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Journal of Chromatography B, 790 (2003) 79–90
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Protein purification by affinity precipitation

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Abstract

Developing the most efficient strategy for the purification of a (recombinant) protein especially at large scale remains a challenge. A typical problem of the downstream process of mammalian cell products is, for instance, the early capture of the highly diluted product from the complex process stream. Affinity precipitation has been suggested in this context. The technique is known for over 20 years, but has recently received more attention due to the development of new materials for its implementation, but also because it seems ideally suited to specific product capture at large scale. The present review gives a comprehensive overview over this technique. Besides an introduction to the basic principle and a brief summary of the historical development, the main focus is on the current state-of-art of the technique, the available materials, important recent applications, as well as process design strategies and operating procedures. Special consideration is given to affinity precipitation for product recovery at large scale.

Keywords: Affinity precipitation; Affinity macroligands; Reviews; Proteins

Contents

1. Introduction

Precipitation occurs when a previously soluble substance becomes insoluble, e.g. due to a change in a crucial chemical or physical parameter of the environment. Since the recovery of a precipitate

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1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(03)00081-3

requires only a simple solid–liquid separation (filtration, centrifugation), intentional precipitation of the substance (class) of interest has long been a powerful tool in bioseparation. A well-known example from analytical chemistry is the quantitative protein precipitation that can be achieved by the addition of sodium deoxycholate and trichloroacetic acid to an aqueous solution. After removal of the precipitate the reminder of the sample can be analyzed without any disturbance from the protein fraction. Concomitantly and depending on the analytical question, the precipitate can also be resolubilized and the protein concentration can be quantified by a suitable protein assay without interference from the other sample components (e.g. [1]). Ammonium sulfate precipitation, on the other hand, is still a widely used method for preparative protein separation, because it is simple, rapid and easy to use even when large volumes have to be processed (scale up potential). In sequential combination with caprylic acid precipitation it was even possible to achieve a crude antibody purification by precipitation alone [2]. One of the largest, if not the largest purification scheme for therapeutic proteins, the fractionation of human plasma, is based on a series of precipitation steps, enforced by changes in the temperature and the addition of ethanol. Chromatography and especially affinity chromatography are nearly useless at this scale and enter only to solve isolated and very specific recovery problems [3].

In general however, protein separation by precipitation lacks specificity. This has led in the late 1970s to the development of two concepts for an affinity precipitation, one by the group of Klaus Mosbach and the other by Schneider et al. The two principles have little in common, however the same nomenclature was used. The objective was to develop an alternative affinity technique, which might overcome some of the known disadvantages of affinity chromatography. At that time affinity chromatography had already supplied the bioseparation community for more than 10 years with an efficient one-step technique for the specific isolation and concentration of proteins. The method was based on immobilizing so-called affinity ligands, i.e. molecules capable of a (bio)-specific interaction (molecular recognition) to the chromatographic support. These ligands then selectively retained and separated the target molecules even from a very diluted and complex feed. Elution could be achieved by an eluent which no longer supported the noncovalent interaction (often a pH shift, a chaotropic, respectively competing agent was used). Affinity chromatography had become very popular in laboratories as well as in bioproduction plants, but certain limitations had also become known. Scale-up, column fouling, and flow-rate limitations frequently caused problems. A particular problem with affinity chromatography was the sometimes inconveniently slow association rate of the target protein molecule with the immobilized biospecific ligand due to diffusion limitations, but also the low available capacity of the stationary phase (porous affinity beads) due to steric hindrances. Affinity chromatography also was awkward to use with raw process streams. As a result many groups tried to develop alternative affinity techniques, which retained the principle of biospecific interaction up to the use of the same affinity ligands, but which were hoped to overcome the disadvantages of affinity chromatography while keeping its main advantage, namely the highly specific enrichment of only the target protein in a single and easy to perform process step.

Precipitation was an attractive concept in this context, since the required solid–liquid separation was extremely well understood. The two concepts developed for affinity precipitation were later distinguished as primary effect and secondary effect affinity precipitation, see Ref. [4] for a review. At present however, the term affinity precipitation is used almost exclusively for processes employing the more general concept developed by Schneider et al., i.e. the formerly called secondary effect or indirect affinity precipitation.

2. Primary effect affinity precipitation

Primary effect affinity precipitation was developed by Mosbach and co-workers in the late 1970s (see [5]) based on the observation that the tetrameric enzyme lactate dehydrogenase (LDH) and bifunctional N_2,N_2'-adipodihydrazido-bis-(N^6-carbonylmethyl-NAD) (Bis-NAD) form at low temperature and in a certain concentration ratio a crosslinked, macromolecular network, which becomes insoluble

and precipitates when the aggregates have grown sufficiently large. In primary effect affinity precipitation the formation of a precipitate is therefore the direct consequence of the affinity interaction between the multivalent enzyme and the bifunctional ligand. The spacer length between the two NAD units in the Bis-ligand had to be chosen in such a way, that it allowed the simultaneous interaction with the active sites of two LDH molecules, but prevented the competing intramolecular crosslinking reaction of the Bis-NAD molecule. It was later found useful to add substrate analogs during complex formation in order to increase the strength and the specificity of the binding between the enzyme and the NAD affinity ligand, i.e. by exploiting the so-called ''locking-on'' effect of ternary complex formation [6]. After separation of the precipitate by centrifugation the aggregates could be redissolved by the addition of NADH, which competes with the Bis-NAD for the binding sites of the enzyme. Once the complex has dissociated, contaminations such as Bis-NAD, NADH and ternary complex agents such as pyruvate or oxalate can be removed by a subsequent gel-filtration step.

In order to reach the maximum precipitation yield, the molecular ratio adjusted between the LDH and the Bis-NAD was found to be important. A yield in the range of 90% could be achieved under nearly equinormal conditions (1.1 NAD equiv. per LDH subunit). Small deviations from this ratio significantly diminished the extent of precipitation. The effect was more pronounced for under – than for over – saturation with Bis-NAD. If the Bis-NAD concentration was too low, no precipitation was observed. The enzyme concentration itself, but also the substrate analog concentration were also found critical for optimal precipitation yield [7,8].

With slight modifications, primary effect affinity precipitation has been used for the purification of other enzymes. Yeast alcohol dehydrogenase is like LDH a tetrameric enzyme, which precipitates slowly in the presence of Bis-NAD, pyrazole and NaCl. Without NaCl no precipitation occurs. Hexameric glutamate dehydrogenase, however, precipitates in contrast to LDH and yeast alcohol dehydrogenase spontaneously with high precipitation yield over a wide Bis-NAD ratio. The resulting precipitate is difficult to redissolve requiring high NADH concentrations. The dimeric liver alcohol dehydrogenase, on the other hand, does not form crosslinked macromolecular networks even when pyrazole is added, due to a deadend formation of a dimeric complex of two Bis-NAD and two enzyme molecules [9].

Certain triazine dyes imitate the NAD coenzymes and exhibit affinity to LDH. With Bis-Cibacron Blue, 90% of LDH could be recovered under optimized conditions, i.e. in the presence of pyruvate [10]. For bovine serum albumin (BSA) and chymosin the yields were 50 and 20%, respectively. Rabbit muscle LDH but not the pig heart isoenzyme could be efficiently precipitated at 97% (purification factor of 6) with a methoxylated derivative of the triazine dye Procion blue H-B. In this case the methoxytriazinyl ring and the terminal *p*-aminobenzenesulfonate ring were presumed to interact with the target molecule, while the central *p*-phenylenediaminesulfonate ring residue serves as the interlinking spacer [11].

Bis-ATP has also been employed for primary effect affinity precipitation, e.g. for the purification of bovine heart phosphofructokinase from tissue extracts (precipitation in the presence of citrate [12]). The application of Bis-ATP is however limited by its instability. Bis-borate ligands have also been tested for cell separation [13]. In addition, borate was used as additive to induce the precipitation of polysaccharide protein A–IgG affinity complexes [14]. Biotinylated phospholipids can be strongly binding ligands for the tetrameric avidin. In aqueous solution such phospholipids form micelles by hydrophobic interaction, which can also be used for the separation of the captured target molecule from solution. Phospholipids have been successfully used for the isolation both of avidin [15] and of antibodies [16]. In 1993 Morris et al. proposed so-called polyligands for the same purpose [17].

The manifold prerequisites for performing primary effect affinity precipitation efficiently, in particular the prerequisite for multivalency in the target protein, but also the limited number of known Bis-ligands reduces the application of this method mainly to the dehydrogenases. In an attempt to increase the versatility of primary effect affinity precipitation Van Dam et al. synthesized bis-copper chelates, which were supposed to crosslink the target proteins via accessible surface histidine residues into a precipitating macromolecular network [18]. At a Cu:his-

tidine ratio near 1, human hemoglobin, which has 26 histidine residues, is precipitated at 100% with $Cu(II)_2$EGTA. Sperm whale myoglobin has six histidine residues and requires a Cu:histidine ratio near 50 for a 100% precipitation yield. The precipitation efficiency increases with increasing spacer length of the Bis-ligand, as shown by using $Cu(II)_2$PEG 20 000$(IDA)_2$. In the case of precipitating hemoglobin, however, Cu^{2+} ions ($CuSO_4$) alone were just as effective as the Bis-ligand $Cu(II)_2$EGTA in precipitating this particular protein. Horse cytochrome c (one histidine residue), on the other hand, cannot be precipitated by either the Bis-ligand or $CuSO_4$. The application range of metal affinity precipitation can be extended even further by genetically engineering of proteins, which allows adding so-called Histidine-tags to the recombinant proteins. Lilius et al. [19] fused five histidine residues as an affinity tail to galactose dehydrogenase, an enzyme, which forms homodimers. Each recombinant homodimer hence carried two of these tags. Addition of $EGTA(Zn)_2$ then resulted in affinity precipitation by linear polymer chain formation. A yield of 90% and a purification factor of 11 were achieved. The amount of metal chelates added was found to be critical. Above 10 m*M* the native protein was found to precipitate spontaneously.

2.1. Preparative-scale application of primary effect affinity precipitation

A few preparative-scale applications of primary effect affinity precipitation are known. LDH was recovered from a crude mixture with yields of 91% recovery and a purification factor of 40 [9]. However, several elaborate pilot precipitation tests were necessary in order to determine the optimal ratio between the LDH concentration/activity and that of the Bis-NAD. Deviations from the optimum ratio caused significant product loss. At the same time it was found that a Bis-NAD addition based only on enzyme activity measurements (the easiest and most common quantification method for an enzyme) gave only a low precipitation yield [20]. These difficulties render the standardization of primary effect affinity precipitation at preparative scale rather complex. Concomitantly, the kinetics of complex formation and dissociation are slow [21] and entrapment of impurities in the aggregates is a very common problem, which contributes to the moderate purification factors obtained.

3. (Secondary effect) affinity precipitation

Affinity interaction and precipitation are directly linked in primary effect affinity precipitation. While this makes the method very straightforward, it is also the cause of many of the above mentioned disadvantages of this form of affinity precipitation. In secondary or indirect affinity precipitation, these two aspects of the process are no longer linked and hence can be performed and controlled independently. Affinity and stimulus-responsiveness are instead combined in an agent called the affinity macroligand (AML), generally a polymeric substance to which one or several affinity ligands are conjugated and which precipitates reversibly from aqueous solution upon an external stimulus such as a change in the ambient pH or temperature. In a recent review Mattiasson et al. discuss the design criteria for an ideal AML [22]. According to him, the ideal polymers for affinity precipitation

(i) contain reactive groups for ligand coupling
(ii) do not interact strongly with the ligand or impurities, to make the ligand available for interaction with the target
(iii) give complete phase separation upon external stimulus
(iv) have a sharp and well-characterized transition
(v) have a very narrow molecular mass distribution
(vi) form compact precipitates
(vii) exclude trapping of impurities
(viii) are easily resolubilized
(ix) have repeatable cycles many times with good recovery, and
(x) are available and cheap

However, to date this ideal remains somewhat elusive and many successful affinity precipitations were performed with AML that failed to meet one or several of these criteria.

3.1. pH-Sensitive affinity macroligands

The first attempt of an indirect affinity precipitation—again of an enzyme—was published in 1981 by Schneider et al. [23]. They synthesized an affinity macroligand (AML) composed of a copolymer of acrylamide, *N*-acryloyl-*p*-aminobenzoic acid, and *N*-acryolyl-*m*-aminobenzamidine. This AML is water-soluble above a pH of 4. Below a pH of 4 the acidic residues on the polymer backbone become neutralized (protonation) and hydrophobic interactions between the uncharged polymer backbones enforce aggregation. Hence the AML precipitates reversibly when the pH is reduced below this value. The benzamidine units in the AML serve as affinity ligands and depending on the pH represent strong and specific inhibitors of the protease trypsin. Contrary to the above discussed enzymes such as LDH, trypsin contains only one binding site for the inhibitor (monovalent enzyme) and hence could not have been purified by primary effect affinity precipitation, where multivalency is required.

The procedure of purifying the protease from a crude pancreatic extract at pH 8 can be considered typical. The AML is added to the extract at a load of 0.5% (w/w). The biospecific interaction between the trypsin and its inhibitor (AML) takes place in homogeneous solution. The AML–trypsin complex is then

(i) co-precipitated by lowering the pH to 4,
(ii) separated by centrifugation from the supernatant, in which putative impurities remain dissolved,
(iii) washed once with water, and
(iv) resuspended at pH 2 for dissociation of the affinity complex.

The latter point is especially important, since at a pH of 2 the affinity interaction is no longer possible, while the AML still forms a precipitate. Alternatively, in some affinity precipitation schemes, the complex is first redissolved, whereafter the affinity interaction is interrupted (elution) followed by reprecipitation of only the AML. In both cases however, after elution the purified product stays in solution, while the precipitated AML is easily removed, e.g. by centrifugation. In the case of the trypsin purification, a supernatant containing 83% of the initial trypsin activity and 7% of the initial chymotrypsin activity was collected after the removal (centrifugation) of the AML. The AML itself was recovered at 99% yield by the centrifugation step. It could be resolubilized at pH 8 and re-used for further purification tasks of crude trypsin batches. The repeated use of AML causes therefore a loss of about 1% of the AML per cycle. The AML performance was found to diminish only slightly with recycling. Affinity precipitation as designed by Schneider et al. became the basic concept for indirect affinity precipitation permitting in essence the one-step purification of a wide variety of proteins by homogeneous affinity interaction between the protein and its AML.

An indirect affinity precipitation of the enzyme LDH was described by Senstad and Mattiasson in 1989 [24]. For this purpose Blue Dextran 2000, i.e. a dextran modified with a triazine dye, was allowed to interact with the LDH in solution. The precipitation and separation of the affinity complex was then initiated by the addition of Concanavalin A (Con A), a tetrameric lectin known for its ability to crosslink carbohydrate units. Here it was used to crosslink the Blue Dextran 2000 molecules into an insoluble network. Compared to primary effect affinity precipitation of LDH, the new method was much less dependent on the target molecule's concentration and a titration of the LDH concentration in the raw solution prior to the separation was no longer necessary. Elution of 25% of the original LDH activity from the precipitate was achieved by adding KCl. Handling of the precipitate however turned out to be difficult, since it had a gel-like structure and was rather voluminous.

In 1994 Kumar and Gupta [25] reported the purification of trypsin from a crude sample using a pH responsive conjugate of Eudragit S-100 and soybean trypsin inhibitor as AML. 83% of the enzyme activity could be recovered in this process. Other examples of Eudragit-based affinity precipitation are the purification of monoclonal antibodies [26,27] and of Concanavalin A [28,29]. Eudragit S-100 is a commercially available high-molar-mass copolymer of methacrylic acid and methyl methacrylate (M_w 135 000 g/mol), which precipitates sharply below pH 4.5. The precipitation is again

fully reversible. Conjugation with affinity ligands shifts the transition to higher pH values, because conjugation reduces the number of charged carboxylic acid groups in the Eudragit backbone. The same effect occurs after addition of ammonium sulfate or PEG 8000 to an Eudragit solution [30]. In cases where the enforcement of a precipitation by low pH is not possible, e.g. in the case of a pH sensitive product, Eudragit S-100 based AML can also be phase-separated by the addition of Ca^{2+} together with an increase of the temperature to 40 °C. This has, e.g. been demonstrated by Guoqiang et al. for the purification of yeast alcohol dehydrogenase in connection with zinc ion-promoted binding (affinity interaction) of the enzyme with Eudragit-Cibacron Blue [31]. Ethanolamine modified Eudragit S-100 has also been used to precipitate protein impurities [32].

Eudragit S-100-based AML are known to show nonspecific protein binding during the affinity precipitation process. These nonspecific interactions are generally enforced or even caused by the modifications of the polymer backbone structure during AML synthesis. The control with pure Eudragit S-100 in an affinity precipitation process gives therefore not the correct picture in regard to the extent of nonspecific binding [33]. Depending on the application, the addition of high salt concentration, organic compounds or PEG may decrease the nonspecific electrostatic or hydrophobic interaction between protein impurities and the polymer (the AML). Nonspecific binding can also be kept low if the minimum AML load is used. While nonspecific interactions generally present a nuisance in affinity purifications, the effect can also be exploited in a beneficial way. In the case of Eudragit, some exemplary applications for nonspecific protein enrichment by secondary effect precipitation have been published [34,35].

In addition to Eudragit, several pH sensitive polymers known in nature have also been adapted to the affinity precipitation of proteins. These include alginate [36–39], chitosan [40–42], and derivatized cellulose [43–45]. A general drawback of pH sensitive polymers like Eudragit S-100 or alginate is that they precipitate at a pH, which is outside the stability range of many proteins. Polyelectrolyte complex (PEC) formation was therefore proposed as a means to shift the precipitation pH of such polymers into the physiological range [46,47]. Polyethyleneimine–Cibacron Blue AML were used in such an approach for the purification of LDH [46]. Upon the addition of the negatively charged polyacrylate, the AML precipitated at a pH between 6.5 and 8.9 depending on the AML:polyacrylate ratio and the dilution factor (ionic strength). The recovery of the PEC was, however, low, and the co-precipitation of negatively charged protein impurities and nucleic acids was esteemed very likely [48]. Concomitantly, the formation of a PEC to achieve precipitation of the affinity complex stands in contradiction to the general philosophy of downstream processing, which says to keep the number of intentionally added substances to the absolute minimum.

3.2. Thermosensitive affinity macroligands

Another class of reversibly precipitable polymers are the so-called thermoresponsive ones, i.e. polymers which precipitate if a certain critical solution temperature (CST) is passed. In aqueous solution, precipitation often occurs as the temperature is increased. A number of poly-*N*-alkylacrylamides such as poly-*N*-isopropylacrylamide (polyNIPAM) and poly-*N,N*-diethylacrylamide possess this property [49]. Most co-solutes lower the critical solution temperature. However, the pH has usually only very little influence on such a CST. This fact has important consequences for the application of affinity precipitation as a bioseparation tool, because it permits a clear disconnection of the pH as a means to modulate the affinity interaction and the temperature as a stimulus for reversible precipitation–solubilisation of the AML (the affinity complex). However, other possibilities do exist. Fong et al. described, e.g. a possible strategy for sequential affinity separation of proteins using pH- and thermoresponsive AML [50].

Chen and Hoffman [51] synthesized a copolymer of NIPAM and *N*-acryloxysuccinimide having a molar mass of around 24 000 g/mol, an average of 5.5 activated ester groups per polymer chain, and a CST of 32 °C in pure water. Conjugation of protein A to this copolymer enabled the capture and recovery of IgG by thermoprecipitation of the formed AML–IgG complex. The dissociation constant of

this affinity complex was $3 \cdot 10^{-6}$ M and the binding capacity of the AML was 25% of the theoretical one. The authors concluded that sterical interference of the protein A binding sites with the polymer might be the reason for the low binding capability of the copolymer-AML, whereas they could exclude the occurrence of nonspecific binding between IgG and the polymer backbone.

Thermoresponsive AML based on polyNIPAM copolymer functionalized with glycidyl methacrylate groups were also used in the Ca^{2+} depending affinity complex formation between *p*-aminophenylphosphorylcholine and rabbit C-reactive protein and thus allowed the purification of this protein at high yield [52]. Further applications of AML based on polyfunctionalized, thermoresponsive copolymers are the purification of trypsin [53], protein A [54], alkaline protease [55] and of egg white lysozyme [56]. The early applicants of temperature-enforced affinity precipitation generally chose to introduce the interactive groups (functionalization) into the thermoresponsive polyNIPAM by statistical radical copolymerization of NIPAM with suitable hydrophilic or hydrophobic acryl monomers. However, this approach soon reaches its limits, since high precipitation efficiency and predictable precipitation behavior become difficult to achieve. A typical example is the attempt to use a Cu(II)-AML for the purification of α-amylase [57]. High salt concentrations were necessary in this case to promote precipitation.

A second generation of thermoresponsive polymers for affinity precipitation became available as a result of recent developments in polymer synthesis. Especially the methods to create linear, homogeneous, low-molar-mass polymers containing one reactive end-group opened many new possibilities. Such ''smart'' oligomers can, e.g. be synthesized by group transfer [58] or chain transfer polymerization [59]. In the latter case the polymer chain length is essentially controlled by the ratio between the monomer and the chain transfer agent. When 3-mercaptopropionic acid is used as chain transfer agent the final oligomer carries a reactive carboxylic acid end group, which may easily be used for the conjugation to the affinity ligand. Interestingly and contrary to the above-mentioned copolymer-based AML, such end group linked affinity ligands have little to no influence on the precipitation behavior of the AML/the affinity complex. This is even the case when the ligand is large and/or charged [60,61].

Oligomeric polyNIPAM usually has a CST at 32–34 °C, but it can also be synthesized in a more ordered isotactic structure with a CST at 41–42 °C [62,63]. In a first application Takei et al. [64] coupled polyNIPAM (M_w 6100 g/mol; M_w/M_n = 1.22, where M_w = weight average molar mass and M_n = number average molar mass) to the outer surface of anti-HSA goat IgG. Upon temperature increase a rapid-response precipitation of the conjugates occurred at around 34 °C. They also found that if the polyNIPAM conjugation is not too excessive, the IgG antigen binding specificity and capacity remain unchanged. However, especially when relatively long oligomers were used at high density, these were found to sterically interfere with the IgG binding sites presumably due to a restriction of the mobility or the accessibility of the free oligomer end.

Hoshino et al. proposed poly-*N*-acryloylpiperidine as AML-precursor [65]. Due to the low CST of this precursor (around 4–8 °C), poly-*N*-acryloylpiperidine-based AML should be especially useful for the purification of thermolabile proteins. In an exemplary application, 68% of the α-glycosidase activity, respectively 80% of the Concanavalin A could be recovered from their crude solutions by using maltose as affinity tag. The affinity constant of association of this tag to the targets was in both cases around 10^3 M^{-1}.

3.2.1. Oligomeric affinity macroligands

Immobilization of an affinity ligand generally reduces its affinity to the target protein compared to that of the free ligand by up to a factor of 1000 [25]. This presents a real problem, since it has, on the other hand, been postulated that affinity precipitation as a one-stage process requires for efficient protein purification affinity constants that should be at least one order of magnitude higher than those normally used in affinity chromatography [58]. The binding strength may, off course, benefit from multipoint interactions between target and AML (concept of avidity [57]). High-molar-mass polymers are known to form highly coiled and entangled (viscous) structures in solutions that hinder the access of incoming macromolecules, such as the target molecules of

many affinity separation strategies. Vaidya et al. [66] found that spatial separation of affinity tags from the polymer backbone by introduction of spacers increases the binding to proteins. This effect increases with increasing spacer chain length in synergism with a more favorable microenvironment for the affinity tag. However, a ligand accessibility comparable to that of a free molecule, can only be achieved by coupling the ligand—preferably in terminal position—to a low-molar-mass polymer (oligomer). Then the crowding effect is almost negligible [58,66,67]. The use of low-molar-mass AML-precursors also reduces the probability of steric interference of the mobile polymer chains with the ligand binding sites [64].

Recently, Garret-Flaudy and Freitag [67,68] reported for the first time the application of oligomeric AML for protein purification. The AML-precursor was a polyNIPAM synthesized by chain transfer polymerization and characterized by low molar mass (M_n 4100 g/mol) and a low polydispersity (M_w/M_n 1.15). Conjugation of 2-iminobiotin to this AML precursor resulted in an AML, which could very successfully be used for the purification of avidin from a cell culture supernatant containing 5% FCS. The avidin recovery was 90% and the purification factor 14. The remaining protein contaminations of the product were below the detection limit. The affinity between the AML and the avidin could modulated by the pH, since binding is enforced at high pH (>9), while the affinity is greatly reduced at low pH (<4). The pH does not affect the CST in the range from 1 to 12. The phase transition of the AML (precursor) as well as that of the affinity complex was very sharp, despite the low molar mass of the oligomer involved. 99% of the AML molecules could be recovered by thermoprecipitation and centrifugation. The AML could be reused several times and nonspecific adsorption was of no concern.

The concept of using linear, low mass, and homogeneous oligomers in affinity precipitation was recently extended to the purification of plasmid DNA [63]. Oligomers with a molar mass of 2300 g/mol and a polydispersity below 1.1 were used to create AML for triple helix affinity precipitation of the target DNA. The oligmers were first activated by avidin and then conjugated to biotinylated 21-mer oligonucleotides. The ligand efficiency of such AML was one order of magnitude higher than the one normally measured in triple helix affinity chromatography, confirming Eggert's thesis [58] that an affinity ligand placed in a terminal position in a low molar mass AML retains a maximum of activity.

Low-molar-mass AML are therefore superior to the previously described polymeric molecules in terms of homogeneity, ligand efficiency and flexibility, making affinity precipitation a very valuable purification method. The fact that the addition of such oligomeric molecules will not lead to a pronounced increase in viscosity—as is the case for polymeric ones—constitutes another strong argument for the use of small AML, especially at large scale, where mixing may otherwise become a problem.

3.3. Preparative-scale affinity precipitation

The attractiveness of affinity precipitation stems from the fact that a well-understood and manageable unit operation ''precipitation'' is combined with the specificity and efficiency of the affinity approach in homogeneous solution. In their perennial review [69] Labrou and Clonis placed (secondary effect) affinity precipitation highest among the major affinity purification technologies in terms of purifying ability and also large-scale potential. The method also shows broad flexibility towards variation in the process parameters. Despite these advantages, however, affinity precipitation is to date not an established downstream processing technique. One reason is the lack of a suitable large-scale separation technology for the precipitated polymers, respectively the affinity complex. High-speed centrifugation is an efficient method except for oligomeric materials [59]. The resulting precipitate, however, tends to be a compact gel, the resolubilisation of which can be time consuming. Entrapment of impurities in the wet gel is almost unavoidable making as a rule of thump three precipitation–resolubilisation washing–cleaning steps necessary [67].

Senstad and Mattiasson [41] were the first to address the problem of developing alternative modes of precipitate recovery at large-scale that avoid centrifugation steps. They proposed flotation as a relatively mild operation. In particular, chitosan-based AML were precipitated by raising the pH of the solution above 8. Afterwards the mixture (liquid

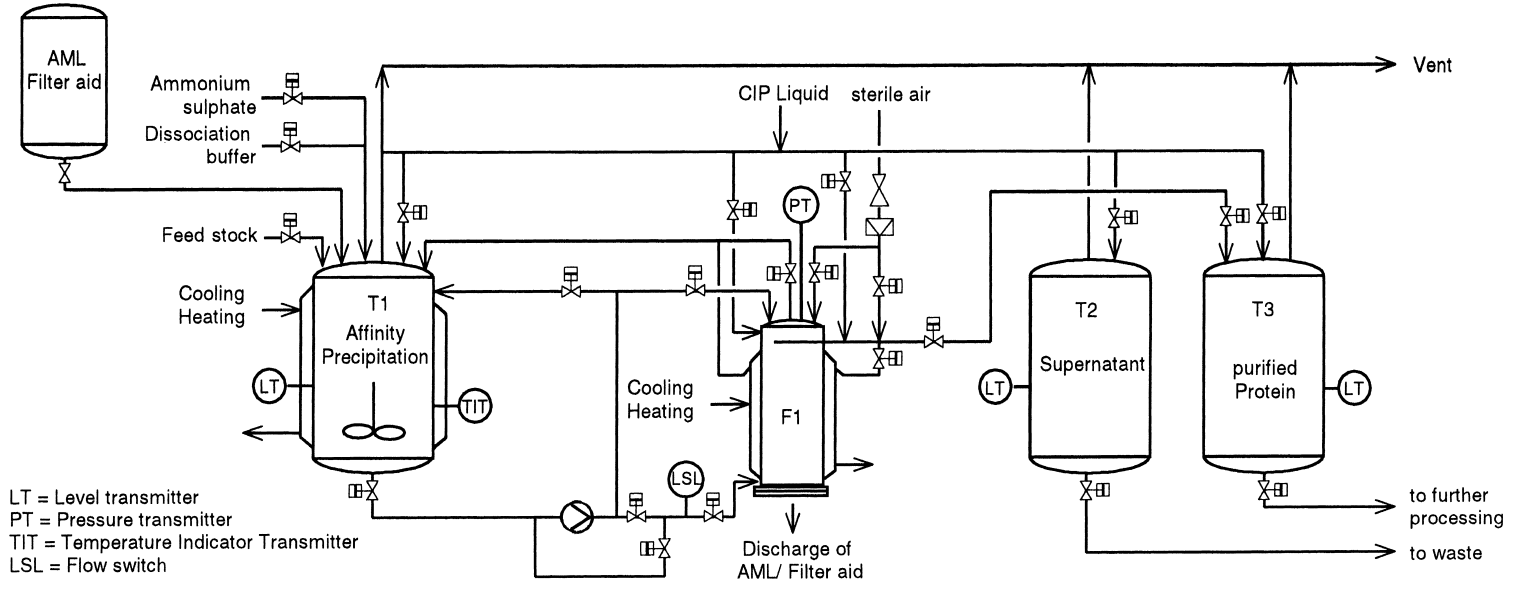

Fig. 1. Process scheme—affinity precipitation for protein purification at large scale (kindly provided by polyTag Technology, Switzerland). Procedure: (1) Addition of feed stock (binding buffer) into T1, (2) adjustment of temperature to 4 °C, (3) addition of AML and filter aids (cellulose fiber and ammonium sulfate), (4) adjustment of temperature to 30 °C co-precipitation of the affinity complex, (5) filtration of the complex-filter aid blend at 30 °C in F1 and collection of filtrate in T2, (6) washing of the filter cake with an ammonium sulfate solution at 30 °C and collection of filtrate in T2, (7) elution of targets from the filter cake by dissociation buffer at 30 °C and collection of filtrate containing purified protein in T3, (8) washing of the recovered AML/filter aid blend for re-use in step 3.

containing the precipitate) was put under pressure (3 bar, 15 min) and then transferred into a flotation chamber at atmospheric pressure. Air bubbles, which formed as result of the pressure expansion, adsorbed to the surface of the precipitated material causing the flotation of the particles and thereby their accumulation at the surface, where they can easily be recovered.

Unfortunately, flotation technologies are not applicable for large-scale separations of polymers like polyNIPAM, since polyNIPAM has a strong tendency to foam. At the same time polyNIPAM thermoprecipitates tend to be unstable [70]. Filtration as separation operation is an alternative, but for oligomers with a molar mass below 5000 g/mol at a concentration of 1% (w/w) a very high loss upon precipitation has been reported [59,65]. The recovery of oligomeric AML by precipitation can be improved by using a higher oligomer concentration, e.g. 10% (w/w). However this will also entail a higher viscosity of the solution and mass transfer limitations similar to those observed for high-molar-mass polymers.

Most recently, a procedure has been proposed, where filtration—by the addition of filter aids and salts—becomes a very efficient separation operation for such oligomers [70] (Fig. 1). In particular it has been found that the thermoprecipitation of oligomeric polyNIPAM (M_n 2300 g/mol) from a 0.5% (w/w) aqueous solution in the presence of short cellulose fibers and salts gives a suspension of compact aggregates that filter (and subsequently wash) very well. The precipitated mixture is readily resolubilized, and the oligomer recovery was higher than 97%. Nonspecific adsorption (entrapment of impurities) was not detected. Obviously, the self-association of the oligomeric polyNIPAM, which is already observable before macroscopic precipitation occurs [62], can be sufficiently promoted by filter aids/salts to allow efficient filtration. The type of salt used plays an important role, since it determines besides the predictable salting-out effect [61,62] also the aggregation behavior and hence the consistency of the precipitate. The determination of the most suitable salt and its optimum concentration is, however, still done empirically.

Even under optimized conditions, only between 97 and 99% of the oligomeric polyNIPAM are recovered and separated by the filtration step. Some contamination of the product by the remainder is hence possible even after washing. However, this should be acceptable in an initial capturing and first purification step for a target protein from a cell culture supernatant. The pronounced difference in size between the oligomeric polyNIPAM and the target protein should allow easy separation during an additional chromatographic polishing step. PolyNIPAM itself is considered a safe material [71,72], if it is sufficiently purified from the neurotoxic monomer. One point, which needs to be further investigated, is the mode of target molecule elution. The process scheme presented in Fig. 1 assumes that the target protein is eluted directly from the filter cake. It might also be possible to remove the filter cake from the filter, redissolve the precipitate in cold dissociation buffer, and to subsequently retrieve the AML by thermoprecipitation in the presence of the filter aid. The final step would then be a separation by filtration of the solids (AML, filter aid) from the solution containing the highly purified target protein.

4. Perspectives

Affinity precipitation is a relatively simple, convenient and reproducible technique that results in high target molecule recovery at high specificity. Using AML based on homogeneous oligomers the ligand efficiency is very high, i.e. the (precious) affinity ligands are effectively used during the purification procedure. The method shows broad flexibility towards variation in the process parameters. Since only mixing of AML into cell culture supernatants is involved, the scale-up potential is high. At preparative scale the most appropriate separation operation for the precipitate is filtration. Future work should be directed to further optimization of the AML (precursor), the establishment of more applications and towards the development of a robust, integrated purification process.

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