PURIFICATION OF UNTAGGED RECOMBINANT PROTEINS: A REVIEW OF RECENT TRENDS

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ABSTRACT

The use of tags is a common strategy to facilitate the purification process of recombinant proteins, allowing easy detection of molecules and high purities in the final product. However, this choice has as the main disadvantages the modification of the target protein structure, what may affect its function, in addition to elevate the cost of the purification process due to the operations required to remove the tag. Hence, the development of purification processes for untagged proteins is a relevant issue, mainly considering the demand from the industrial recombinant protein production sector. In the present work, a literature review is presented, addressing applicable purification processes of untagged proteins reported lately. Studies addressing purification of recombinant proteins with different characteristics, secreted or accumulated into the host cells and delivered by different platforms, were selected. In all works, purities next to 100% were achieved, with reasonable global recoveries, even without the presence of tags. Cryoprecipitation poses as a promising alternative to replace additional chromatographic steps.

Keywords: Chromatography. Purification. Untagged proteins.

1 INTRODUCTION

The use of tags consists of a strategy of genetic modification in which an additional sequence of amino acids is fused to the N- or C-terminus of the protein of interest, making the purification process easier. The most common tag is the tail containing six histidines (His-tag), which has a pseudo affinity to bivalent metals, such as Ni, Co, Cu, Fe and Zn, and permits the purification of the His-tagged protein by immobilized metal affinity chromatography[1]. The strategy of adding a tag to the protein of interest simplifies the purification process, allowing easy detection and separation of molecules from host cell proteins. It may also contribute to increase protein stability and solubility. However, the addition of tag to the target molecule can cause many disadvantages, affecting the conformational structure, biological activity and toxicity of the protein and consequently, changing its function[2].

Besides these negative effects, regulation issues related to tag addition have to be faced for industrial recombinant protein production by pharmaceutical companies. Regulatory agencies do not allow the presence of tags in the final product, thus protease cleavage sites between the tag and the protein of interest must be included and, after cleavage, an additional step must be added to the purification process to eliminate tag residues, uncleaved molecules and the protease enzyme[3]. However, the removal of the tags has technical difficulties, and it is possible that the cleavage is not complete, with some amino acids remaining in the protein even after purification. Furthermore, tag separation may have low yield and depends on expensive chromatographic resins due to the similarity between the tagged molecules and the hydrolyzed ones[4]. Industrially, adding a tag to a protein is only viable when it presents therapeutic or production advantages, because it requires additional time and cost associated with subsequent purification operations, with the aim of removing the tag[5].

In this way, the development of a sequence of purification steps for the target molecule without the addition of tags (untagged) is a very relevant research subject. On the other hand, academic studies addressing the purification of untagged recombinant protein are still scarce. The present review addresses applicable purification processes for untagged proteins, which were reported in the literature lately, highlighting the results obtained of purity and global recovery of the protein of interest.

2 MATERIAL & METHODS

Three articles were chosen to perform the literature review about purification processes of untagged recombinant proteins. In these works, purification processes for target proteins with different characteristics and produced in different host cells are studied.

In the first selected work, the purification of a protein secreted by the host cells, that is recovered from the culture broth, is addressed. The untagged anti-nerve growth factor nanobody (anti-NGF Nb) is the target protein. The untagged anti-NGF Nb is a fusion of two anti-NGF Nb and one anti-human serum albumin Nb, having a molecular weight of 42 kDa and an isoelectric point of 6.5. Nanobodies are single-domain antibodies, which have potential application value in tumor-targeted therapy, immunotherapy, diagnostic probe, and molecular imaging. Nerve growth factor (NGF), which regulates the growth and development of peripheral and central neurons, maintains neuronal survival and plays a key role in both arthritis and acute and chronic pain. An untagged anti-NGF Nb was expressed in stably transformed *Pichia pastoris* cells, which were grown in a defined medium using a 5 L bioreactor. The final culture broth was harvested by centrifugation at 8000 g for 15 min and the supernatant was collected and filtered through a 0.45 µm filter membrane to obtain a clarified harvest at an anti-NGF Nb concentration of 2.3

mg/mL. A method for capture and purification of the anti-NGF Nb by mixed mode weak cation chromatography and cation exchange chromatography was established. The first process used two mixed mode weak cation resins (Capto MMC and Eshmuno HCX) to capture the protein of interest; the second one used a Capto S ImpAct column to remove impurities[6].

The second and third articles chosen describe the purification of untagged recombinant proteins which accumulate inside the cell of the host organism. This is the case of the pneumococcal surface protein A (PspA) from family 2, clade 4, containing the entire N-terminal alpha-helix region, the clade definition region and the first block of proline rich region (PspA4Pro). This protein has a molecular weight of 43 kDa and an isoelectric point of 4.8. This recombinant protein is used in the development of pneumococcal vaccines with broader serotype coverage. The PspAPro was obtained through a ClearColi® cultivation, a strain of *Escherichia coli* free of endotoxic activity, which was grown in a defined medium using a 5 L bioreactor to obtain a biomass containing 146 mg of PspA/g of dry cells. The purification process incorporated cell disruption using a high-pressure homogenizer, precipitation by adding 0.1% (v/v) of cetyltrimethylammonium bromide (CTAB) followed by centrifugation at 17696 g and 4ºC for 120 min. The resulting supernatant was homogenized to obtain the clarified cell extract, which was processed through anion exchange chromatography with a Q-Sepharose column and cryoprecipitation to obtain purified the PspA4Pro[7].

In the third article, the target molecule was the untagged termination factor Rho from *Azospirillum brasilense* (AbRho), which monomer has a molecular weight of 47 kDa. The transcription termination factor Rho is a ring-shaped, homohexameric protein that causes transcript termination by interaction with specific sites on nascent mRNAs[7]. The total molecular weight is estimated at 282 kDa. Bacteria of the genus *Azospirillum* are microorganisms capable of growing using atmospheric nitrogen as the sole source of nitrogen (diazotrophic) and are found in association with the roots of several plants of agricultural importance such as corn, wheat, sorghum and rice. The process of transcription termination is essential for proper expression and regulation of bacterial genes. Hence, it is important to obtain information that can help to understand transcriptional regulation in this particular bacterium. The AbRho was obtained through a culture of *Escherichia coli* cells on 200 mL LB medium. The purification process comprised cell disruption by sonication on an ice bath and a two-step chromatography procedure. The first chromatography was based on chemical affinity and used a Heparin column; the second one used a Sephacryl S-200 gel filtration column[8].

3 RESULTS & DISCUSSION

Table 1 presents the results of purity and recovery obtained in each purification process developed in the three articles, with their respective proteins of interest.

Table 1 Results of the three purification processes.

Work	Protein of interest	1st chromatographic step	2nd chromatographic step or cryoprecipitation	Clarified purity (%)	Purity after 1st step (%)	Purity after 2nd step (%)	Overall recovery after 1st step (%)	Overall recovery after 2nd step (%)
LI *et al*. (2022)	Anti-NGF Nb	Mixed weak cation chromatography	Cation exchange chromatography	11.4	93.0	99.8	76.9	64.6
CARDOSO *et al*. (2022)	PspA4Pro	Anion exchange chromatography	Cryoprecipitation	56.8	78.3	98.3	28.2	25.3
PARIZE *et al*. (2022)	AbRho	Affinity chromatography	Gel filtration chromatography	44.0	92.0	>99.0	46.2	45.7

The three proteins described in the works have some different characteristics. In the case of Anti-NGF Nb and PspA4Pro, the molecular weight was similar and the main difference was related to the isoelectric points of the proteins, which were, respectively, 6.5 and 4.8. Observing these values, the purification processes were developed using ion exchange chromatographies, in which the system pH was regulated to promote the adsorption of the protein to the chromatographic resins. In the case of Anti-NGF Nb, the pH was maintained in the range of 4-6, whereas, for PspA4Pro, the pH was set at a value of 6.5. The main difference between AbRho and the other two proteins was related to its arrangement as a homohexameric protein, having a total molecular weight of 282 kDa. Due to this characteristic, it was interesting to add the gel filtration chromatography as one of the main steps in the purification process, according to its principle of separating molecules based on their size.

Table 1 shows that it is possible to develop efficient purification processes for recombinant proteins with different characteristics, secreted or accumulated intracellularly, and achieve high purities even when tags are not added. The first and the third works reached purities next to 100% using two chromatographic operations. However, it is important to mention that the use of more than one chromatographic operation can make the purification process more expensive, due to the high cost of the chromatographic resins.

Using the reasoning presented, the process developed in the second work is probably less expensive, since only one chromatographic step was utilized. The authors used cryoprecipitation to replace a second chromatographic step, which has presented excellent purification efficiency, increasing the purity of the material obtained after the anion exchange chromatography in, approximately, 1.3 times.

Nevertheless, it was also noticed that the process in the second work showed the lowest global recovery value in the initial chromatographic step, that is, there was a greater loss of mass of the protein of interest compared to the other two works. Also, the purity achieved after the anion exchange chromatography was lower than the purities after the first chromatographic operation in the other two works. In the second and third works, it can be observed that the two global recovery values were similar, in other words, the second purification step utilized in both processes presented high yield of the protein of interest. These differences in the recovery among the three purifications compared are mainly related to the characteristics of both target protein and contaminating proteins present in the clarified samples. Even though it is always desirable to develop a purification process which deliveries a product with high purity and recovery, the cost of adding several steps to remove the contaminants more surgically has to be taken into consideration.

4 CONCLUSION

This short review shows that it is possible to develop efficient purification processes for recombinant proteins with different characteristics, secreted or accumulated intracellularly, and achieve high purities even when tags are not added. It is necessary to study deeply the physical-chemical properties of the target molecules in order to select appropriate chromatographic operations and establish the adequate arrangement of them. The efficiency of the purification process may vary depending on these studies and, in some cases, the desired high purity is achieved at expenses of lower recovery. Thus, a techno-economic analysis is recommended to find the most profitable purification strategy. Cryoprecipitation poses as a promising operation that could be studied more frequently as an alternative to elevate the purity of target proteins after the first chromatographic step, because it has presented great purification efficiency, low loss of mass of the target molecule and it is not expensive.

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