Potential for Using Histidine Tags in Purification of Proteins at Large Scale

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Attachment of oligo-histidine tag (His-tag) to the protein N- or C-terminus is a good example of early and successful protein engineering to design a unique and generalized purification scheme for virtually any protein. Thus relatively strong and specific binding of His-tagged protein is achieved on an Immobilized Metal-Ion Affinity Chromatography (IMAC) matrix. Most popular hexa-histidine tag and recently also deca-histidine tag are used in combination with three chelating molecules: iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), and carboxymethyl aspartic acid (CM-Asp), covalently attached to the chromatographic matrix. The following combinations with divalent metal ions are preferentially used: (Cu, Zn, Ni, Co)-IDA, Ni-NTA, and Co-CM-Asp. At large scale, regarding cost and product purity, a decisive step is precise and efficient cleavage of His-tag by the cleavage enzyme. Two-step IMAC followed by a polishing step appears to be a minimum but still realistic as an approach to generic technology also for more demanding products. Possible drawbacks in using His-tags and IMAC, such as leaching of metal ions, inefficient cleavage, and batch-to-batch reproducibility must be carefully evaluated before transferred to large scale. Although a great majority of reports refer to small laboratory scale isolations for research purposes it appears there is much higher potential for more extensive use of His-tags and IMAC at large scale than currently documented.

1 Introduction

By genetic manipulations it is easy to fuse an oligo-histidine tag to the N- or C-terminus of any protein to be produced in recombinant microorganisms at laboratory or large scale. The tag should simplify purification and reduce the number of chromatographic steps. Consequently, this should significantly increase the final yield and substantially reduce the investment and production costs.

In research reports it is often quoted that a particular protein is purified in His-tagged form in a single purification step. Although this statement might be true in the majority of cases, this information is of little use when the experimental data on tag removal, accuracy of the cleavage and final yield are not provided.

Comprehensive information on various aspects of using histidine tags and IMAC have been summarized in recent review articles [1–4]. By all means, usage of histidine tags has really become widespread for purification of recombinant proteins but in this review we would like to evaluate the potential for large-scale protein purifications. Additionally, we would also want to stress that the final step, tag removal, is really a critical one and must be carefully evaluated in assessing the applicability of histidine tags in large industrial purification schemes.

2 Using IMAC as an Efficient Isolation Technique

IMAC, nowadays widely used for quick and effective isolation of recombinant proteins in almost every research laboratory, dates back to the seventies. In 1975 Porath [5] and coworkers introduced a new approach to protein fractionation based on differential affinity and respectively differential binding properties of certain amino acid residues to immobilized metal ions and called it Metal Chelate Affinity Chromatography. The method was introduced as a group-specific affinity separation and became popular through the research work of Porath [5, 6] and Sulkowski [7,8]. Hundreds of papers have since been published describing the method as highly selective purification tool for isolating target proteins from complex biological mixtures, serving for preparative and analytical purposes. In the very beginning, IMAC techniques were used for separating proteins and peptides with naturally present exposed histidines. But at the end of the eighties, with the work of Hochuli [9, 10], who introduced the engineered histidine affinity handles and a special matrix (Ni(II)-nitrilotriacetic acid commonly known as Ni-NTA) for selective binding of numerous adjacent histidines, the new era for efficient purification of recombinant proteins began. Stretches of several neighboring histidine residues are quite uncommon among naturally occurring proteins, therefore, the engineered proteins with oligo-histidine affinity handles usually called histidine tags differ significantly from the majority of the proteins in biological samples, and due to high affinity for IMAC matrices they can be easily and selectively purified by the relatively simple isolation procedures.

In IMAC the adsorption of proteins is achieved through coordination among immobilized metal ions serving as affinity ligands and electron donor groups from the protein surface. Metal ions are immobilized by coordinative binding to the chelating molecules that are covalently bound to the chromatographic support. Most commonly used in IMAC are the intermediate metal ions such as Cu(II), Ni(II), Zn(II), and Co(II), which can coordinate nitrogen, oxygen

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and sulfur. Although the amino terminus of the protein and numerous amino acid residues, such as Glu, Asp, Tyr, Cys, His, Arg, Lys, and Met can participate in binding, the actual protein retention depends mostly on the availability of surface exposed histidine and cysteine residues. The latter are rarely present in the reduced state on the protein surface, which leaves histidines as the major targets. Aromatic side chains of Trp, Phe and Tyr, when positioned in the vicinity of histidine residues, somehow contribute to binding [8, 11].

The chromatographic scheme depends on the type of metal affinity ligand used. With hard metal ions, protein binding via carboxylic groups, tyrosine or phosphorylated side chains of serine, threonine and tyrosine is performed at acidic or neutral pH while the elution is achieved by rising pH gradients or addition of competitive substances, such as organic acids or phosphate. On the contrary, with intermediate metal ions (Cu(II), Ni(II), Zn(II), and Co(II)), adsorption to chromatographic support is accomplished at pH values supporting the imidazole nitrogens of histidine residues in the non-protonated form, usually in neutral or slightly basic medium. Relatively high-ionic strength buffers containing from 0.1 up to 1.0 M NaCl are usually employed with the aim of reducing unspecific electrostatic interactions with the matrix and restricting the binding to metal coordination. Elution is accomplished either by protonation, using buffers with lower pH or reducing pH gradients, or by ligand exchange with the competitive molecules, such as imidazole or ammonium salts. Strong chelating agents, such as EDTA can also be used but with this elution mode the metal ions are completely removed and binding capacity of the column is lost. To achieve higher selectivity for the target protein, screening of various parameters, e.g., variation of metal ions, usage of different supports with various chelating compounds and different spacer arms is usually performed. Even ligand density can be used to influence the separation efficiency [12]. Although many different chelators are described in research papers, the commercially available matrices usually employ a tridentate chelator iminodiacetic acid (IDA), tetradentate chelators nitrilotriacetic acid (NTA) and carboxymethylated aspartic acid (CM-Asp). Tridentate or tetradentate refer to the number of occupied coordination bonds formed between the metal ion and surface immobilized chelating compound, while the remaining coordination sites in the octahedral arrangement around a divalent metal ion are normally occupied by water molecules and can be exchanged with appropriate electron donor groups from the protein. Consecutively, in tetradentate chelators metal ions are more firmly bound and less leakage occurs but tridentate chelators display higher binding affinities for proteins. The structure of IDA, the mostly used chelator in IMAC is depicted in Fig. 1. While IDA matrices are available by numerous producers and can be charged with various metal ions, Ni-NTA of Qiagen is usually used with Ni(II) and CM-Asp known under the trade name of TALON (Clontech) with Co(II) ions.

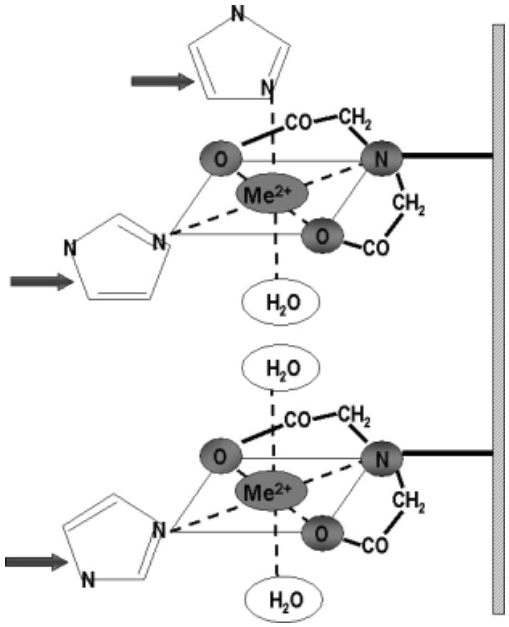

Figure 1. Putative structures of octahedral complexes formed around metal ion (Me^{2+}) bound to the iminodiacetic acid (IDA), the most frequently used chelator in large scale IMAC. Covalent attachment of IDA to the chromatographic matrix can be achieved through spacers of various lengths and chemistries. Often used metal ions are Zn (II), Cu(II), Ni(II), Co(II). Histidine ligands, donored by the protein or its polyhistidine tag, are shown in complex with two neighboring Me-chelated IDA molecules. Arrows point to imidazoles, arising from side chains of histidines coming from His-tag fused to the target protein. Although flexible histidine tag can acquire numerous conformations, most probably only one or two consecutive histidines from the same tag can co-operatively interact in the formation of the complex. Other spaces are occupied with water molecules as shown in the figure or with other molecules bearing electron donor groups, e.g., buffer components or competitive molecules like imidazole added in order to elute the attached His-tagged protein. By using His-tags of various lengths one can modulate the affinity, however, most popular His-tags are designed within the range of six to twelve consecutive histidines.

3 Choosing the Proper His-Tag Attachment Site

In general, proteins are chemically and structurally diverse molecules, which make them unsuitable for generic approaches in purification methodology. On the other hand, especially in large-scale industrial purification schemes, generic approaches would be most favorable as an important means to achieve quick and cost-effective production of recombinant proteins. For this purpose, genetically engineered affinity tags with the possibility for precise and efficient tag removal could play an important role. Among numerous affinity tags commonly used today, histidine tags offer many advantages for scale-up applications, such as low metabolic burden, inexpensive affinity matrices, mild elution conditions, etc. [13].

First purifications of proteins by IMAC were carried out with proteins containing native-surface exposed histidine residues in appropriate proximity and orientation to enable multi-point attachment to immobilized metal ions that is a prerequisite for high affinity [14]. However, multi-point attachment can be much more efficient when the proteins are

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appropriately genetically engineered to contain long stretches of consecutive histidines or surface patches rich in histidine residues.

Histidine affinity tags similarly to other fusions used for purposes of rapid protein isolation, can be attached either to the amino or carboxyl terminus of the polypeptide chain. Which position is better, depends on the specific protein and its intended usage and is usually determined experimentally. If the protein part close to the N- or C- terminus is involved in interaction with other proteins such as specific receptors or antibodies and takes part in its biological function, the addition of the histidine tag to this end will most probably interfere with biological activity. Similarly, attachment of the histidine tag makes no sense if the chosen terminus is hidden in the protein interior. For instance, the carboxyl terminus of the tumor necrosis factor alpha (TNF) is not surface accessible and additionally this region is very susceptible to any change in the primary amino acid sequence. Only one example of the histidine tag attached to the C-terminus of TNF was reported, which resulted in insoluble and inactive protein [15]. On the other hand, the N-termini of the trimeric TNF molecule are surface exposed and completely flexible, which makes them suitable for histidine tagging. Besides commercially available His6, His10, and oligo-histidine tag from TAGZyme system (see Tab. 2), various non-commercial tags, all attached to the N-termini, were tried for TNF purifications [16]. With all tags purification in a single step to over 95 % purity was possible but good chromatographic features with sharp chromatographic peaks were not obtained. This could be achieved with TNF analogs bearing genetically engineered histidine clusters, composed of three or six histidine residues in the tip of the trimeric TNF molecule [16, 17]. Surface clusters were formed by introducing one or two histidines into the flexible loop of each TNF subunit. Such histidine-rich surface clusters represent another example how to design proteins for efficient IMAC purification or immobilization. In a similar way, in glutathione-transferase a metal coordination site forming a four-histidine motif on the surface of the dimeric protein was constructed [18]. Based on the knowledge about protein 3D structure and active sites, such histidine-rich surface motifs can be very attractive and useful but it has to be borne in mind that proteins with newly introduced histidine clusters are mutants differing from the authentic protein structure. Due to eventual differences in biological activity, immunogenicity and stability, engineered histidine motifs cannot be used for purification of therapeutic proteins. On the other hand, they are widely applicable for research, industrial enzymes and proteins, which are used for diagnostic purposes.

4 His-Tag Removal and Concept for Two-Step Isolation

Introduction of histidine residues into the protein structure, either by numerous histidine residues attached to the protein termini or inside the amino acid sequence with the aim of constructing surface histidine cluster, has often been reported not to compromise the biological properties of proteins. Even some applications of histidine-tagged proteins in clinical trials have been reported [19–22]. Furthermore, in the preclinical studies, a TNF-alpha mutant with engineered histidine patch exhibited lower systemic toxicity than the natural TNF-alpha [23]. The usage of histidine tagged proteins is still much broader in the research, e.g., in structure and structure-reactivity studies, in structural genomics, rapid screening of novel mutant proteins, determination of protein localization inside the organelles, etc. With widely commercially available expression plasmids containing the sequences for His-tags, the usage of His-tagged proteins and IMAC has become a routine for quick and simple isolation of recombinant proteins in almost every research laboratory. On the other hand, there are more and more reports in the literature describing to a greater or lesser extent altered biological or physicochemical properties of the histidine tagged proteins as compared to the natural untagged counterparts [24–31].

Doubtlessly, in the case of pharmaceutical-grade proteins the authentic protein structure is usually required and histidine tags have to be removed after the separation has been accomplished. Chemical cleavage under relatively harsh conditions is usually avoided, instead various proteolytic enzymes are broadly used. Historically, natural proteases capable of recognizing specific amino acid sequences, such as thrombin, coagulation factor Xa, enterokinase and carboxypeptidase were among the first enzymes used for cleavage of fusion proteins with appropriately engineered recognition sites. However, even proteolytic enzymes recognizing long and rarely occurring recognition sequences, such as AspAspAspAspLys in the case of enterokinase, are not ideal and can lead to nonspecific cleavage resulting into micro-heterogeneity and loss of protein [2, 32, 33]. Similarly, cleavage by trypsin-like proteases, thrombin and factor Xa, can often result in significant non-specific proteolysis [34].

Efficient removal of proteolytic enzymes after the cleavage is even of greater concern. When high purity of the protein is requested, the often quoted “single-step” IMAC isolation is in reality transformed into a sequence of several steps usually accompanied by much lower yields. Tab. 1 shows some commercially available proteolytic enzymes, however, for high purity of the final protein, recombinant cleavage enzymes containing uncleavable His-tags by themselves, are most appropriate.

Single-step IMAC purifications do not assure sufficient purity for high quality requested in biopharmaceutical proteins. Based on histidine-tagged recombinant cleavage enzymes we envisage as much more realistic a minimum core purification scheme involving two IMAC steps (see Fig. 2). Under appropriately chosen conditions, in the first IMAC-1 step contaminants are efficiently removed and the His-tagged protein isolated with a high purity. After enzymatic cleavage, the protein mixture is subjected to the second chromatographic step, IMAC-2, which strongly binds His-tags, His-tagged cleavage enzyme(s), the remaining non-processed target protein and eventual non-specifically bound host

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Table 1. Some commercially available cleavage enzymes.

Cleavage enzyme	Protease source	Recognition site	Protease removal	Manufacturers
Factor Xa	Natural	Ile-Glu/Asp-Gly-Arg▼	Benzamidine-Agarose	Amersham Biosciences, New England Biolabs, Roche
Thrombin	Natural	Leu-Val-Pro-Arg▼ Gly-Ser	Benzamidine-Agarose; Streptavidine agarose for biotinylated enzyme of Novagen	Amersham Biosciences, Novagen, SIGMA, Roche
PreScission	Recombinant GST fusion enzyme	Leu-Glu-Val-Leu-Phe-Gln▼ Gly-Pro	GSTrap	Amersham Biosciences
Enterokinase	Recombinant or natural	Asp-Asp-Asp-Asp-Lys▼	Trypsin Inhibitor-Agarose	New England Biolabs (rec), Novagen (rec), Roche (calf intestine)
TEV protease	Recombinant His6-enzyme	Glu-Asn-Leu-Tyr-Phe-Gln▼ Gly	IMAC (Ni-NTA, HisTrap)	Invitrogen - Life Technologies, Amersham Biosciences
TAGZyme	Recombinant His6-enzymes	Exoproteolytic digestion	IMAC (Ni-NTA)	Qiagen
HRV 3C Protease	Recombinant His6-enzyme	Leu-Glu-Val-Leu-Phe-Gln▼ Gly-Pro	IMAC (Ni-NTA)	Novagen
SUMO Protease	Recombinant His6-enzyme	Tertiary structure of the ubiquitin-like (UBL) protein (SUMO)	IMAC (Ni-NTA)	Invitrogen - Life Technologies, LifeSensors

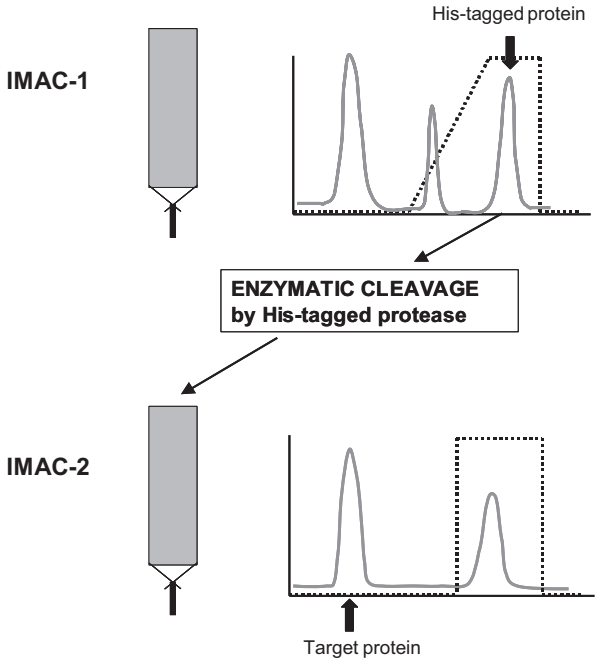

Figure 2. Schematic presentation of a two-step IMAC purification involving histidine tag removal by a recombinant His-tagged cleavage enzyme. The third, in general optional polishing step is not shown, although for high purity pharmaceutical proteins it is regularly used for final removal of aggregates formed during the downstream processing.

contaminants co-eluted during the first step. In contrast, the target protein, now untagged, is obtained in the contaminant-free window, usually in the flow-through fraction of the IMAC-2 step. This concept of two-step IMAC isolation is widely applicable, from research and high-throughput purposes on the small scale to large-scale industrial purifications.

For instance, in the TAGZyme system the efficient and precise exoproteolytic removal of histidine tags is achieved by histidine-tagged dipeptidyl-aminopeptidase I (DAPase) alone or in combination with glutamine-cyclotransferase (Qcyclase) and pyroglutamylaminopeptidase (pGAPase) [35]. In this case the protein must contain a specific stop point amino acid at the appropriate site, which might differ from the authentic structure of the target protein. In addition, in the final product the absence of all cleavage enzymes must be proven and documented.

According to published data as well as our own experiences the decisive steps determining the economy of the purification process are the precise and efficient enzymatic cleavage and final isolation of really pure, contaminant-free target protein. The role of cleavage efficiency of two widely used enzymes, enterokinase and DAPase, with an emphasis on oligomeric proteins, has been thoroughly discussed in a recent paper [32] showing that progressively reduced final yields can be expected with increasing degree of oligomerization. Thus, when using histidine tags with oligomeric proteins, a special care is recommended to avoid significant product loss by carefully optimizing cleavage conditions, e.g., buffer composition, reaction time, and temperature, the amount of cleavage enzyme, etc.

For small scale, the above outlined approach of two-step IMAC has still been further elaborated resulting into the automated tag removal ÄKTAxpress system [36]. After the capture step, the on-column cleavage of the His-tagged protein is performed by dispensing the AcTEV protease solution onto the IMAC column to allow the cleavage reaction to proceed. After the incubation, the cleaved, untagged protein is recovered first, while the affinity tags, His-tagged cleavage enzyme and the remaining uncleaved protein are

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eluted during column regeneration. With ÄKTAxpress system further polishing steps are usually required.

Most recently quite interesting self-cleavable fusion construct containing several structural elements: His6-Cleavage enzyme sortase-Recognition sequence LPXTG-Target protein has been reported [37]. Isolation of His-tagged fusion protein is followed by on-column self-cleavage induced by Ca(II) ions and/or triglycine, however, an extra glycine remains attached to the N-terminus of the target protein. This approach is claimed to be simple, robust and inexpensive thus leading closer to really single-step purification, however, its general applicability for a range of proteins and for large scale use has to be confirmed.

5 Recombinant Proteins Purified by His-Tags at Industrial Scale

5.1 Chromatographic Media for Large-scale Applications

Ideal features of a chromatographic medium for efficient protein separation are high selectivity, high binding capacity, high mass transfer, low unspecific adsorption, incompressibility, chemical stability of matrix and immobilized ligands, non-toxic leachables as well as capability of withstanding many operation and sanitization cycles [38]. For industrial production of biopharmaceuticals sanitization by sodium hydroxide is usually mandatory. When above technical criteria are generally fulfilled, the medium cost plays the most important role in large-scale application.

Classical stationary phases for protein separations are based on biologically widely compatible polysaccharides, such as agarose or cross-linked dextran, which are easily activated and have been widely used for small-scale applications. Nevertheless, these soft-gel matrices possess low mechanical strength and high-pressure drop, which both make them less attractive for large-scale separations. Inorganic adsorbents, such as silica, and synthetic polymers exhibit much better mechanical properties but, on the other side, they can lead to irreversible non-specific adsorption. Due to their relative hydrophobicity, the surface must be usually coated with a hydrophilic polymer. For example, POROS MC (Applied Biosystems) media consist of cross-linked poly(styrene-divinylbenzene) flow-through particles with bimodal pore size distribution for very rapid mass transport. The particles are surface-coated with a cross-linked polyhydroxylated polymer functionalized with imidodiacetate groups. Very high flow rates are the main advantage of this type of perfusion chromatography resulting in high throughput and processing capacity when scaling up (see Tab. 2).

Table 2. Some commercially available IMAC chromatographic media for large-scale purifications.

Trade name	Manufacturer	Matrix structure	Bead size/ distribution	Chelating compound	Applicability	Linear velocity (cm/h)	Max. operating pressure
Chelating Sepharose Fast Flow	Amersham/General Electrics Health Care	Highly cross-linked 6 % agarose	45–165 μm, mean 90 μm	IDA	Industrial production of marketed biopharmaceuticals	200–400	0.3 MPa
Chelating Sepharose Big Beads	Amersham/General Electrics Health Care	Highly cross- linked 6 % agarose	100–300 μm	IDA	Capture of target molecules in packed bed chromatography	1200–1800	0.3 MPa
Streamline Chelating	Amersham/General Electrics Health Care	Macroporous cross-linked 6 % agarose with crystalline quarz core	100–300 μm	IDA	Capture of target molecules in expanded bed adsorption	200–300	0.1 MPa
BD Talon Cell Thru	BD Biosciences	Cross-linked 4 % agarose (Uniflow)	300–500 μm	TALON (carboxymethyl aspartate)	Preparative	Up to 800	0.06 MPa
BD Talon Superflow	BD Biosciences	Cross-linked 6 % agarose	60–160 μm	TALON (carboxymethyl aspartate)	Production	Up to 3000	0.97 MPa
Profinity IMAC	Bio-Rad	UNOsphere polymeric support	Mean 60 μm	IDA	Process separation	800–2000	0,69 MPa
Prosep-Chelating	Millipore	Controlled pore glass	74–125 μm	IDA	Process separation	Up to 1000	20 MPa
Toyopearl AF-chelate-650M	Tosoh Bioscience	Copolymer of ethylene glycol and methacrylate polymers	40–90 μm	IDA	Laboratory to process scale	60–600	0.3 MPa
Ni-NTA Superflow	Qiagen	Highly cross-linked linked 6 % agarose	60–160 μm	NTA	Small to production scale	Up to 3000	0,97 MPa
POROS® 50 MC	Applied Biosystems	Cross-linked poly(styrene-divinylbenzene)	50 μm	IDA	Preparative and production scale	1000	10 MPa
Sartobind® Metal Chelate Membrane Adsorbers	Sartorius	Stabilized reinforced cellulose	–	IDA	Small to production scale	> 4800	0.6 MPa

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Combination of polysaccharide on inorganic beads is used for expanded-bed adsorption (EBA) technique enabling the recovery of proteins directly from unclarified mammalian cell suspensions and bacterial cell homogenates. By applying an upward reverse flow of the crude initial suspension, the bed is expanded allowing the cell debris and eventual whole cells to flow through, while the protein of interest is adsorbed to the chromatographic support and later eluted in the normal flow. Application of this technique reduces the number of steps and thus decreases process time, which makes it especially appropriate for scaling up and industrial use. A Streamline Chelating adsorbent was found suitable for pilot-scale capture of proteins allowing processing of 100 L cell broth in a single run [39].

Glass, most commonly used as controlled-pore glass, also exhibits excellent flow properties. In the Millipore series ProSep the adsorbent with immobilized iminodiacetic acid (IDA) is typically applicable for large-scale separations (see Tab. 1).

Immobilized Metal Affinity Membrane Adsorbers (IMA-MA) represent an interesting alternative to conventional chromatographic media with porous particles packed into columns. In classical chromatographic media biomolecules typically diffuse into the nanometer pores of the particles, which represents the rate-limiting factor of the process. In contrast, the micro-porous membranes are characterized by the convective flow and less diffusion limitations, enabling high flow rates, high dynamic binding capacity and very short separation times. Membrane type adsorbers are based on organic natural or synthetic polymers, such as various forms of cellulose, hydrocarbon polymers, aromatic copolymers, etc. Most of the membranes reported in the literature employ IDA as the chelating compound [4]. Although metal affinity membrane adsorbers are available from various producers, they are mostly designed for small-scale or high-throughput isolations of recombinant proteins for research or screening purposes. However, Sartobind-IDA from Sartorius is also available in form of Sartobind MultiSep reusable modules, which withstand harsh cleaning procedures and allow scaling up to the production format.

IDA-derivatized hollow-fibre polysulfone membranes are reported as better compared to flat-sheet membranes allowing easy scale-up and processing of particle containing solutions [40].

Regarding new approaches one should also mention Convective Interaction Media monolithic discs (CIM discs) from BIASeparations, reported to enable immobilization of IDA [41], although the more elaborated larger scale modules are available for ionic and hydrophobic chromatography [38,42]. Currently the applicability of CIM disc monolythic columns for large scale cannot be yet fully assessed, however, good chemical stability, high resolution and especially low backpressure at high flow rates make this material promising for rapid purification of proteins especially when short residential time is needed.

5.2 Purification of Therapeutically Relevant Proteins

In the literature numerous reports on IMAC used for purifying pharmaceutically and therapeutically interesting proteins can be found. Typical examples are isolations of various types of antibodies or their fragments [43–45], purifications of interferons [46], albumin [47], protein C [48–50], Factor IX (US 6,627,737 B1), etc.

Although above cited papers deal with IMAC purifications of therapeutically relevant proteins, they all describe natural proteins, which are capable of coordinative binding to the chelated metal ions due to the presence and appropriate arrangement of naturally present histidine residues. For efficient isolation of recombinant proteins histidine tags offer numerous advantages but in the case of pharmaceutically useful proteins, the authentic structure is usually compulsory, therefore, histidine tags if employed for purification purposes must be removed for final usage. Typical example is a high-level production of human growth hormone in the form of a fusion protein with a His10-tag and the enterokinase recognition site included to enable the enzymatic cleavage and thus generation of the authentic structure [51]. On the other hand, histidine-tagged antibodies as such, without tag removal, have been described as useful for diagnostic purposes [52] and for cost-effective production of biosensors, in which histidine tags serve for oriented immobilization [53]. Purification of His6-scFv-antibody fragments was accomplished by metal chelate affinity precipitation with Cu(II) and Ni(II) copolymers of vinylimidazole and N-isopropylacrylamide [54]. Histidine-tagged recombinant vaccines represent a broad area of modern therapeutically relevant proteins, which can be often used without tag removal. A typical example is a malaria-transmission-blocking vaccine candidate, based on the *Plasmodium falciparum* predominant surface protein Pfs25. The protein with a His6-tag attached to the carboxyl terminus was produced by secretion from *Seromyces cerevisiae* and purified on large scale by Ni-NTA [20]. Recombinant vaccine candidates yielding protection against *Pseudomonas aeruginosa* infections have also been described, with histidine tags added to the N- or C- terminus [22]. Recombinant enteropathogenic *Escherichia coli* vaccine candidates BfpA and EspB were overexpressed as N-terminally tagged His6 proteins and purified by Ni-agarose affinity chromatography [55]. The bacterial superantigen staphylococcal enterotoxin A (SEA), a potent inducer of CTL activity and cytokine production and as such interesting for cancer therapy, was expressed in the form of a transmembrane fusion protein and purified by IMAC due to the presence of a histidine tag [56]. VP8* fragment of the VP4 protein from bovine rotavirus with a His7-tag on the C-terminus was produced in the plant *Nicotiana bethamiana*, purified by IMAC and tested on mice [57]. Furthermore, the chimeric rVP2H protein bearing a His6-tag at the C-terminus, a vaccine candidate for the prevention of infectious bursal disease [58], was expressed in insect larvae in the form of virus-like particles and affinity purified.

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In spite of a vast number of reports on histidine-tagged proteins, in recent reviews on the process chromatography [59] as well as on the industrial application of affinity ligands [60], the large scale application of histidine tags and IMAC has only been superficially mentioned.

In general, from the literature data it is difficult to judge whereas the usage of the particular histidine tagged protein is only of scientific relevance or it has application and commercialization capabilities. It is inevitable that purification tags will be broadly accepted for protein products in the future [1]. Market pressure for shorter product release times and economical production will force the companies to introduce effective strategies enabling high purity and high recovery of the target proteins. In comparison to other affinity tags added for purification purposes, the usage of relatively short histidine tags and cheap IMAC columns that are easily regenerated and withstand numerous production cycles, is particularly advantageous. Many applications of histidine tags disclosed in the patent literature support these expectations. Some examples are: production of the thermostable Taq polymerase fragment in the form of the histidine tagged polypeptide with a factor X protease cleavage site (EP 1 507 002 A2), preparation of the vaccine for treatment of prostate tumors in the histidine-tagged form (US 2003/0143240 A1) or recombinant papilloma virus vaccine with a His6 C-terminal tag (US 6,342,224 B1), preparation of His-tagged intimin for stimulating the immune response (US 2002/0006407 A1) or production of thrombin inhibitory agents with His6 and His5 carboxyl terminal extensions (US 6,207,419 B1).

6 Drawbacks and Obstacles in Transferring IMAC to an Industrial Scale

Although drawbacks and obstacles listed below refer equally to small laboratory and large industrial scale their potential influence related to process economy, Good Manufacturing Practice (GMP) and regulatory requests (e.g., in the case of biopharmaceuticals) should be carefully analyzed and taken into account before transferred to pilot and large industrial scale. Some parameters, for instance, inefficient cleavage and cost of cleavage enzyme, leaching of metal ions and batch-to-batch reproducibility, not so important at the laboratory scale, may become decisive at large scale.

Toxicity of Ni(II)

Within the group of divalent cations most often used (Zn, Cu, Ni, Co), nickel is the most toxic [61, 62]. Although for Ni-NTA leaching of Ni(II) is stated to be low, usually up to 1 ppm [63], large amount of this metal released into the waste waters during column regeneration and recharging, should be taken into account in large scale isolations.

Batch-to-batch Reproducibility

Due to already discussed leaching of metal ions, as well as other undesired effects, for instance deposition of non-specifically bound or denatured proteins, the reproducibility is usually more critical in comparison to some other, e.g., ionic matrices. Using only 50–75 % of the full column capacity it is often possible to perform several consecutive runs without a column regeneration cycle and still having the required resolution. However, when using harsher elution conditions, e.g., low pH gradient or high concentration imidazole gradient, a regeneration cycle might be needed after each run to obtain reproducible results.

Patents

We cannot really assess the possible influence of valid patents on exploitation of His-tags for commercial large-scale applications. The basic patent US 4,569,794 known as "His-Tag Patent" already expired (February 2003), however, for some other non-US patents the expiration date would be November 2005. Therefore, we may expect further expansion of using His-tags when patent(s) comprising also the most popular hexa-histidine tag will be fully expired.

7 Potential for Using His-tags and IMAC at Large Scale

Optimization of downstream processing costs including the investment costs will be a driving force for further reduction of number of chromatographic steps. On the other hand the widespread opinion is that regulatory and registration authorities, e.g., in the case of biopharmaceuticals, would accept three chromatographic steps, but not less, as reliable, being well on the safe side. Thus the progressive cost reduction trend in most demanding pharmaceutical processes is in conflict with the regulatory requests, which are more conservative by definition and by practical implications. This might be one of very important obstacles in moving towards two-step or even single-step purification schemes using, not only His-tag/IMAC, but any other Affinity-handle/ Affinity column combination.

Reduction of the process validation costs and time is another driving force aiming at standardization and creation of a generic downstream process composed of fixed number, type and sequence of chromatographic columns to be used for several or even many different proteins. Currently, this seems to be possible only within the family of very similar proteins, such as monoclonal antibodies. However, a generalized approach would be exactly what His-tag/IMAC combination offers - a standardized procedure for purification of proteins otherwise differing significantly in physical and chemical properties. The exploration of the full potential for

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using His-tag/IMAC as a standardized chromatographic step at large scale is only at its beginning. Another possibility not yet fully explored at large scale is coupling the processes of renaturation and purification for proteins produced in *E. coli* as inactive aggregates in the form of inclusion bodies. His-tagged proteins can be selectively bound to IMAC column directly from the denaturation buffers used for solubilization of inclusion bodies. Renaturation is performed on the same column by gradually removing the denaturant [64, 65]. In some cases better refolding yield is achieved in comparison to classical renaturation by dilution.

Finally we have to mention also the integration of upstream and downstream processes using expanded bed adsorption (EBA) techniques [66, 67]. His-tagged proteins and IMAC matrices specially designed for EBA seem to ideally fit this concept. Another type of integrated purification and subsequent usage of immobilized His-tagged enzyme, e.g., D-hydantoinase [68], is a good example for industrial enzymes applied in biotransformation where a robust and cost effective process is requested. An interesting integrated process for capture and purification of His6-tagged antibody fragments from undiluted and unclarified *E. coli* culture cell broth has also been described using mechanically stable supermacroporous cryogels with Cu(II) charged IDA [69]. The process is claimed to be scalable and represents an alternative to expanded bed adsorption technique.

Based on the review presented we conclude there is a huge amount of experimental data at the laboratory scale that will help developing also large scale processes in the future. Major suppliers of chromatographic media are already offering good quality IMAC matrices for large scale and EBA applications.

It is also expected that new improved His-tags as well as new more robust IMAC matrices will be designed in the future thus enabling more large scale applications.

8 Conclusions

Single-step isolation of His-tagged proteins is a reality in many research laboratories, where removal of His-tag and the extreme purity are often not required. We have not found any well documented case of single-step large scale isolation of therapeutic protein without additional chromatographic steps to achieve the requested purity. Even an efficient two-step chromatographic isolation at large scale is still a challenge and might be an attractive goal in many cases where both IMAC steps as well as cleavage of the tag are efficient enough. Here we must stress again that cleavage precision and efficiency is the decisive step and must be considered as a possible bottleneck, often destroying the expected good overall yield and economy of the process. At present, a realistic scheme for using IMAC at large scale would include two-step IMAC followed by a third polishing step to remove traces of aggregates, which is regularly requested for biopharmaceutical proteins to be used in human therapy. On the other hand there is a great opportunity for broader use of His-tags and IMAC at industrial scale for many other proteins, e.g., process and diagnostic enzymes, proteins for research and development such as in the field of genomics and proteomics especially in cases where tag removal is not required. At last but not least, one should be aware of limitations due to valid patents in using His-tags as well as various metal-chelating matrices and cleavage enzymes. Based on literature search and our own experience we conclude there is much higher potential for more extensive use of His-tags and IMAC at large scale than currently documented. Finally, one can imagine that a lot of information is kept by the companies as a trade secret especially for products and processes of strategic importance.

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