Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 848 (2007) 40–47
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Protein A chromatography for antibody purification ☆

Sophia Hober [a], Karin Nord [b], Martin Linhult [c,*]

[a] *Royal Institute of Technology, Stockholm, Sweden*
[b] *Affibody AB, Bromma, Sweden*
[c] *Octapharma AB, Nordenflychtsvägen 55, 112 75 Stockholm, Sweden*

Received 2 May 2006; accepted 20 September 2006
Available online 9 October 2006

Abstract

Staphylococcal protein A (SPA) is one of the first discovered immunoglobulin binding molecules and has been extensively studied during the past decades. Due to its affinity to immunoglobulins, SPA has found widespread use as a tool in the detection and purification of antibodies and the molecule has been further developed to one of the most employed affinity purification systems. Interestingly, a minimized SPA derivative has been constructed and a domain originating from SPA has been improved to withstand the harsh environment employed in industrial purifications. This review will focus on the development of different affinity molecules and matrices for usage in antibody purification.

Keywords: Affinity chromatography; Cleaning-in-place (CIP); Deamidation; Protein A; Protein engineering; Stabilization

Contents

1. Introduction 40
2. Protein A 41
3. Improvement of proteinaceous affinity ligands for industrial applications 42
4. Development of synthetically derived ligands for affinity purification 43
   4.1. SPA mimics 43
5. Comparison studies of SPA media for large scale applications 43
6. Concluding remarks 46
   References 46

1. Introduction

Lately, the number of therapeutic monoclonal antibodies (mAb) in clinical trials has been extensively increased and affinity chromatography purification is commonly used in their downstream processing. Affinity chromatography has several advantages since it is an easy, fast and selective procedure for capturing of the target protein. Due to its selectiveness, an affinity-purification step early in the purification chain is commonly introduced. Thereby, the number of successive unit operations can be reduced [1,2].

The demand for cost efficient production processes has led to the necessity of optimization of the downstream purification, including the affinity step. Larger volumes to be processed and harder requirements for the cleaning-in-place (CIP) protocols are some of the features that need to be solved.

The most applied affinity system for the purification of antibodies is the Staphylococcal protein A (SPA) and smaller ligands derived thereof. The affinity between protein A and IgG was one of the first native interactions to be explored for the development of an affinity system for protein purification [3]. Another affinity ligand, also used for IgG-purification and originating

☆ This paper is a part of a special issue entitled "Polyclonal and Monoclonal Antibody Production, Purification, Process and Product Analytics", guest edited by A.R. Newcombe and K. Watson.

* Corresponding author. Tel.: +46 8 5564 3103; fax: +46 8 5664 3022.
E-mail address: martin.linhult@octapharma.se (M. Linhult).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.09.030

from a bacterial species, is the streptococcal protein G (SPG) [4]. Despite the widespread use of protein A and protein G in antibody purification, the ligand stability is an issue that has limited their use. In order to improve the tolerance of ligands used in affinity chromatography, different methods have been used. A protein engineering strategy was employed to improve the SPA and SPG stability towards CIP treatment [5,6]. Due to the superior stability of protein A compared to protein G, the use of protein G in industrial purifications is very restricted. However, affinity purification using protein G is the normally the first choice for the purification of serum and human IgG subclass III, due to protein A's low affinity towards that subclass.

Alternatives to the protein-based ligands of bacterial origin have also been developed. Lowe and co-workers have managed to develop a triazine-based ligand for the purification of antibodies [7]. In addition, peptide based ligands also have been developed for the capture of antibodies.

This review article will present different protein A-based ligands and sorbents for the purification of mAb. Moreover, advantages as well as disadvantages for protein A, triazine and peptide based affinity ligands will be discussed.

2. Protein A

SPA is a cell wall associated protein domain exposed on the surface of the Gram-positive bacterium *Staphylococcus aureus*. SPA has high affinity to IgG from various species, for instance human, rabbit and guinea pig but only weak interaction with bovine and mouse (Table 1) [8,9]. The gene encoding SPA was sequenced by Uhlén et al. [10]. SPA consists of three different regions; S, being the signal sequence that is processed during secretion [11], five homologous IgG binding domains E, D, A, B and C [12] and a cell-wall anchoring region XM [13], see Fig. 1.

Table 1
IgG binding of SPA [3,4,8,9]

Species	Subclasses	Protein A
Human	IgG1	++
	IgG2	++
	IgG3	−
	IgG4	++
	IgA	Variable
	IgD	−
	IgM	Variable
Rabbit	No distinction	++
Guinea pig	IgG1	++
	IgG2	++
Bovine		+
Mouse	IgG1	+
	IgG2a	++
	IgG2b	+
	IgG3	+
	IgGM	Variable
Chicken	IgY	−

Strong binding ++, medium interaction +, weak or no interaction −.

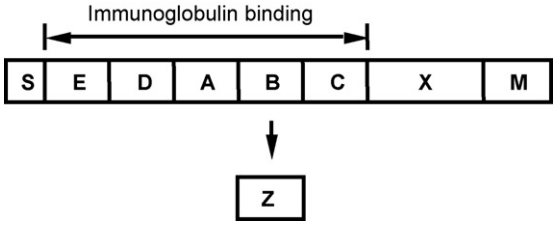

Fig. 1. The Staphylococcal protein A shown here is a cell-wall associated protein, consisting of a signal sequence (S) processed during secretion, five homologous IgG-binding domains (E, D, A–C), and a cell-wall attaching structure (XM). Also shown is the commonly used Z domain, corresponding to an engineered version of the B domain of SPA [14].

Each of the five domains in SPA is arranged in an anti parallel three α-helical bundle of approximately 58 aa and the three dimensional structure is stabilized via a hydrophobic core. The domains are independently capable to bind to the Fc-part of IgG1, IgG2 and IgG4, with an estimated affinity constant (K_A) of 10^8 (M^{-1}), but shows only weak interaction with IgG3 [15]. In addition, each domain has high affinity for the Fab part of certain antibodies [16]. The binding site for the Fc part of the IgG molecule has been determined in a study of the B domain and was shown to involve 11 residues of helix 1 and helix 2 [17]. In addition, another study using the D domain showed that the Fab-binding part was located distinctly apart from the Fc-binding. The 11 residues involved in the Fab interaction are located on the second and third helices [18].

In order to increase domain B's tolerance towards site-specific chemical cleavage of fusion proteins using hydroxylamine, the sensitive Asn-Gly dipeptide at residues 28–29 was changed by site directed mutagenesis to Asn-Ala, resulting in an engineered domain denoted Z [14]. While introducing the single mutation G29A to the B domain the Fab interaction diminished, probably due the fact that the Ala disturbed the interaction between the two molecules [16,18]. For the application as immobilized affinity ligand for capture of IgG, previous work indicate that a head-to-tail construct of the Z domain has similar molar binding capacity for IgG as the native SPA molecule [19]. The Z scaffold has also been used in a combinatorial protein engineering approach for the introduction of novel binding specificities. Thirteen surface-exposed amino acids at the binding site were randomized in a phage display library and several new binders were possible to select [20]. Furthermore, by exchanging or prolonging the loop region between helix one and two, novel variants of Z allowing for milder elution conditions have been constructed. By this approach elution of bound IgG could be accomplished at pH 4.5 instead of the normally used pH 3.3 [21].

Several issues have to be considered when attaching protein ligands to matrices, for instance coupling chemistry, direct versus non-direct immobilization and ligands density. Immobilization of a protein to a resin can be done by different chemistries, such as NHS, thiol and epoxy. A non-directed immobilization may provide multiple attaching points that could be beneficial for the ligand stability as well as minimization of ligand leakage. However, a disadvantage with that approach is that multiple attaching points may interfere with the functional regions of the

protein. One strategy to overcome the problem is to use directed immobilization, for instance by introducing a thiol group in the protein [19]. The capacity of the resin is dependent on ligand density [19,22], but steric effects will also play a role. Earlier it has been shown that the dynamic capacity of a matrix is highly dependent on the orientation of the ligand [19,22]. In addition, different studies have shown that mono and dimer of protein ligands have similar binding capacities for the targets proteins [19,22].

3. Improvement of proteinaceous affinity ligands for industrial applications

As mentioned previously, the naturally existing protein ligands normally have high specificity but often the stability towards high pH is lower than desired for large-scale applications. Inactivation of germs as well as pyrogen removal and antiseptic management are important issues in large-scale purifications to ensure no carry-over and avoid cross-contamination of proteins from different separations [23]. A cleaning-in-place (CIP) procedure must be set for each process and the protocol should be specifically designed according to what contaminations are present in the purification mixture. Sodium hydroxide (NaOH) in concentrations from 0.1 to 1 M is a commonly used agent for cleaning of the equipment as well as the resins since it is a potent remover of tightly bound, precipitated, or denatured proteins, lipids and nucleic acids. In addition sodium hydroxide is effective in inactivation of most microorganisms including bacteria, viruses, yeast and also endotoxins [24,25]. Furthermore, it is easy to remove, simple to monitor and associated with low cost. Thus it is advantageous to have equipment and matrices that can tolerate high pH.

Since most proteins are sensitive to alkaline solutions the utilization of protein-based affinity media in large-scaled applications is hampered by the limited stability of such media. To further improve protein domains for large-scale purification, a protein engineering strategy was used to stabilize and optimize the IgG binding domains of proteins A and G [5,6]. Asparagines are known to be susceptible to high pH, through covalent modifications such as deamidation or backbone cleavage. The extent of modifications are highly sequence and conformation dependent [26–28]. These reactions are spontaneous and require only water to occur. The modifications take place at physiological solvent conditions but since the reaction is dependent on hydroxide ions, the reaction rate is elevated by increasing pH. [29]. For improvement of an IgG-binding domain from protein A, several site specific mutations were performed to analyze the necessity of the different asparagine residues, as well as these residues impact on the tolerance of high pH. All new protein variants were analyzed using surface plasmon resonance to assess the possible changes in affinity. Moreover to challenge the mutants in affinity chromatography, the domains were covalently attached to chromatographic matrices and used for affinity purification with an integrated CIP procedure between the purification cycles. This procedure is optimal since studies in free solution do not always reflect the constraints imposed by the matrix environment. An excess of material was loaded onto the column and the amount of eluted material was measured to determine the capacity. Among the new, engineered variants, domains showing higher tolerance towards alkaline pH could be found [6]. Due to the relatively high tolerance to harsh conditions that protein A already exhibits, a bypass mutagenesis approach was used. The experiment was designed to use a destabilized Z domain, namely ZF30A, as comparison instead of the parental Z domain. This approach was supposed to more easily report on the differences achieved by the mutations. In ZF30A a phenylalanine taking part in the hydrophobic core was exchanged for an alanine giving a domain with lower conformational stability compared to the parental Z. However, the structure as well as the affinity was almost unaffected. In Fig. 2 the alkaline tolerance of the ZF30A domain and different mutants thereof is shown. These protein domains were repeatedly exposed to high pH and the retained capacities of the columns were measured. The deactivation data in Fig. 2 suggest the four of the exchanged aspargines (N6, N11, N43 and N52) are less important for the sensitivity to alkaline conditions since their deactivation patterns are very similar to the parental molecule Z(F30A). Asparagines N11, N43 and N52 are all situated in helices and therefore probably less sensitive to deamidation. Moreover, N6 is located in the N-terminal region of the protein, hence, possible transformation of this amino acid might not be detected with this approach [6]. Asparagine N23, is located in the loop between the first and the second helix and the experiments showed that the alkaline tolerance of the Z(F30A) was enhanced by exchanging aspargine 23 for a threonine [6]. In addition, when grafting the N23T mutation to the Z scaffold an increased tolerance to alkaline treatment compared to the native Z molecule could be seen. Interestingly, the variant carrying a N21A mutation in the loop region between the first and second helix showed lower alkaline resistance than all other Z-variants. The increased deamidation rate in alkaline conditions might have had a structural explanation, since N21 is postulated to interact with N52 and by breaking this interaction the structural stability might have been impaired. These experiments showed that by exchanging aspargine 23 for a threonine the alkaline tolerance

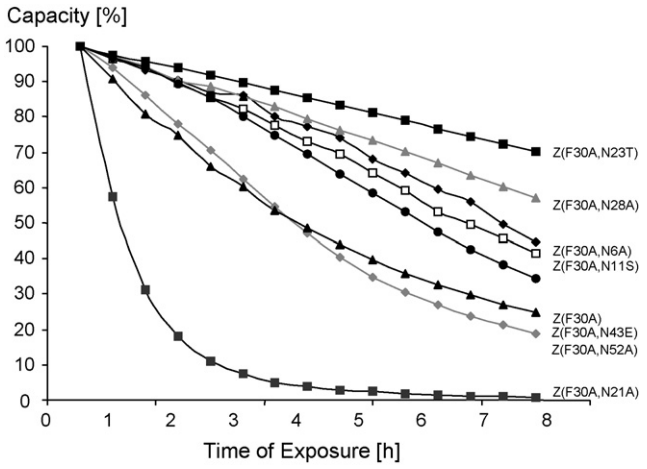

Fig. 2. A comparison of the capacity of Z(F30A) and different mutated variants following an ordinary affinity chromatography scheme with an integrated CIP-step. The protocol was run 16 times with 0.5 M NaOH as cleaning agent and the duration for the alkaline exposure was 30 min in each round.

of the Z(F30A) was enhanced. Additionally, when grafting the N23T mutation to the Z scaffold an increased tolerance to alkaline treatment compared to the native Z molecule could be seen. Both in the investigation of the C2 domain from protein G as well as the Z domain developed from protein A the most sensitive asparagines were found to be located in the loop regions [5,6]. This agrees with previous reports that the fragility of asparagines is dependent on flexibility of secondary structure elements [26].

4. Development of synthetically derived ligands for affinity purification

Protein-based affinity ligands normally have high specificity but often suffer from low stability and low tolerance to alkaline conditions, leading to limited life cycles that might result in low scale-up potential due to high cost. Therefore, large efforts have been put in the development of chemically synthesized affinity molecules where triazine commonly is used as backbone for further modifications. Selective matrices based on other molecules for example mercaptoethylpyridine or peptides have also been developed to replace the commonly used protein A matrix.

4.1. SPA mimics

In order to further develop affinity chromatography for IgG purification, different peptide-based ligands have been created and different approaches have been used for the construction. Peptides specific for a certain antibody showing affinity to the antigen binding part have been developed [30,31]. Furthermore, peptides that mimic the interaction between protein A and the Fc part of the IgG have been generated by selection [32–35]. The great advantage with the more general binders is of course the broad field of possible applications. Two different methods have been used for selection of the binding peptides; screening of peptide libraries and selection using phage display. By screening of peptide libraries, a number of different affinity peptides have been discovered [31,34,35]. Fassina et al. produced a peptide library with the aim to mimic an IgG-binding domain from SPA [34]. By coupling of the peptides to a solid phase, they could successfully show that one of the new peptides (TG19318) was functional as affinity ligand for IgG purification. In addition, they were able to show that the peptide also possessed affinity to other immunoglobulins [36,37]. Interestingly, in order to avoid proteolytical degradation, Verdolivia et al. chose to synthesize the selected peptide using D-amino acids. The new molecule was shown to be very resistant against proteolytical enzymes and also usable as affinity ligand [35]. By using phage display, several different research groups have developed peptides with affinity for IgG molecules. Also here, peptides with affinity for the antigen binding part of IgG have been enriched [30,32]. By sequence comparison between the selected peptides and protein A, Erlich et al. were able to conclude that the newly selected peptides showed homology with protein A. Two of four peptides challenged in affinity purification were able to capture an IgG-molecule although with rather low capacity [7].

Triazine dyes, such as cibacron blue F3G-A, have been used in affinity chromatographic applications in protein purification for about 30 years. Triazine has been used as backbone to synthesize new biomimetic ligands and a number of ligands have been developed by the use of enzyme inhibitors in combination with structural information [38]. As the knowledge about three-dimensional structures of proteins increases, the design of useful affinity ligands will be more successful. By careful analysis of the natural binding surface between protein A and IgG, the group of Professor Lowe was able to design a ligand with affinity for IgG. The molecule was constructed by mimicking the key dipeptide Phe-132:Tyr-133 motif of protein A. These two residues create a hydrophobic core, which lies on a helical twist of the B-domain of SPA and are oriented towards a shallow groove in IgG. A triazine scaffold that has similarities with the helical twist showed structural likeness with the dipeptide. The resulting molecule was shown to be able to capture human IgG selectively [7]. In a following study the ligand could be further refined and the resulting lead ligand displayed an affinity constant (K_A) for human IgG of $1.4 \times 10^5\ M^{-1}$ and the produced matrix a capacity of 51.9 mg IgG/g moist weight gel [39].

In a recent publication by Newcombe et al., a synthetic protein A adsorbent was used in the initial purification step for capturing of polyclonal antibodies [40]. Due to the crudeness of serum a strong cleaning agent between batches is needed. Hence, a synthetic ligand would be advantageous for large-scale applications. The synthetic ligand, MAbsorbent® A2P binds all subclasses of human IgG. The binding capacity of the matrix was found to be approximately 27 mg/ml adsorbent. Moreover, the initially achieved purity was approximately 80%. By changing the elution profile and introducing a washing step with caprylic acid to remove residual albumin it was possible to increase the purity to approximately 95% [40].

5. Comparison studies of SPA media for large scale applications

Several different SPA sorbents have been developed for industrial purposes. The most common is conventional chromatographic matrices although other systems exist, such as expanded bed adsorption (EBA). Other affinity ligands exist as well both with bacterial origin as well as peptides and triazine based ones. A commonly used affinity ligand is based on the Streptococcal protein G [9]. This ligand is preferred when purifying truncated versions IgG molecules such as Fab and sc-Fv since the ligand has the ability to bind to the Fab region. Also, protein G has another binding profile regarding subclasses and species origin of the IgG molecules compared to protein A [9].

An optimal matrix that has great dynamic capacity in combination with tolerance for high flow velocity is difficult to manufacture [41]. When scaling up, process optimization includes several different demands, for instance low ligand leakage, high resin rigidity, possible re-use of the resin, short operational time, as well as low host cell protein (HCP), and DNA content in the eluate. In each case, studies have to be carried out to examine which parameters are essential for a cost effective process. In the downstream purification process of IgG one of the largest costs is often the protein A resin. However, when looking at the total cost of a process, big savings primarily is achieved by shortening

Table 2
Different protein A-based affinity media investigated in references [36–41]

Sorbents	Manufacturer	Matrix	Ligand[a]	Reference[b]
Protein A-Sepharose 6 FF Protein A-Sepharose 4 FF rProtein A-Sepharose FF	GE Healthcare	Crosslinked agarose	nProtein A/rProtein A	[37,38,41]
Streamline protein A	GE Healthcare	Exbanded bed	rProtein A	[36,37]
MabSelect MabSelect Xtra	GE Healthcare	Crosslinked agarose	rProtein A	[38–40]
MabSelect SuRe	GE Healthcare	Crosslinked agarose	Engineered rProtein A	[39,40]
Immobilized rProtein A IPA 500	RepliGen	Crosslinked agarose	rProtein A	[38,41]
ProSep A ProSep rA High capacity ProSep A High capacity ProSep-vA Ultra Prosep-vA High Capacity	Bioprocessing/Millipore	Controlled pore glass	rProtein A/nProtein A	[36–41]
Protein A-Sepharose CL-4B	Fermentech	Sepharose	n.a	[41]
Affi-gel-Protein-A gel	Bio-Rad	Crosslinked agarose	nProtein A	[38,41]
Affi-gel-Protein-A support	Bio-Rad	polymeric	nProtein A	[38]
Protein A-Ultrogel	IBF	2% agarose 2% acrylamide	n.a	[41]
Eupergit C-Protein A	Röhn Pharma	Copolymer of methacrylamide *N*-Methylene-bis-methacrylamide	n.a	[41]
POROS 50 POROS LP POROS 50 A High Capacity	PerSeptive Biosystem	Polystyrene/divinylbenzene	rProtein A	[37,38]
Protein A Ceramic HyperD F	Biosepra/Pall	Polyacrylamide in ceramic macrobead	rProtein A	[38]
Ultra link Immobilized Protein A	Pierce	Polymeric	nProtein A	[38]
Protein A Agarose 4XL	Affinity Chromatography Ltd.	Crosslinked agarose	rProtein A	[38]
Protein A cellthru 300	Sterogen	Agarose	rProtein A	[38]
AF-protein A Toypearl 650 M	Tosoh Biosep	Polymethacrylate	n.a	[38]

n.a.: Not available.
[a] nProtein A, natural protein A; rProtein A, recombinant protein A; Engineered rProtein A, an alkaline stabilised ligand.
[b] For further details, please see reference as indicated.

the time for the development phase from research to industrial scale and manufacturing. After that the targets for cost saving are re-use strategies, limitation of the non-productive time and time reduction of process steps. Here, resin stability and flow rates are of great importance [42].

Several protein A based affinity media are available on the market and comparison studies between these have been published in several articles [43–48]. A summary of the most frequently used matrices that have been analysed in different studies is presented in Table 2.

Füglistaller published the first comprehensive comparison study of commercial SPA media. Evaluation on capacity and leakage of eight different ligands were performed. The capacities among the evaluated resins ranged from 0.5 to 20 mg/ml gel, and protein A Sepharose Fast flow exhibited the highest capacity. Moreover, leakage of the ligand was investigated in the presence and absence of IgG, at pH 3.0, 4.0 and 8.9. A considerable leakage could be detected at pH 8.9 in the presence as well as absence of IgG, whereas a significant ligand leakage at pH 4.0 only could be detected in the presence of IgG [48]. However, many of the media analysed in this study recently have been replaced with improved sorbents. Characteristics that have been improved are resin rigidity and specific immobilization of the ligands. Old resins with low rigidity have been improved by better cross-linking techniques. Also, the immobilization of the ligands have been improved by protein engineering techniques were specific amino acids have been introduced to be used in the coupling chemistry [6,11].

For large-scale applications, optimisation regarding cost effectiveness is important, and one interesting strategy is outlined by Farhner and co-workers [43]. In their study five different sorbents; Poros 50 (PerSeptive Biosystems), Poros LP (PerSeptive Biosystems), Sepharose A Fast Flow (GE Healthcare), Streamline (GE Healthcare) and Prosep (Bioprocessing) were investigated (Table 1). To optimise the process they defined the production rate as the amount of antibody produced in one cycle divided by the time used for the purification. The acquired figure was divided by the cross-sectional area to make the equation independent of scale. Production rate was thereby defined as a function of five factors; dynamic capacity, antibody concentration in the load, the number of column volumes (CV) for elution/equilibration/wash, the load

flow rate and the elution/equilibration/wash flow rate. The dynamic capacity was expressed as a function of flow rate and the concentration of the feed was known and fixed. The CV for elution/equilibration/wash was also fixed and elution/equilibration/wash was expressed as a function of column length. Thus, it was possible to express production rate as a function of flow rate and column length. Interestingly, they were able to conclude that Poros LP had higher production rate compared to Poros 50, although a lower dynamic capacity. The fact that Poros LP had a lower pressure drop (higher permeability) gives it a higher production rate. Furthermore, the study also showed that it can be advantageous to increase the flow rate despite the fact that the maximum dynamic capacity is lowered. More cycles are needed but on the other hand each cycle is completed in less time. This strategy might be helpful when optimizing the down stream process in a production facility. However, other considerations also have to be done for large-scale applications and different optimizations are needed in different processes.

An expanded bed adsorption (EBA) matrix, Streamline, was also included in their study. This is an interesting technique enabling cell removal, capture and initial purification in one step. Such a reduction of the number of unit operations would be very beneficial in large-scale applications. Despite the crude feed stock, the technology showed similar properties as conventional affinity chromatography regarding HCP, DNA and protein A content in the eluate [43,44]. In a study performed by Blank and co-workers, three different process alternatives were compared regarding the recovery of monoclonal antibody. Two processes using EBA were compared to a conventional recovery scheme using conventional chromatography. In alternative number one, an EBA matrix with immobilized protein A was used as a first step. This was followed by one cation and one anion-exchange chromatographic step. In the second strategy, a cation exchange chromatography step using the EBA technology was followed by a protein A-based affinity chromatography step in a packed bed mode and finally an anion-exchanger chromatographic step. And last, a conventional affinity recovery step based on protein A was followed by a cation and finally an anion-exchange chromatographic step. In the study, process number one with the protein A EBA column as the first step gave comparable purity, regarding host cell protein and DNA contamination as well as, ligand leakage and antibody aggregation, to the more conventional process number three. However, the protein A EBA column showed a decrease in dynamic capacity after 80 cycles. The decreased capacity of the column is of course a disadvantage for the EBA technology and highlights the importance of the development of more stable affinity matrices, especially when loading very crude feed stocks on the column [43,44].

Recently Jungbauer and co-workers published a comprehensive study where several different commercial protein A media were evaluated regarding dynamic bindning capacity (DBC), dependence on residence time, mass transfer properties and life time [45–47]. In the first paper, 15 different commercially available protein A media were studied regarding equilibrium and dynamic binding capacity using polyclonal IgG. To also assess the selectivity of the media the capture of IgG from crude feed-stock was investigated. The media analysed in the study were made of several different materials, such as crosslinked agarose, polyacrylamide in ceramic macrobeads, porous glass, polystyrenedivenylbenzene, polymeric and polymethacrylate (Table 1) with the mean particle diameter ranging from 50 to 300 μm [45]. Due to the use of polyclonal antibodies in the study, the first plateau in the chromatogram that is caused by early eluted IgG3, which has very low affinity for SPA, was neglected when estimating the DBC. After the initial small scale screening, matrices with highest DBC were selected for further studies with larger columns. In this case, the agarose-based media showed higher DBC along a broader velocity range compared to the smaller columns in the pilot study. For the other materials the DBC was almost identical in the two experiments. The dependence of the DBC on residence time was also analysed for different media, and it was concluded that for residence times longer than 3–5 min, MabSelect and rProtein A Sepharose FF exhibited superior DBS's. These data indicate that the residence time is essential for efficient capture, especially for the agarose based media, and that a residence time of more than 3 min is highly relevant for industrial processes [45].

The selectivity of the gels was investigated by spiking cell culture medium with polyclonal IgG and loading this onto the different sorbents. After extensive washing the bound material was eluted and the sorbents were equilibrated. The regenerated sorbents were boiled with SDS buffer and the supernatant was analysed with SDS-PAGE. The data revealed that some of these gels exhibited strong interactions with other media components and could not be completely regenerated [45].

Further evaluations were focused on the mass transfer properties of new improved protein A-based media [46]. The agarose-based media MabSelect Xtra and MabSelect SuRe (GE Healthcare) as well as the porous glass media ProSep-vA Ultra (Millipore) were included in the study. MabSelect Xtra has been designed to exhibit larger binding capacity while MabSelect SuRe (Superior Resistance) has been designed to withstand alkaline treatment. Focus for the development of ProSep-vA Ultra was increased binding capacity. MabSelect Xtra is an affinity media based on highly cross-linked agarose with higher ligand density, larger pore size but decreased particle size compared to MabSelect. MabSelect SuRe is a highly cross-linked agarose with an optimized ligand. One of the protein A domains has been modified by protein engineering to withstand alkaline conditions, where alkali sensitive amino acids have been substituted with more stable ones, and the final construct is a tetramer of the engineered domain. The ProSep-vA Ultra surface area is increased by reduction of the pore size compared to ProSep-vA High Capacity. Adsorption equilibrium data for the different media were collected, and the results showed that MabSelect Xtra exhibited the highest capacity, about 10% above the three others that also show high binding capacity and low dissociation constants. Since ProSep-vA Ultra has reduced pore size compared to the earlier developed media, ProSep-vA High Capacity, the capacity was higher but the mass transfer velocity was decreased. The conclusion of this study was that all of these newly developed protein A media had comparable dependence of DBC on flow velocity, and at residence times above 4 min a DBC approaching

equilibrium, which are highly desirable properties for designing process affinity chromatography protocols. [46].

A life time study has also been performed by Hahn et al., where the same three new protein A sorbents were investigated [45]. The media were investigated according to the performance in 50 chromatographic cycles. Data regarding ligand leakage, carry-over between batches, HCP content in eluate and IgG binding capacity were collected. Between each cycle, regeneration (strip) and/or CIP were carried out according to the manufacturers' recommendations. Since the material for the different media differ, different CIP and strip protocols were applied. The silica backbone for ProSep-va is very sensitive towards alkaline conditions compared to the sepharose media used for the other two. The results of the study emphasize that the alkaline resistant protein A sorbent exhibits the highest stability with the lowest leakage of the three tested matrices, even though it was regenerated with the highest concentration of NaOH. The HCP content in the eluate was about 10 times higher when using the ProSep-vA Ultra sorbent compared to the agarose-based media. An interesting observation for the MabSelect SuRe media was that the elution profile was sharper than for the other two media, indicating a weaker interaction between the protein A ligand and the antibody under the elution conditions [46]. This weaker interaction has by Ghose et al. been shown to be beneficial for milder elution conditions and thereby decreasing the risk for protein aggregation [49].

6. Concluding remarks

For several years protein purification with affinity chromatography has been widely used in the downstream processing of monoclonal antibodies. The far most employed affinity systems are based on the protein A ligand despite the high costs. Due to an increased requirement for better process economies and higher feedstock titers, new demands are put on the downstream processing of mAb, and especially on the affinity chromatography step. This has led to the development of both improved affinity ligands and improved media.

Alternative ligands have been developed by using structural information for the educated design of triazine based moleules. Moreover, by using combinatorial approaches, such as phage display libraries, specific peptides have been selected with the ability to bind IgG. Some of the acquired peptides have also been shown to function as affinity ligands.

Another strategy used to improve affinity matrices is protein engineering methods for design of enhanced SPA molecules [6]. In a protein engineering study where the most sensitive amino acids were replaced it was possible to create an improved SPA molecule for large-scale applications enabling cleaning-in-place with 0.5 M NaOH, which is a remarkable improvement for a protein ligand.

EBA technology could be beneficial to decrease the number of unit operations in the process. However, due to the crude cell extract feeded directly onto the SPA/EBA matrix, difficulties to clean the matrices are encountered. Therefore, packed bed format are still the most commonly used approach. There are several different suppliers of protein A based matrices, and new improved products have been launched lately. All of these new launched resins, independent of base matrix, have met the new requirements regarding shorter residence time and higher flow rates.

However, features like carry over between batches, ligand leakage, and stability over multiple rounds and residence times are important factors in large-scale purifications and the demands will increase as the products improve. Hence, both ligands and base matrices can hopefully be optimized even further in the future.

References

[1] J. MacLennan, Biotechnology (NY) 13 (1995) 1180.
[2] N.K. Harakas, Bioprocess Technol. 18 (1994) 259.
[3] R.C. Duhamel, P.H. Schur, K. Brendel, E. Meezan, J. Immunol. Methods 31 (1979) 211.
[4] L. Björk, G. Kronvall, Immunol. J. 133 (1984) 969.
[5] S. Gülich, M. Linhult, S. Ståhl, S. Hober, Protein Eng. 15 (2002) 835.
[6] M. Linhult, S. Gülich, T. Gräslund, A. Simon, M. Karlsson, A. Sjöberg, K. Nord, S. Hober, Proteins 55 (2004) 407.
[7] R. Li, V. Dowd, D.J. Stewart, S.J. Burton, C.R. Lowe, Nat. Biotechnol. 16 (1998) 190.
[8] D.D. Richman, P.H. Cleveland, M.N. Oxman, K.M. Johnson, J. Immunol. 128 (1982) 2300.
[9] Amersham Pharmacia Biotech, Handbook, Antibody Purification, 2000.
[10] M. Uhlén, B. Guss, B. Nilsson, S. Gatenbeck, L. Philipson, M. Lindberg, J. Biol. Chem. 259 (1984) 1695.
[11] L. Abrahmsen, T. Moks, B. Nilsson, U. Hellman, M. Uhlén, EMBO J. 4 (1985) 3901.
[12] T. Moks, L. Abrahmsen, B. Nilsson, U. Hellman, J. Sjöquist, M. Uhlén, Eur. J. Biochem. 156 (1986) 637.
[13] B. Guss, M. Uhlén, B. Nilsson, M. Lindberg, J. Sjöquist, J. Sjödahl, Eur. J. Biochem. 138 (1984) 413.
[14] B. Nilsson, T. Moks, B. Jansson, L. Abrahmsen, A. Elmblad, E. Holmgren, C. Henrichson, T.A. Jones, M. Uhlén, Protein Eng. 1 (1987) 107.
[15] L. Jendeberg, P. Nilsson, A. Larsson, P. Denker, M. Uhlén, B. Nilsson, P.Å. Nygren, J. Immunol. Methods 201 (1997) 25.
[16] B. Jansson, M. Uhlén, P.Å. Nygren, FEMS Immunol. Med. Microbiol. 20 (1998) 69.
[17] J. Deisenhofer, Biochemistry 20 (1981) 2361.
[18] M. Graille, E.A. Stura, A.L. Corper, B.J. Sutton, M.J. Taussig, J.B. Charbonnier, G.J. Silverman, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 5399.
[19] C. Ljungquist, B. Jansson, T. Moks, M. Uhlén, Eur. J. Biochem. 186 (1989) 557.
[20] K. Nord, E. Gunneriusson, J. Ringdahl, S. Ståhl, M. Uhlén, P.Å. Nygren, Nat. Biotechnol. 15 (1997) 772.
[21] S. Gülich, M. Uhlén, S. Hober, J. Biotechnol. 76 (2000) 233.
[22] M. Linhult, S. Gülich, T. Gräslund, P.Å. Nygren, S. Hober, Protein Eng. 16 (2003) 1147.
[23] M. Asplund, M. Ramberg, B. Johansson, Proc. Biochem. 35 (2000) 1111.
[24] P. Girot, Y. Moroux, X.P. Duteil, C. Nguyen, E. Boschetti, J. Chromatogr. 510 (1990) 213.
[25] R.F. Burgoyne, M.C. Priest, K.L. Roche, G. Vella, J. Pharm. Biomed. Anal. 11 (1993) 1317.
[26] A.A. Kosky, U.O. Razzaq, M.J. Treuheit, D.N. Brems, Protein Sci. 8 (1999) 2519.
[27] A.A. Kossiakoff, Science 240 (1988) 191.
[28] R. Lura, V. Schirch, Biochemistry 27 (1988) 7671.
[29] T. Geiger, S. Clarke, J. Biol. Chem. 262 (1987) 785.

[30] P. Bellofiore, F. Petronzelli, T. De Martino, O. Minenkova, V. Bombardi, A.M. Anastasi, R. Lindstedt, F. Felici, R. De Santis, A. Verdoliva, J. Chromatogr. A. 1107 (2006) 182.
[31] S.A. Camperi, N.B. Iannucci, G.J. Albanesi, M. Oggero Eberhardt, M. Etcheverrigaray, A. Messeguer, F. Albericio, O. Cascone, Biotechnol. Lett. 25 (2003) 1545.
[32] G.K. Ehrlich, P. Bailon, J. Biochem. Biophys. Methods 49 (2001) 443.
[33] G.K. Ehrlich, P. Bailon, J. Mol. Recognit. 11 (1998) 121.
[34] G. Fassina, A. Verdoliva, G. Palombo, M. Ruvo, G. Cassani, J. Mol. Recognit. 11 (1998) 128.
[35] A. Verdoliva, F. Pannone, M. Rossi, S. Catello, V. Manfredi, J. Immunol. Methods 271 (2002) 77.
[36] G. Palombo, S. De Falco, M. Tortora, G. Cassani, G. Fassina, J. Mol. Recognit. 11 (1998) 243.
[37] G. Palombo, M. Rossi, G. Cassani, G. Fassina, J. Mol. Recognit. 11 (1998) 247.
[38] Y.D. Clonis, N.E. Labrou, V.P. Kotsira, C. Mazitsos, S. Melissis, G. Gogolas, J. Chromatogr. A 891 (2000) 33.
[39] S.F. Teng, K. Sproule, A. Husain, C.R. Lowe, J. Chromatogr. B Biomed. Sci. Appl. 740 (2000) 1.
[40] A.R. Newcombe, C. Cresswell, S. Davies, K. Watson, G. Harris, K. Donovan, R. Francis, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 814 (2005) 109.
[41] A. Jungbauer, R. Hahn, Curr. Opin. Drug Discov. Dev. 7 (2004) 248.
[42] G. Jagshines, A. Grönberg, T. Björkman, K. Lacki, H.J. Johansson, Biopharm. Int. (June 2006).
[43] G.S. Blank, G. Zapata, R. Fahrner, M. Milton, C. Yedinak, H. Knudsen, C. Schmelzer, Bioseparation 10 (2001) 65.
[44] R.L. Fahrner, D.H. Whitney, M. Vanderlaan, G.S. Blank, Biotechnol. Appl. Biochem. 30 (Pt 2) (1999) 121.
[45] R. Hahn, R. Schlegel, A. Jungbauer, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 790 (2003) 35.
[46] R. Hahn, P. Bauerhansl, K. Shimahara, C. Wizniewski, A. Tscheliessnig, A. Jungbauer, J. Chromatogr. A 1093 (2005) 98.
[47] R. Hahn, K. Shimahara, F. Steindl, A. Jungbauer, J. Chromatogr. A 1102 (2006) 224.
[48] P. Füglistaller, J. Immunol. Methods 124 (1989) 171.
[49] S. Ghose, M. Allen, B. Hubbard, C. Brooke, S.M. Cramer, Biotechnol. Bioeng. 6 (2005) 665.