is normally high makes the sorption step easy, but on the other hand, it necessitates the use of harsh elution conditions which may invalidate the purification. Common practise is to use extreme pH values, detergents, chaotropic salts, or the mixtures of the above as eluting agents. It should be noted that if the antigen is also a large protein, the affinity material may equally be destroyed by the elution process.

There are few methods that can be applied to facilitate elution. The conformation of the bound epitopes can be changed to lower their binding strength to the antibody. Multipoint immobilization of antigens can weaken the affinity as well as the change of the immobilization material and method. Changes in the conditions of elution affect antigen and/or antibody.

Polyclonal antibodies can be eluted from the column under mild conditions, but it should be noted that these antibodies may constitute only low-affinity antibodies while the high-affinity fraction remains bound. Low-affinity antibodies are not useful for many of the applications of antibodies except for purification of antigens by immunoaffinity chromatography itself [62,63].

Alkaline (pH 11.0) and acidic (pH 2.5) buffers [59], solutions of high ionic strength, and detergents are commonly used [2,64] for the elution of antibodies. In some fortunate cases, specific interactions can be found to disrupt the antigen–antibody interaction without significant changes in the structures of the partners. For example, although 0.1 *M* Li-3,5-diiodosalicylate causes denaturation of antibodies leading to the breakdown of the antigen–antibody complex, their native structure is recovered after the removal of the denaturant [65]. In other cases, antibodies can be eluted from the immunosorbent with the use of soluble antigens of the same nature [55] or other compounds competing with the antibodies for the same antigen binding sites. For example, monoclonal antibodies prepared against TEM-1 β-lactamase was found to compete with penicillins and cephalosporines for binding to the enzyme [66]. Consequently, penicillin and its derivatives can be used to mildly elute for the mild elution of these antibodies. A drawback is that these additives (as well the denatured antibody) are impurities and require removal from the final product.

2.6. Isolation of Fab fragments of antibodies

The isolation of mono- and bivalent Fab fragments of antibodies can be performed by the classical method of Porter using papain cleavage and subsequent ion-exchange chromatography [67]. Improvements have been done in the chromatographic separation of the Fab fragments from the reaction mixture [68–73]. Advances in affinity chromatography [69,74,75], immobilized proteolytic enzymes [75], immobilized protein A for separation of Fc fragments [28], and even the use of anti-papain antibodies for removal of contaminations of the proteolytic enzymes [76] have been described. The problems and strategies for Fab fragments produced by gene technology [77,78] involve primarily a lack of adequate protein expression and reassembling of the peptide components. When a correct Fab fragment is obtained, the purification by affinity chromatography is similar to conventional methods.

The main problem for the production of Fab fragments from antibodies is the design of the proteolytic degradation so that the splitting of antibodies into Fab and Fc fragments is nearly complete without damaging the Fab moiety. The optimization of hydrolysis requires time and materials. Unfortunately such an optimization is required for most cases. The non-hydrolyzed antibodies and Fab and Fc fragments can be easily separated from the reaction mixture by gel chromatography, but unfortunately, the Fab and Fc fragments have about similar molecular masses and they do not separate. Since the final Fab product is monofunctional and no longer contains the Fc fragment, standard immunoassays cannot be applied, requiring the use of competitive inhibition enzyme-linked immunosorbent assay (ELISA) [71].

The proteolytic cleavage of antibodies can be performed during their purification on the immunosorbent to protect the active site of the antibodies from damages [79]. Antigens were immobilized on

Sepharose and allowed to bind to the immobilized antigens and then cleaved by papain. The soluble products being removed were the Fab fragments. In this particular case, proteolysis of the matrix bound antibodies was not as effective as that of the soluble ones. Probably, the matrix imposed some diffusional limitations and hence hydrolysis proceeded more slowly. The yield of the active Fab fragments was also not very high because half of the Fab fragments were lost while washing the column.

Fab fragments can be obtained by employing nonstoichiometric polyelectrolyte complexes (NPECs) with attached antigens [60]. A 1.8-fold purification of antibodies was achieved without a chromatographic step yielding a preparation with more than 95% purity. The NPECs with attached antigen were used to solve three main problems of the Fab fragment production, namely, (i) protection of the Fab binding site from proteolytic degradation; (ii) affinity separation of Fab fragments from the reaction mixture; (iii) selective separation of only those Fab fragments which have affinity to the antigen, hence avoiding the need to determine the affinity of the Fab fragments to the antigens after the purification procedure. The method has the advantage of immunocomplex formation of the antibodies with antigen–polycation conjugate. The conjugate was prepared by the covalent attachment of the antigen to polycation, poly(*N*-ethyl-4-vinylpyridinium) bromide, containing 7% hydroxyethyl groups. Proteolysis of monoclonal antibodies bound to the conjugate was followed by: (i) precipitation of the conjugate induced by polyelectrolyte complex formation with added polyanion, poly(methacrylic) acid and pH-shift from 7.3 to 6.5, and (ii) elution at pH 3.0 resulted in 90% immunologically competent Fab fragments. The required papain concentration was 10-fold less than that in the case of free antibodies in solution. Rather small damage of the antibodies and high yield of the Fab fragments strongly suggest that the antigen–polycation conjugate not only protects binding sites of monoclonal antibodies from proteolytic damage but also facilitates the proteolysis probably by exposing the antibody molecules in a way convenient for proteolytic attack by the enzyme. Compared to the procedure which digests free antibodies in solution and uses chromatographic isolation of the Fab fragments [67–73,80], the described method is gentler toward antibodies because their binding sites are protected by binding to immobilized antigens. On the other hand, the Fc fragments are exposed to papain resulting in an efficient hydrolysis with much smaller concentrations of the enzyme.

2.7. Differences in purification strategies of mono- and polyclonal antibodies

The strategies of affinity chromatography of antibodies on immobilized antigens differ for mono- and polyclonal antibodies. The procedure for purification of monoclonal antibodies includes their separation from non-immunoglobulin proteins, like albumin, in the ascites or culture supernatant. Consequently, the principal task is to separate the immunoglobulin fraction from other proteins. Both immobilized antigens and proteins A, G, and L are convenient. The cell wall proteins of *S. aureus* are more convenient because they do not necessitate the immobilization of a new specific antigen every time enabling a standard procedure to be applied [28,29]. Thus, the main usage of immobilized antigens for the isolation of monoclonal antibodies is when *S. aureus* proteins have insufficient affinity [30–32,42].

For the isolation of polyclonal antibodies, not only does the immunoglobulin fraction need to be separated from other proteins, but also a variety of antibodies with different specificities must be isolated from each other. At the first stage, antibodies can be purified using protein A affinity chromatography (in the case of IgG class), but for the final purification, chromatography on immobilized antigens is the method of choice. It allows not only the separation of a specific antibody from other proteins and nonspecific antibodies, but also the fractionation of certain antibodies according to their affinities. They can be obtained by eluting the affinity column with buffers of different pH values and salts stepwisely or with a gradient. The antibodies with a low affinity are eluted with the buffer of pH in the range from 3.5 to 4.5, and the antibodies with a high affinity are eluted with the buffer of pH 2.0–2.2. Other fractions of the antibodies can be eluted with a basic buffer solution (pH 10.5–11.0). Sometimes, even after such procedures a part of the antibodies remains bound to the immunosorbent and can be

eluted only with detergents. This material may not be exploited because of denaturation, but washing with detergents allows a complete regeneration of the immunosorbent. Treatments with urea, or extreme pH values do not necessarily result in changing in the antibody-binding properties of the sorbent, since immobilization protects the antigen conformation. Thus, it is possible to isolate several fractions of antibodies differing in their affinity to the antigen using the same antiserum. These fractions are applicable for different purposes that will be described in Section 3. Moreover, the use of the differential fractionation, different immobilized forms of antigens, or different immobilized antigens allows isolation of homogeneous fractions of antibodies with different properties from polyclonal antisera.

As an illustrative example of the use of large-scale immunoaffinity chromatography is the recovery of antibodies from whey, a by-product of cheese production, which is a potential source of antibodies. Immunization of a dairy cow in the mid-lactation with murine IgG and dinitrophenyl-keyhole limpet hemocyanine resulted in the formation of antibodies to these antigens in both blood and milk as well as its downstream products [81].

2.8. Analysis of the specificity of antibodies towards various antigen forms

In the routine generation of monoclonal antibodies, the selection of clones is carried out with an immunoassay, usually ELISA. A desired antigen is adsorbed overnight onto polystyrene surfaces of microtiter plates followed by removal of the non-adsorbed antigen with solutions containing a buffer, NaCl, and often a non-ionic detergent [82]. The polystyrene surface is highly hydrophobic and unnatural for biomolecules and obviously conformational changes of antigens take place during the adsorption. Therefore, there remains a moderate probability that hybridomas producing antibodies to non-native antigen forms are chosen [83,84]. Although such antibodies can be useful for the separation of proteins from their non-native forms [60,84], or for creation of the artificial chaperones [60,84–86], it should be recognized that the antibodies may not be suitable for diagnostics purposes, for example. Antibodies to the native forms can be revealed by using a modification of the ELISA procedure based on the competition between the free and adsorbed antigens [87].

To answer the question, what forms of the antigens interact with poly- or monoclonal antibodies, different immobilized antigen forms can be prepared, ranging from the native to completely denatured forms. In the case of oligomeric proteins, different polymerization degrees can be prepared. Analyzing the interaction of the antibodies with various preparations enables the specificity of the antibodies to be precisely determined.

2.9. Separation of antibodies against different antigen forms

Antibodies capable of interacting with a certain antigen form are necessary for research purposes (investigation of the mechanism of protein folding, study of intracellular localization of antigen forms) and practical applications (removal of non-native proteins, preparation of artificial chaperones). In the case of monoclonal antibodies, it is sufficient to select critically the required hybridomas.

Recently, systematic studies for obtaining antibodies recognizing only non-native molecular forms of a protein were carried out [83,84]. Clones of antibodies against the enzyme GAPDH were selected with plates coated with non-native enzyme forms. Analysis of two selected clones by affinity chromatography on immobilized tetrameric, dimeric and monomeric forms of the protein, including unfolded protein subunits, showed that both clones of the antibodies effectively bound to active and inactive monomers and dimers, as well as to the unfolded polypeptide, but not to the native tetrameric protein [88–91]. The antibodies were successfully exploited for studying pathways of protein folding.

Polyclonal sera inherently contain a set of different forms of antibodies against an antigen. Native and denatured forms of antigens are immobilized and used to obtain different fractions of antibodies. This approach was used to isolate antibodies to the both native and denatured GAPDH from polyclonal serum from a rabbit immunized with GAPDH from *Bacillus stearothermophilus* with little cross reaction [92]. In general, populations of antibodies that are rather

homogeneous in terms of their properties against certain antigen forms can be separated from polyclonal sera.

2.10. Immunization of an animal with two antigens and a two-step separation of the produced antibodies

It is common to immunize an animal with a single antigen, because this simplifies the downstream of antibody separation. However, if there are effective ways available for the separation of the antibodies from sera, it is possible to immunize with two or more antigens. By example, an animal was immunized with two different antigens and then the antibodies were isolated from the serum in two steps [93]. The enzyme GAPDH from *B. stearothermophillus* and GroEL from *Escherichia coli* were used as the antigens. Rabbits were immunized by a mixture of the proteins with Freund's adjuvant according to standard procedures. The immunization resulted in a pronounced immune reaction that made it possible to perform further immunization without the adjuvant. Immunoblotting revealed a high titre of antibodies to both GAPDH and GroEL in the serum. First, the antiserum was passed through the column containing the immobilized GAPDH, removing the antibodies to this antigen. The antibodies to GAPDH were eluted with a buffer of pH 2.2. Then the antiserum was passed through the next column containing the immobilized GroEL and the bound antibodies were eluted in the same way. Analysis of the two fractions of antibodies by different methods showed that they did not exhibit cross reaction, allowing their application not only for the isolation and identification of the corresponding antigens, but also for the investigation of complexes between GAPDH and GroEL [93]. Thus, the isolation of two types of antibodies from serum is rather simple and a convenient method and it is quite likely that animals can be immunized with more than two antigens and still produce relatively high titers to all. It is also possible to inject new antigens into animals that were previously immunized by other antigens. This approach is convenient for obtaining small amounts of antibodies against different antigens.

3. Isolation of antigens by using immobilized antibodies

The use of immobilized antigens for the isolation of antibodies seems to be an optimal method in the field of the affinity chromatography, since it is based on the extremely specific interactions. Nevertheless, there are only a few examples of effective antigen purification by this method. There are three main factors limiting the use of antibodies for affinity chromatography: (i) high specificities of the antigen–antibody interactions (species- and tissue-specificity) requires isolation of specific antibodies for the isolation of antigens from a certain source; (ii) immobilization of antibodies on a support often results in a decrease or complete loss of their antigen-binding properties; and (iii), the tight binding of antigens to antibodies, being extremely convenient for isolation of the antigens from the protein mixture, complicates their elution from the immunosorbent. Approaches for avoiding or diminishing these drawbacks are discussed below.

3.1. Methods of preparing insoluble antibodies

Antibodies can be directly immobilized onto solid support, or the trivalency (two Fab and one Fc fragments) is exploited. Since the Fc-part is not involved in antigen recognition, it is a beneficial target for binding [94]. In the following section the main focus will be on the IgG class.

3.1.1. Covalent immobilization of antibodies on insoluble sorbents

Usual methods of covalent immobilization result in binding of all three fragments in a random way. Possible variants are presented in Fig. 1. It is seen that only a part of the immobilized antibodies retains the ability to interact with antigens, and a part of the antibodies loses the ability to interact with two antigens typical of soluble antibodies. Nevertheless, some approaches can be used to increase the yield of the active antibodies. A number of studies describe immobilization of antibodies through their carbohydrate fragments, which are located a moderate distance from the active sites. The carbohydrate can be activated by periodate oxidation to yield aldehyde

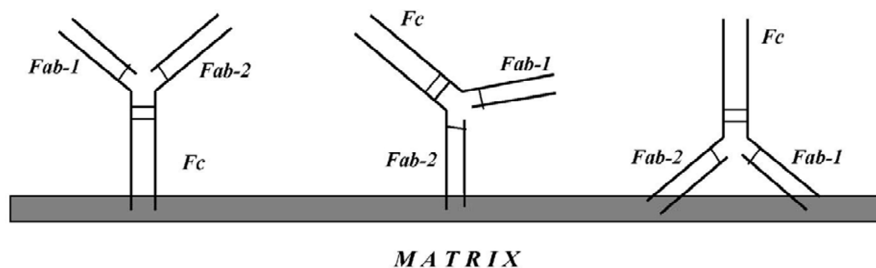

Fig. 1. Schematic diagram demonstrating the possible variants of covalent immobilization of antibodies on an insoluble matrix. Fab-1, Fab-2 and Fc are the Fab and Fc fragments of antibodies.

side groups, allowing the antibodies to be attached to amino-derivatized supports by imine linkages [95–97].

Immobilization onto CNBr-activated Sepharose is the most widespread method used and, in standard conditions, yields 10–35% of active antibodies. The yield of active antibodies may be increased by using a lower pH during the immobilization [55]. Presumably, while carrying out immobilization according to the standard procedure (pH 8.3), all the surface lysine residues interact with the support with the same effectiveness. The content of the immobilized protein decreases slightly by lowering the pH (with using Sepharose of the same activation degree), but the yield of active antibodies increases severalfold. Therefore, lowering the pH to 6.5–7.0 may result in selective interactions of the lysine residues of the Fc fragments with the activated support. The phenomenon is not necessarily universal and may be adversely affected by a decreased stability of the bound antibody.

Covalently immobilized IgGs can be digested with papain and the nature of the products released into solution can be determined [98]. If the products are solely Fab fragments, it is most probable that the coupling has taken place at the Fc unit. This provides a method for choosing reaction conditions for the binding of antibodies through the Fc fragment only.

The yield of active antibodies can be increased by prior chemical modification of the antibodies. To accomplish this, the most reactive amino groups are masked with a reaction of dimethylmaleic anhydride resulting in a 1.6–1.8-fold increase in the immunoactivity of the antibodies (polyclonal goat anti-mouse antibodies) immobilized on different epoxy-containing carriers. A 3–10.7-fold increase in the activity of the immobilized monoclonal antibodies against carboxypeptidase A [99] was obtained.

Activated diol-silica support has been used for the covalent immobilization of antibodies. Using immobilized monoclonal antibodies against interferon-α, a 100-fold purification of the recombinant human interferon-α was achieved in one step [100].

3.1.2. Sandwich sorbents

A rather simple immobilization method for antibodies is the so-called sandwich sorbent while its use for purification of antigens is more sophisticated. The sorbent is based on the ability of antibodies to interact with two antigens simultaneously due to the existence of two spatially separated active sites connected with a flexible hinged site (Fig. 2) [55,101–103]. First, an antigen is coupled to a matrix, and then antibody against it is added. This matrix–antigen–antibody complex can be used for the isolation of antigens, since one of the active sites of the bivalent antibody molecule remains free. The main advantage of the sorbent is that the purification and immobilization of the antibody is not necessary.

Sandwich sorbents are widely used for analytical purposes. It is not necessary to isolate the antigen investigated, and this approach yields similar results as which use covalently bound antibodies. The application of the sorbents for preparative purposes is more complex. The sandwich sorbent is prepared with subsequent elution of the antigen to be separated using conditions under which the primary antigen–antibody bond remains uncleaved. The strength of the interaction between two active sites of an antibody molecule and two antigens (immobilized

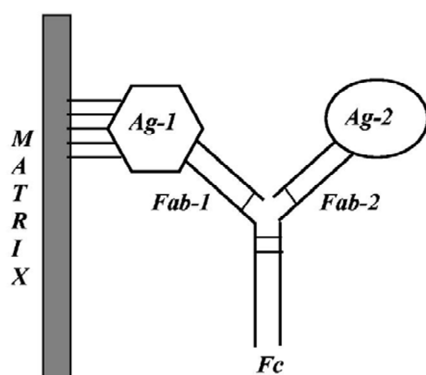

Fig. 2. Schematic diagram showing the purification or immobilization of antigens by the use of the sandwich sorbents. The first antigen (Ag-1) is immobilized covalently on an insoluble matrix. Antibody is bound to Ag-1 through one Fab fragment; the second antigen (Ag-2) can then be bound through the second Fab fragment.

or soluble) depends mainly on the two processes. First, it is known that the binding of an antibody to an antigen is often characterized by a positive cooperativity, i.e., after binding of an antigen to the first active site, the affinity of the second active site towards antigens increases. This will fortify the sorption of the antigen from solution. After binding of the secondary antigen, both of the sites will bind equally. Regardless, the antigen being captured from the solution is difficult to elute from the sorbent without destroying the bond between the antibody and the primary antigen. This situation is aggravated by probable weakening of the interactions between the primary antigen–antibody interactions as a result of steric and conformational hindrances due to the immobilization process with the matrix. As a consequence, the affinity of an antibody to the immobilized antigen tends to be lower than that to free native antigen. While passing soluble antigen through the immunosorbent containing covalently immobilized antigens coupled to antibodies, only the elution of the antibodies with the antigens was observed [55]. Presumably, the free antigens rapidly displaced the primary antigen–antibody bonds. In analytical uses of sandwich sorbents, the displacement of antibodies from the complex with the immobilized antigens is less significant, because the concentration of the free antigen is very low.

Noteworthy, the standard method of preparation of sandwich sorbents can be modified by introducing cross-linkages between the immobilized primary antigen and the antibody with bifunctional reagents. After the treatment of the binary complex with glutardialdehyde, the complex does not dissociate even under rather stringent conditions [102]. After adsorption of the antigens from the solution, they can be eluted with acidic or basic solutions without dissociation of the antibodies.

3.1.3. Insolubilization of antibodies by binding to immobilized protein A

One method for immobilization of antibodies is their selective binding through the Fc fragment to immobilized protein A [38,45,104,105]. The cell wall proteins of *S. aureus* often exhibit high affinity to Fc fragments of antibodies (for example, protein A binds to Fc fragment of IgG). It is conceivable that immobilization through the Fc fragment is optimal, because it leaves two active sites accessible to interacting with free antigens. A disadvantage of this approach is the possibility of a cleavage of the protein A–antibody complex during the elution of the antigens. The antibodies immobilized through protein A efficiently bind not only to antigen proteins, but also to cells. Antibodies adsorbed on covalently bound protein A were successfully used for the isolation of erythrocytes from human blood [106]. It was demonstrated that upon the immobilization of protein A on glycine–Sephadex G-10 with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide/*N*-hydroxysuccinimide, binding took place only at the surface of the particles of the matrix. On the other hand, protein A could diffuse into the particles of the matrix and was bound both at the outer and the inner surface of the CNBr-activated Sepharose. To obtain the same level of the binding efficiency of the cells to the sorbent, one can use smaller amounts of protein A in the case of protein A–glycine–Sephadex G-10 system than in CNBr-Sepharose system. That is why immunosorbents constructed of protein A–glycine–Sephadex G-10, are more suitable than those based on protein A immobilized on CNBr-Sepharose [106]. A specific bifunctional fusion protein A with a cellulose-binding domain was constructed and bound to cellulosic microtitre plates followed by its use for purification of human IgG [104]. Bayer et al. were the first who employed the

avidin–biotin interaction for affinity chromatography [107]. Carriers covered with avidin (e.g., glass beads) can be used for a very firm immobilization of biotinylated antibodies [108].

When working with antibodies immobilized by different methods, the possibility of their leakage from the sorbent should be taken into consideration. Such a leakage results in the contamination of the protein being purified and degradation of the sorbent properties. A non-competitive ELISA is convenient for evaluating leakage [109].

3.2. Selection of low affinity antibodies applicable for antigen purification

To purify antigens, it is preferable to use antibodies of low affinity. In the case of monoclonal antibodies, such hybridomas can be selected by standard procedures [110,111]. In the case of polyclonal antibodies, the fraction of low affinity antibodies can be isolated during their purification. Usually the fraction eluted at pH 3.5–4.5 containing antibodies of the low affinity to the antigens is discarded, although it can be useful for purification of antigens allowing their elution under rather mild conditions [84,85,112].

Antibody fragments (mono- and bivalent Fab fragments), as well as mini-antibodies (fragments of the hypervariable sites) usually exhibit a lower affinity to antigens [113,114]. A synthetic immobilized 10-residue peptide with an amino acid sequence analogous to that of the hypervariable L_3 segment of a monoclonal antibody against lysozyme was used for the purification of lysozyme [113].

3.3. Isolation of proteins and other compounds with different kinds of insoluble antibodies

Immobilized antibodies are used for the purification of different substrates [64,115–122], including enzymes [29,62,65,66,123], peptides [124], carbohydrates [63,125], antibiotics [126], hormones [127], receptors [128], and cells [115,129–131].

Compared to standard purification methods, affinity chromatography using immobilized antibodies has certain advantages. First, a protein can be purified in a predictable way by passing it through the immunosorbent, then washing the immunosorbent from non-specifically bound proteins and finally eluting the desired product. Second, the technique allows isolation of a whole spectrum of antigens unlike standard methods which may lose part of them. For example, proteins can exist in different conformational or oligomeric states, or as a complex with other low- or high-molecular-mass compounds, or as a partially denatured protein. Methods which rely on different molecular masses or charges can distribute the components to fractions which can be recovered only by sophisticated analyses. Third, the immunosorbents can be used for analytical purposes, for example, for determination of the content of a certain protein in tissue or organ extracts that is of importance for the investigation of metabolism in both normal and pathological cases.

Even though immobilized antibodies can be unstable, a properly chosen system can be stable. For example, monoclonal antibodies immobilized on Sepharose 4B were used more than 150 cycles for purification [132]. The main restriction for the use of immunoaffinity chromatography for the purification of antigens is the need to isolate and immobilize an antibody for each antigen and the tedious elution of antigens from the immunosorbent.

β-Lactoglobulin [116], prolactin [117], and human protein C [118,119] were isolated with antibodies immobilized by different methods. Alkaline phosphatase was purified with a 95% yield [62] and pyruvate kinase was purified 2400-fold from an erythrocyte lysate with a 83% yield [123] in one step from a crude extract. Successful isolation of recombinant α-amylase [65] and β-lactamase [66] have been also described. It is of importance that immunoaffinity chromatography allows isolation of proteins from fluids under conditions close to those in vivo. For example, a particular dimeric form of transketolase as the complex with RNA was isolated from yeast extract [133]. There were not found any indications for interactions between transketolase and RNA beforehand. They were eluted from the immunosorbent with a buffer with pH of about 11, but completely retained their biological activities. Besides, using antibodies to GAPDH, investigators were able to isolate complexes of GAPDH with phosphoglycerate kinase and 2,3-bisphosphoglycerate mutase from erythrocyte lysates [83]. Although it was impossible to dissociate the active bienzyme

complex into the solution, the active 3-phosphoglycerate kinase and 2,3-bisphosphoglycerate mutase could be eluted separately by changing pH of the medium by 1–1.5 pH units [83]. To elute β-lactamase from the immunosorbent, antibiotics competing with the antibodies for the active site of the enzyme were employed [65]. Immunoglobulin E (IgE) was purified on an immunoaffinity column containing anti-IgE polyclonal antibodies bound to epoxy-activated agarose [134]. IgEs were eluted with 1.75 *M* ammonium sulfate at pH 8.3 with subsequent dialysis yielding a recovery of 50–60% of the immunoactivity. The column was used repeatedly several times.

An important problem that can be solved by immunoaffinity chromatography is the removal of non-native and denatured protein forms. Because of the increasing usage of recombinant proteins, there has emerged a qualitatively new need to separate functional proteins from their non-native forms, which may exist abundantly, say, in bacterial inclusion bodies. As a rule, the quantity and quality of the non-native forms (conformers, separate subunits, unfolded polypeptide, molten globule) are not known and their separation and analysis by traditional methods are not simple. The molecular masses and approximate isoelectric points of the non-native forms and target protein are often near identical. A single-stage affinity step on immobilized antibodies against the non-native forms of the protein can effectively discriminate the contaminants. After such a procedure, specific activity of a renatured enzyme increased from 55 to 95% (relatively to the native enzyme) indicating to a virtually complete recovery of the initial enzymatic activity [84]. Non-native proteins can also be removed with antibodies immobilized on soluble polyelectrolytes [60,85]. In this case, all the reactions take place in a homogeneous medium, and then the immobilized antibodies in the complex with the bound non-native proteins are transformed into a solid phase. Soluble immobilized antibodies were incubated with a mixture of native and non-native proteins and then precipitated by the addition of positively charged poly(*N*-ethyl-4-vinylpyridinium bromide), followed by centrifugation of the non-native proteins. By this procedure, the specific activity of the soluble enzyme increased from 80 to 125 U/mg yielding practically pure protein.

Immobilized antibodies are widely used for the purification of biological fluids (including human blood) from harmful compounds. For example, the use of immobilized specific antibodies allows removal of the excess of low density lipoproteins from the plasma of patients suffering from hypercholesterolemia [122]. Specific immobilized antibodies were also used for the removal of human protein C from plasma of patients with high levels of coagulation factors [118,119], and different toxins, such as enterotoxin for example [120,121].

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