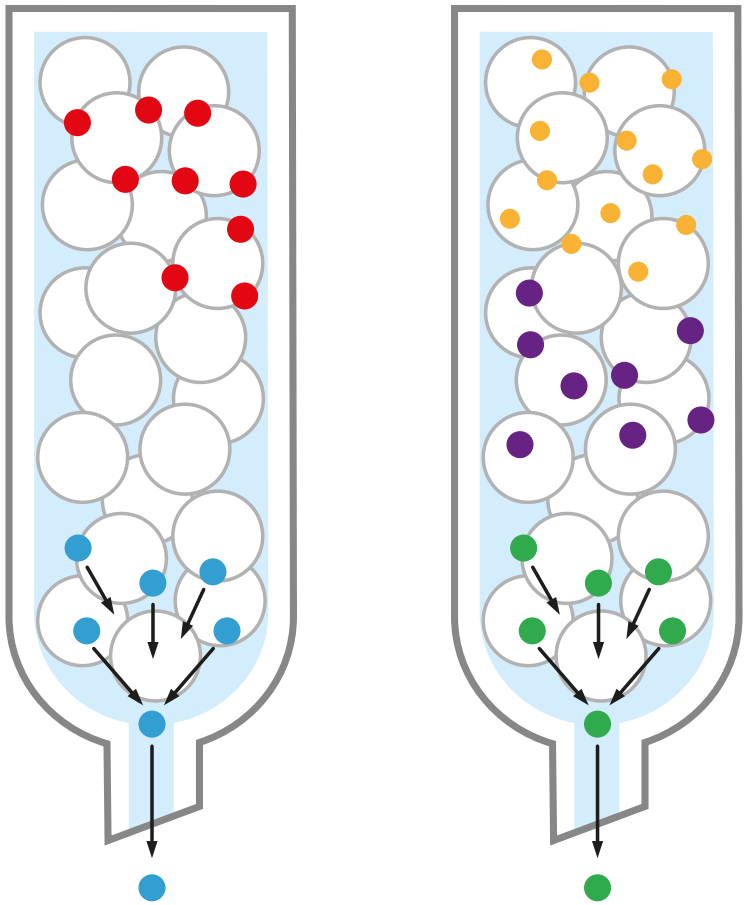
The basic guide to recombinant protein purification

From host choice to protocol design.
Learn everything you need to know before you enter the world of recombinant protein purification

by Dr. José Luis Corchero



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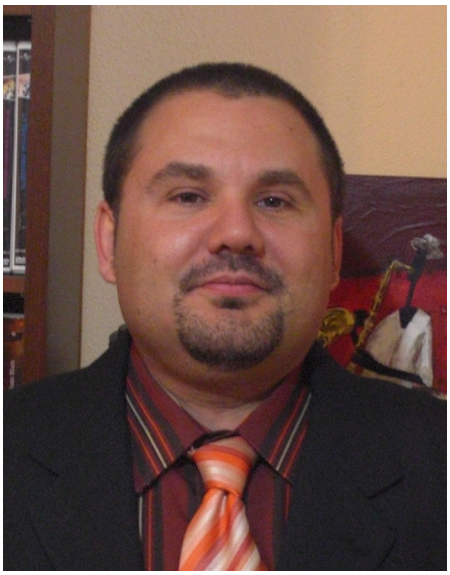
SUMARY

About the author:

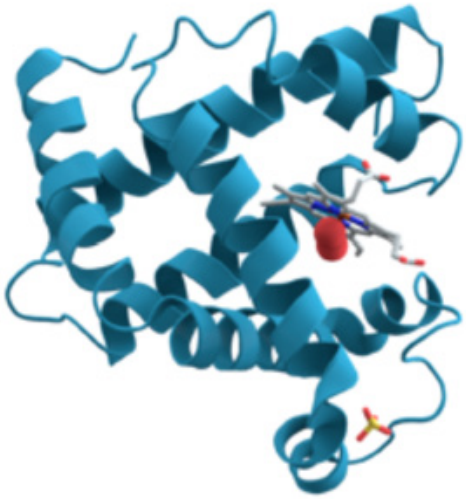
Dr. José L. Corchero graduated in Biology in 1990. After that, he obtained a Master Degree (1993) in Biotechnology and a PhD. (1997) in Biological Sciences. His doctoral thesis dealt with several aspects regarding production of recombinant proteins in E. coli, protein structure, folding and aggregation, and bacterial physiology. Then, he moved to the “”Center for Disease Control and Prevention” (Atlanta, USA) with a post-doctoral fellowship, where he worked for 2 years on detection and diagnostic of herpesviruses. In 2000, Dr. Corchero joined the R+D section of Biokit SA., where he developed diagnostic kits based on magnetic microparticles.

Since 2007, Dr. Corchero is a senior scientist of CIBER-BBN (Networking on Bioengineering, Biomaterials and Nanomedicine) within the research group of Prof. Antonio Villaverde (IBB, Universitat Autònoma de Barcelona). This group is mainly interested in a) the development of non-viral, protein-only vectors for gene therapy, b) the study of protein aggregation in bacterial and mammalian cells, c) production of recombinant proteins for enzyme replacement therapy and d) applications of bacterial inclusion bodies in tissue engineering (“nanoscaffolds”) and as therapeutic agents (“nanopills”). Dr. Corchero current research mainly deals with production of therapeutic proteins for rare diseases and the development of new vehicles for the targeted delivery of such proteins.



Introduction. An introduction to recombinant protein purification

Human beings extensively use proteins for different purposes such as health, industrial production or food. Nevertheless, most of these proteins come from natural sources that do not produce them in sufficient quantities for human use; therefore, we turn to the use of recombinant proteins. Such proteins are obtained from organisms (called “host organisms”) that do not produce them naturally, and in which the gene of the protein of interest is incorporated.



This opens a plethora of possibilities in the production of proteins of interest to humans, but as we can imagine, there are also many questions emerging when planning how to obtain them. What advantages does the use of recombinant proteins have? What is the best host organism to produce them? How do we get this organism to produce the protein?

A key aspect to take into account is that there is always the need to purify a protein before using it for any purpose, since we have to separate it from the rest of the contaminants in the culture medium in order to be able to work properly with it. This purification process can reach various degrees of purity, although the most demanding ones will be usually reserved for the most delicate uses, such as therapeutic purposes.

Purification processes can be performed through several techniques; however, the most commonly used is chromatography, which relies on separating molecules according to differences in their properties such as size or electric charge. Although affinity chromatography is the preferred method, due to its efficiency, we can also note the ion exchange, hydrophobic or gel filtration chromatographies.

In addition to these standard chromatographies, there are alternative methods that are being used, such as purification through magnetic particles, due to the several advantages they offer.

Purification processes are usually composed by various steps, being the final goal to obtain the protein of interest with the highest possible level of purity, starting from the cell culture producing the protein. Thus, previous to the chromatographic methods, there is always an initial preparation step, in which producer cells and the culture medium are separated. If the target protein is located within the cell fraction, there is the need to lyse the cells and clarify the sample in order to eliminate larger particles or aggregates that could interfere in further steps.

After the preparation step, our target protein is ready to be separated from contaminants, in the so called the “capture phase”. Highly specific methods such as affinity chromatography allow us to obtain a high level of purity in a single step. However, some cases might need the combination of several steps in order to achieve the desired results.
For protein purification with delicate uses and that require a very high level of purity, such as therapeutics, there should be a final step called polishing, which separates the protein of interest from the remaining contaminants in order to obtain the final, desired high level of purity.

The planning of a purification process is based on several factors, spanning from the properties of the recombinant protein to its final application. We have included ideas and advices for this planning in this guide.

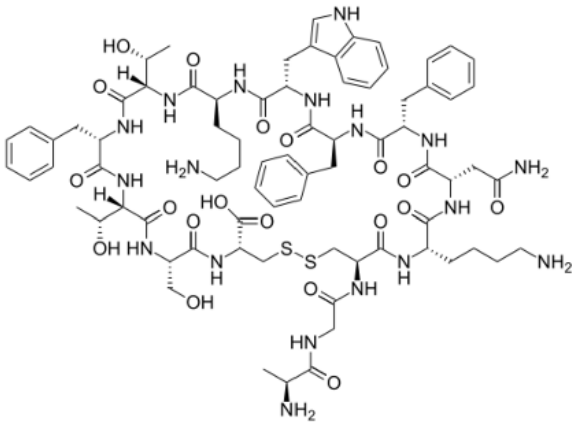
The selection of the purification techniques applied in each step is not accidental, but it's based on the purpose of each of these steps. The resolution, capacity, speed and recovery of each technique are always taken into account.

Finally, we have to consider the existing alternatives for protein purification, for example the magnetophoretic procedures. These techniques, based on the use of magnetic particles as a solid phase, eliminate some of the time-consuming sample preparation steps, such as clarification, and they reduce buffer volumes and time.

Chapter 1. The 2 key reasons to work with recombinant proteins

Proteins are biomolecules of great value to humans, since they have a wide variety of uses in different sectors. They can have therapeutic (such as in insulin cases or blood clotting factors), industrial (such as the lipases that are included in detergents that degrade grease stains) or biotechnological (in the case of certain toxins that are used as pesticides) applications. They also have an extensive application in the research field, in cases when they can be used to study the molecular mechanisms of many diseases, find new drugs, or elucidate tridimensional structures, among others.

A large part of these proteins comes from higher organisms, which produce them in small quantities, making more difficult to obtain them in sufficient quantities to further studies. In order to solve this problem and to produce proteins with high added value in a cheap and simple way, we turn to recombinant proteins. Briefly, a recombinant protein is a protein that has been produced in an organism or a cell that doesn't produce it in a natural way, which is called the host.



A classic example of a recombinant protein is insulin, a protein produced by the pancreatic cells of a healthy person in order to regulate the levels of carbohydrates and fats in the blood. People affected by the types of insulin-dependent diabetes, however, do not produce insulin and they depend on its external intake to survive. The insulin for therapeutic purposes was originally obtained from porcine pancreatic tissue, an expensive and not very effective process. In addition, since the insulin was obtained from a non-human source, it wasn't equivalent and its efficiency and safety were compromised.

Further advances in molecular biology enabled human insulin to be produced in bacterial cells, with Escherichia coli being the most common organism for this purpose. It was a recombinant protein that could be produced in a cheaper and safer way than the use of animals, making its industrial production more profitable.

All recombinant proteins are obtained by expressing a cloned ("recombinant") gene in a cell line that is adequate due to its characteristics. Using current molecular biology techniques allows not only the introduction of the recombinant gene into the host cell but also offers the possibility to modify the recombinant gene.

This is not an essential step, but provides many advantages to the entire production process.

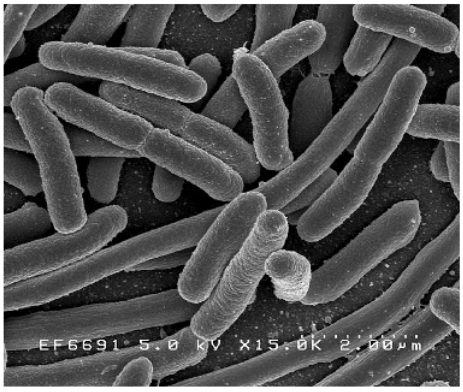
Modifications can be introduced into the gene to obtain an improved recombinant protein, in terms of better activity, solubility or stability, for example. There is also the possibility to add to the protein a specific sequence called tag, which will allow to identify and/or purify the protein.

As we can see, the production of recombinant proteins assumes important improvements in respect to the direct extraction from their natural hosts. In addition to improving the safety and economic aspects (the expressed gene is human, and it is made in the optimal organism for its production), the possibility to modify the gene and the final properties of the protein offer many additional advantages.

Chapter 2. The 4 key hosts for the production of recombinant proteins

Production techniques of recombinant proteins offer multiple options when it comes to available hosts; in other words, the organisms that incorporate the gene of the protein of interest and express it correctly.

Among all possible hosts, we have traditionally turned to bacterial cells for the production of recombinant proteins, especially Escherichia coli. This is due to the fact that it is probably the best-known organism. In addition, bacterial cells can easily grow and multiply, usually doing it in well-established and easily available culture media, involving less difficulties and a reduced cost.
However, when it comes to prokaryotic organisms, they are incapable of carrying out post-translational modification, such as splicing, glycosylation or the formation of disulfide bonds. These modifications tend to be vital for most of the eukaryotic protein functions, meaning that prokaryotic organisms do not allow us to obtain a fully functional product.



These limitations force us to use more complex organisms, turning now to the eukaryotic territory. Yeasts are very useful in this sense, since they are simple enough to work with comfortably while being able to introduce the majority of post-translational modifications to the proteins. We must take into account that not all of these modifications are identical to the ones developed by human cells. For example, glycosylation is slightly different, and therefore it can produce an immunological response if the protein has therapeutic purposes.
Another host cells with sufficient advantages to be used extensively are the insect cells. For the production of recombinant proteins in insect cells, a recombinant baculovirus must be obtained, which will be used as a vector to introduce the recombinant gene into the host cells.

Finally, the most similar host organisms to humans are the mammalian cells. These cells involve more complex work, but their behavior is closer to that of the human cell (when it comes to the production of proteins and their post-translational modification). In certain cases, it is important to induce mutations in the protein to study its effects or to try to improve its functionality. Mammalian cells allow, once you have the recombinant plasmid that encodes for this mutant, the obtention of several mg of recombinant protein in a few days, by means of transient gene expression.

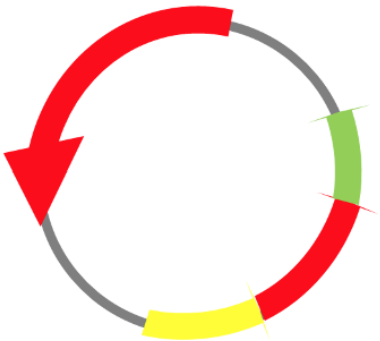
We have here reviewed the host organisms most commonly used for the production of recombinant proteins. Although all of them offer advantages and disadvantages, the final decision about which organism to work with is always made based on the complexity and the application of the produced protein.

In other words, while a simple protein or one not intended for therapeutic purposes could be produced in bacteria or yeasts, proteins that need to maintain their functionality should be synthesized in more complex cell hosts.

Chapter 3. How to design the best vector for the production of recombinant proteins

The host in which a recombinant protein is produced doesn't naturally include the gene of this protein in its genome. Therefore, this gene needs to be introduced in a process called molecular cloning. Successful cloning of a gene requires several elements, which are discussed below.

First, we need a DNA fragment containing the gene of the protein of interest. It will usually be a restriction fragment, although sometimes PCR will be performed in order to create a fragment that contains the gene. The second important element is the vector, a DNA molecule into which the gene will be inserted and that will serve as a vehicle for the genetic information. Any piece of DNA could be used as a vector, as long as it includes an origin of replication in order to be able to replicate following entry into the host cells. Bacterial plasmids and genomes of certain viruses (such as bacteriophages and baculovirus) are the most common vectors, since they can replicate independently from the host cell in which they are introduced. This enables a high amplification of the recombinant DNA, so it can be replicated in a greater number of copies than the chromosomal DNA.



Third, it is necessary to have a marker to differentiate the hosts that have incorporated the gene of the recombinant protein (transformant individuals) from the hosts that haven't. This is because the efficiency of the process can be relatively low.
Several methods exist for that purpose, although the most common one tends to select conditions in which the vector confers a selectable phenotype, such as resistance to antibiotics. By accompanying the introduced gene with another gene conferring resistance to antibiotics, by applying this antibiotic in the culture medium, we will select those host cells that have incorporated the vector and therefore, that will express the recombinant protein. Two examples of these genes are ampR y tetR, which confer resistance to ampicillin and tetracycline, respectively.
Selection can also be performed by using nutritional requirements as a marker, or by directly detecting the presence of the gene (through colony hybridization) or of the protein (through specific antibodies).

One key element to control recombinant gene transcription is the promoter, the DNA region before the gene that regulates its expression. The promoter can be constitutive (always active), although inducible promoters are more frequently used in order to control gene expression.

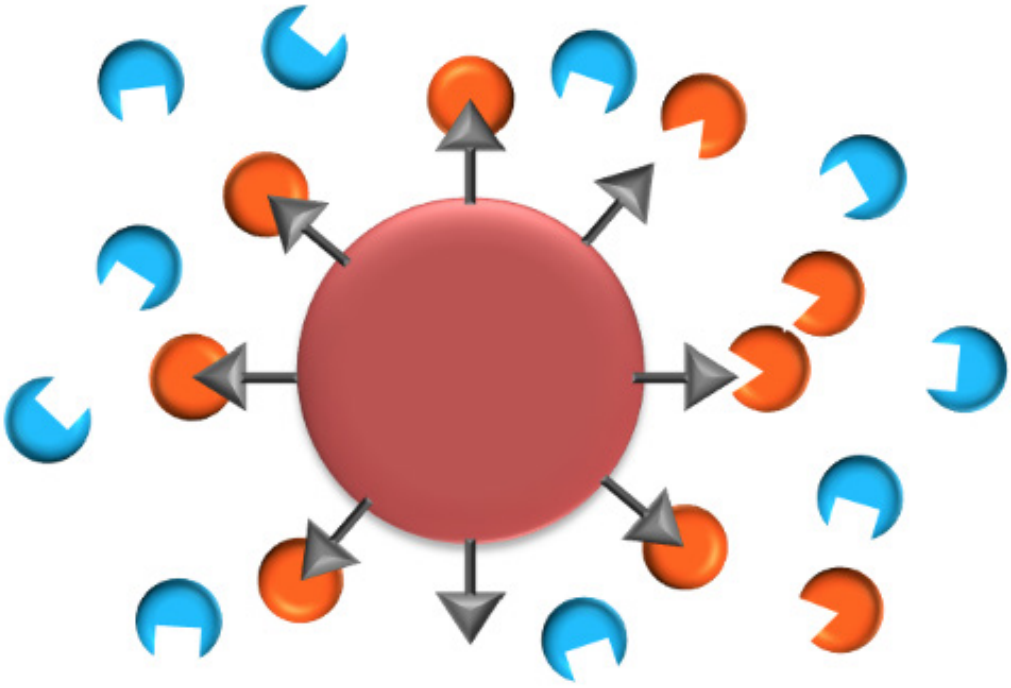
The choice of the promoter will depend upon several aspects, like the toxicity of the recombinant protein for the producing cell, for example.
Another element that is commonly included in current expression vectors is DNA regions encoding for different tags (that will be expressed in frame with our protein and that will facilitate its purification).
The ideal vector for the production of recombinant proteins should incorporate all these elements that have just been discussed. There are many options, since there is a variety of promoters, plasmids and markers, but a specific study for each case will allow us to optimize the process and develop it under the best conditions.

Chapter 4. The 6 most effective methods to purify recombinant proteins

Recombinant proteins are produced in the host cells along with a great variety of molecules that it contains naturally. However, for most of their applications (such as for example in therapeutics), a recombinant protein should be purified and isolated from the rest of cell molecules. This is obtained by the purification processes.

Examples in which purification is necessary is in protein structure or sequence studies. In this case, we cannot have the protein of interest mixed with other molecules in order to ensure that the obtained results are reliable. Another example is a protein with a therapeutic application, a case in which we need to ensure that only the therapeutic protein is injected in the recipient organism to avoid unexpected or confusing results.

Most of the methods for the purification of recombinant proteins are based on chromatography, which is in fact a set of different techniques. Chromatography techniques perform a separation based on differences between the properties of the protein to be purified and the properties of the rest of molecules of the sample.



These properties can be specific structures of the molecule, the electric charge or the size, among others.

The most widely used method for protein purification is affinity chromatography, which separates proteins based on their specific interaction with a matrix. One of these methods is gel filtration, which separates molecules according to their size.

By using a resin with pores of a specific diameter, molecules can be separated by the difficulty they encounter when passing through it. Another commonly used method is ion exchange chromatography, which separates molecules based on the electric charge they have in certain conditions of pH and temperature.

It is one of the most effective techniques, since it takes advantage of the incorporation of a structure of choice (called a tag) onto the protein. Such tag is not present in any other molecule of the sample, conferring to our target protein the specific characteristics that will be used to distinguish and separate from the rest. However, in certain situations, we cannot add a tag to our molecule, and we need to turn to less specific methods (although, they can be equally effective, if they are used correctly).

Two other common methods are those that separate proteins based on their polarity; the most used ones being the hydrophobic interaction chromatography and the reverse phase chromatography. The main difference among these methods is the polarity of the matrix with which the protein to be purified interacts.

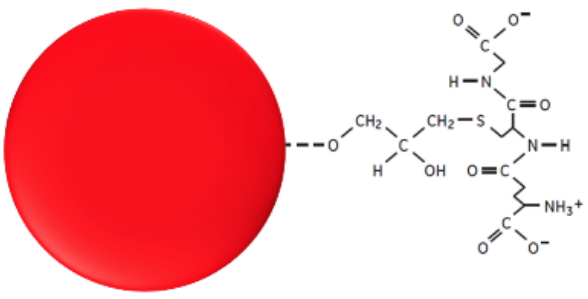
These last techniques are less specific than affinity chromatography, since it is very common to find molecules with similar size and/or charge; therefore, they won't separate very well. Due to this, these techniques are usually performed in combinations of two or more in order to achieve good results. They

Chapter 5. The 4 best tags to purify recombinant proteins

Chromatography is one of the most common methods for the purification of recombinant proteins, and more specifically affinity chromatography is the one that is mostly used due to its high specificity, which allows us to obtain great purity in one single step. When using this technique, a tag is added to the protein of interest, a small structure that is not included in the original protein and that allows us to easily capture it.

Generally, tags are short sequences of 3-4 amino acids (up to a maximum of 15) and are intended to minimize as much as possible the properties of the protein. We have to take into account that there are very sensitive proteins that easily lose their activity when their sequences are modified; therefore, this technique cannot be used for all cases.

An initial study should be performed in order to determine in which region of the protein the tag will be incorporated, out of which the most common and less harmful are the terminal ends and solvent-exposed loops. In some cases, it is convenient to eliminate the tag after the purification in order to obtain a protein as similar as possible to the original; thus, there are enzymatic methods for this function. In other circumstances, if the tag doesn't cause activity loss, there is no need to eliminate it and it can be left in the final structure.



Hence, a tag allows us to separate the protein from the rest of the elements in the sample, but it also allows us to detect the presence of the protein in case there is no specific antibody for it.

In this case, we add the protein, a known tag, and a specific antibody is used to measure its presence during the entire process. At the end, this turns out to be cheaper rather than producing a specific antibody for the protein, which is a very long and expensive process.

The most widely used tags in the purification of recombinant proteins are the histidine tag, which are incorporated either into the C- or N-terminal ends. They consist of a 6-histidine residue motif (at least), an amino acid that has high affinity and selectivity for the ions of nickel and other metals. These ions can be bound in a matrix through chelating agents with which they form complexes.

By this "tagging" process, a protein with the histidine tag is the molecule that presents a stronger binding with the matrix (in comparison to the rest of the molecules of a bacterial extract, for example). That allows us to obtain a relatively pure protein with only one purification step. If we add this effectiveness to the fact that it is a small tag (thus hardly disruptive) and that it only depends on the primary structure of the protein, it is easily understood why it is the preferred option.

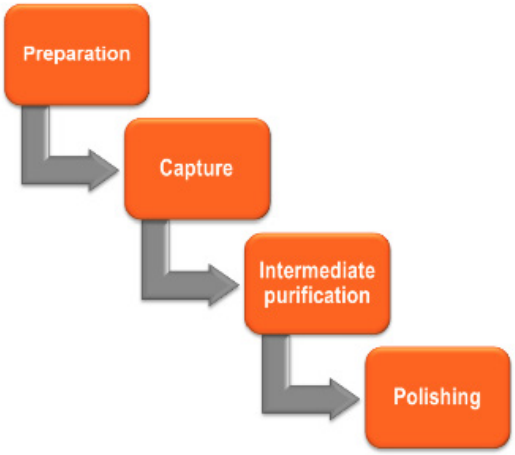
Another popular option is Glutathione S-Transferase (GST) tags, which are based on the affinity of GST by the glutathione ligand (that can be bound to a matrix). This binding to the ligand is reversible, and allows a gentle purification process that doesn't affect the protein structure or function. However, this tag has a larger size than the histidine one; thus, it may be difficult working with it.

We also have the Maltose Binding Protein (MBP) tag and the Strep-tag II. The first one binds to maltose and they can confer to the fused protein increased expression levels and higher solubility, additionally facilitating the protein folding. Regarding the Strep-tag II, it is a small sequence of 8 amino acids, which due to their size rarely interferes in the protein structure; therefore, there is no need to eliminate it. It binds specifically to the streptavidin ligand, which can also be bound in a matrix.

As we have seen, we have a great variety of tags for the purification of recombinant proteins. Although the histidine tags are the most widely used, the rest also offer many interesting advantages that we should consider.

Chapter 6. The step-by-step process of protein purification

Protein purification has as a final goal the isolation of the protein of interest from its environment, in other words, from the remaining molecules that are contained in the host that produce the protein. Although there are several available techniques and many options to consider, the general outline is the same.



The process starts with the preparation of the sample, which consists of cell harvesting, cell disruption (incase our target protein is intracellular) and clarification. Cell harvesting involves separating the cells from the culture medium, usually by centrifugation or filtration. As for the cell disruption, it can be achieved through different methodologies that will be chosen depending on the host.

Not all cells oppose the same resistance to lysis, and this should be taken into account because at times, specific protocols are needed for the more resistant cells (bacterial cells, due to its wall).

The most widely used techniques are chemical methods (such as enzymatic methods or detergents) and physical methods (such as sonication or the French press).

All methods have advantages and disadvantages, and they are never fully effective. Due to this, we tend to use combinations of methods, such as, for example, sonication followed by a detergent treatment. Obviously, in the case of preparing a sample of secreted proteins, cell disruption will not be needed and one step will be eliminated.

The last preparation step is the clarification, which is needed because the protein to be purified will be mixed with other elements such as membrane remains, organelles, cellular debris or insoluble proteins.

This is achieved by filtration or centrifugation, succeeding in separating the liquid fraction with dissolved soluble molecules from the remaining cells. The goal is to obtain a clean medium of particulates, which will allow us to carry out the separation itself. It is during these steps that we try to decrease the working volume in order to deal with more manageable volumes.

The next purification step is recovery or capture, which has as a goal to isolate, concentrate and stabilize the protein of interest. Always preserving its functionality, the protein is concentrated and the most important contaminants are eliminated. This step functions as a simple and fast initial purification that will be refined later, although a high level of purity can be obtained with very specific methods.

As discussed before, affinity chromatography is, currently, the most common method for purifying recombinant proteins.

We should also consider the magnetic particles option, which provides many advantages in regards to chromatography and assumes a general simplification of the process. In case of using less specific methods, an intermediate purification may occur in order to separate the protein of interest from the rest of the contaminants that still accompany it after the clarification.

The last step is polishing, although it overlaps a little with the purification and it's not always needed. This step consists of eliminating as much of the impurities as possible, performing more chromatographies, or eliminating some elements used in the chromatography that are not needed (or even undesired) in the final product. We can do a dialysis or change the buffer in order to have the already purified final protein in adequate conditions, such as pH or salinity. Polishing is done or not depending on the desired purity grade and on the acceptable efforts to be put into the process. As already discussed, this will depend on the use of the purified protein.

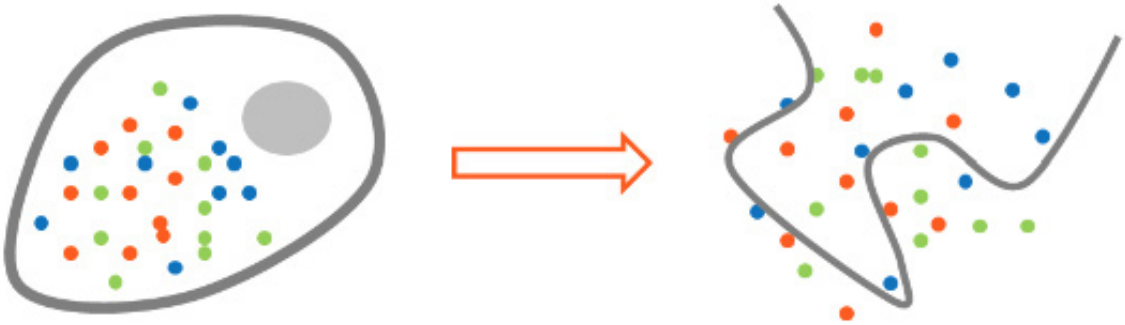
Chapter 7. The 5 factors to take into account in the preparation of samples to purify recombinant proteins

During the sample preparation for the purification of recombinant proteins, there are several considerations that will influence its development. All preparation steps are important and we have to pay attention to them.

For example, cell collection and disruption can be very simple or complicated steps, depending on the cells that we work with. Eukaryotic cells are easily ruptured, but prokaryotic cells have a cell wall and are harder to lyse. This is something to take into account, since an excessive treatment may distort the protein of interest or release proteolytic enzymes that can degrade the protein. Protease inhibitors can help in this aspect, and nucleases can solve viscosity problems.

Protein stability is another factor to take into consideration, since protein structure must be maintained in order to not lose any type of biological activity. Cell lysis produces the release of proteases that affect this structure; therefore, stability tests help us to choose the best, less harmful procedure. Changes in temperature, pH and salinity can also affect the protein. There are proteins that are very stable with which we can work in a more permissive manner, while there are others that are more unstable and that we have to protect with inhibitors and more controlled conditions.

Finally, detection and quantification come into play. We have to consider that in all of the steps we



the amount of the target protein and its recovery after each purification step. It would be also helpful to have an enzymatic assay in order to check if there is some loss in protein activity (we may recover a large protein amount, but it can be useless if we are affecting its biological activity).

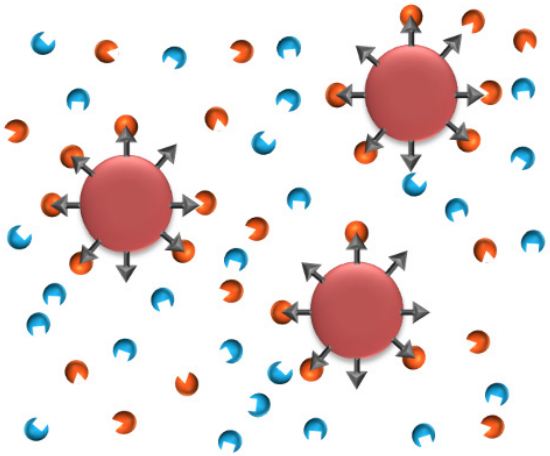
On the other hand, clarification during the preparation for chromatographic purification is also very important, since whole cells or even cell debris, organelles or other cytoplasmic components cannot be passed through a chromatography column. After the clarification step, the sample should be in an adequate buffer so that the interaction with the column is optimal. For this clarification step, centrifugation or filtration are usually performed. Both methods are conceptually easy steps, but they tend to be problematic when working with large volumes. Due to this, it's recommended to achieve maximum concentration during the first stages in order to keep a reduced sample volume and in that way simplify the subsequent work.

We can see that there are steps to take into account before, during and after the purification. It's very important to do a bibliographic search beforehand, in order to have as much information as possible. Due to this, if we have little information, multiple mistakes can be made in the process and the protein might be affected during the purification.

Chapter 8. Four great tips for better capture in the purification of recombinant proteins

The purification of recombinant proteins is a long and complex process, which is influenced by a multitude of variables. As has already been mentioned, the nature of the protein itself, its properties and the characteristics of the host producer will greatly influence the design and development of this procedure. There are also very few available techniques, although it is very well known that the preferred one is affinity chromatography.

After the preparation of the sample to be purified, which consists of the collection and disruption of cells followed by clarification, comes the capture step. In this phase, the goal is the isolation and concentration of the recombinant protein of interest, aside from a correct stabilization of the product. Concentration is needed to avoid additional workload that involves the purification of large volumes of culture medium, since the chromatography column can only withstand limited flows and pressures. Thus, a decrease in the work volume means decrease in the processing time, something that is always beneficial.



On the other hand, there is a need to consider the characteristics of the protein that we are working with. A protein that is very sensitive to the changes in the environment will be easily affected by, for example, the changes in temperature or pH, or by the presence of proteases. This will directly affect the way we work, which will need to be done more carefully, always keeping the temperature at low levels and applying protease inhibitors. However, there are less liable proteins that are affected to a lesser degree by these changes in the environment, so working with them will be more comfortable and permissive.

Obviously, the isolation of the recombinant protein is the main goal of the purification. The separation of the protein from the molecules in its environment, mainly produced naturally by the host organism, assures us that we will obtain a pure extract that can be used in most applications. However, in most cases this is impossible to achieve and therefore we work with purity degrees (a percentage that represents the molecule of interest in relation to the total sample).

In any case, the selected purification method has to be optimized in order to achieve fast, cost-efficient protocols. When we can use highly efficient methods, such as affinity chromatography, we can successfully eliminate almost all of the contaminants, and achieve a high level of purity. However, other times we have to use several types of chromatography, which tend to be less cost-effective.

Additionally, the chosen method needs to present the highest possible capacity and specificity for the recombinant protein. In other words, we need to look for a technique that is capable of binding as many recombinant proteins as possible, while at the same time excluding as many contaminants as possible.
As already discussed, in the capture we need to focus on obtaining a strong and fast selection, without seeking to achieve a completely pure extract at the end of the process.

Chapter 9. The 4 basic principles for an efficient purification of recombinant proteins

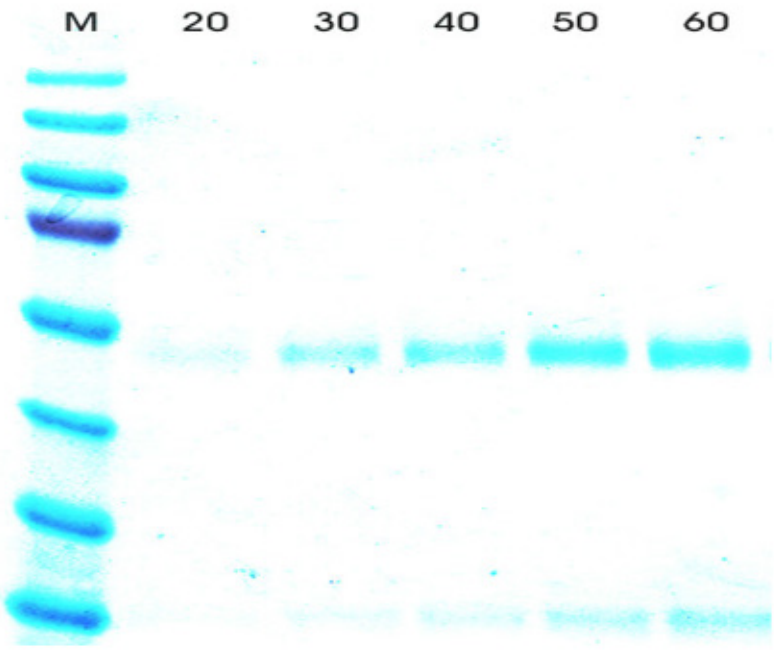
Affinity chromatography allows us to obtain good results and a high level of purity with a single purification step, since a structure that is exclusively found in the recombinant protein is used as a tag. However, this is not possible in all cases.

There are proteins that don't accept changes in their sequence, even if the changes are so minimal as the incorporation of a tag, since the proteins quickly lose their biological activity with any modifications in their structure.

In this case, we turn to less specific methods, such as ion exchange chromatography or gel filtration. By having lower specificity, combining several techniques and creating a purification protocol in several steps is crucial. These steps are capture, intermediate purification and polishing.

The question that derives from this necessity is: How to choose these techniques and apply them in an order that gives the best results? Knowledge of the purpose of each stage and the properties that are offered by each technique are key for the correct development of the purification. Thus, there are 4 characteristics to assess in each stage of the protocol.

First, there is the resolution of the technique, this is, its capacity to differentiate and separate molecules with similar properties.



The resolution depends on several factors such as selectivity of the column or the molecules contained in the sample. It gains importance in the last stages of the purification, when most contaminants have been eliminated and only the most similar ones to the protein to be purified are left.

Second, we need to assess the binding capacity, which refers to the sample amount that is admitted each time by the chromatography matrix. This can depend on the volume or the total amount of molecules that can bind to the column. Therefore, it's interesting to have an increased capacity at the beginning of the purification, since at the end of the process the sample amount is reduced.

We also need to take into account the speed of the process, especially in the first stages. As already mentioned, fast purification minimizes the exposure of the recombinant protein to proteases and other contaminants that can degrade it, optimizing the recovered amount at the end. Additionally, the initial purification stages are the ones that present highest sample volumes, so that velocity speeds up the overall process.

Lastly, recovery gains importance as the purification advances, since at the same time it increases the value of the purified product. It can be compromised by factors that affect the recombinant protein, such as exposure to proteases or extreme conditions of pH and temperature.

The optimization of one of these parameters can affect the others. For this purpose, in the capture stage the efforts are focused on boosting the capacity and speed in order to quickly eliminate a vast majority of contaminants and avoid the degradation of the protein to be purified. It's common to apply affinity chromatography, immobilized metal ion affinity chromatography or ion exchange chromatography.

On the other hand, resolution and capacity gain more importance in intermediate purification. Although on occasions it can be omitted, this stage focuses on eliminating contaminants more similar to the recombinant protein, while always maintaining the capacity. We turn to techniques with good specificity, such as ion exchange chromatography or hydrophobic reaction.

Finally, if we need to refine the results we come to the polishing stage. Only the contaminants most similar to the protein of interest are left, and at the same time the losses of this one are more costly; thus, recovery and resolution become a priority. It's common to apply gel filtration or ion exchange chromatography at this stage.

Chapter 10. How to achieve the maximum purification of recombinant proteins through polishing

In purification of recombinant proteins, highly pure samples are rarely obtained with the initial stages of the process. Whether we perform a highly specific affinity chromatography (with histidine tags, for example) or purification with several stages of capture and intermediate purification, there are always contaminants in the final sample. These contaminants are molecules that are closely related to the protein to be purified since, if a high-resolution technique is not applied, they can be hardly differentiated from the protein to be purified.

This is acceptable when producing recombinant proteins for industrial uses. For example, the lipases that are incorporated in certain detergents, as well as other enzymes that participate in the fermentation process, do not require a high level of purity and thus they allow more permissive work. However, proteins with therapeutic uses, such as insulin or clotting factors, have to be dispensed in a highly pure way in order to avoid adverse reaction or interactions with contaminants.

In these last cases, the acceptable level of purity is more restrictive; therefore, the purification needs to be highly optimized and the present contaminants reduced to a minimum. With this purpose, and after the initial purification stages, a final stage called polishing can be incorporated. Although it can overlap with the previous stages, and it can even apply the same techniques, the purpose of polishing is to obtain the desired final purity.

As is clear, one of the vital characteristics of this stage is the resolution. In the previous steps, the majority of the contaminants were eliminated, which normally are formed by molecules with very different properties from the protein to be purified. Thus, only traces of protein impurity and other undesired substances will be left, such as endotoxins, nucleic acids or viruses. Additionally, it is possible to find structural variants of the protein itself, which also need to be eliminated in order to achieve the highest purity.

Therefore, the election technique should be able to discriminate the recombinant protein and the rest of the molecules with which it shares many properties, but are not needed in the final extract. This is achieved through a technique of high resolution, especially with gel filtration. Good recovery is also very important in polishing, since as the purification advances the loss of molecules of the protein of interest becomes more costly.

To sum up, we can see that polishing is an optional but very important stage for the most demanding purification processes. It allows us to obtain high purity in the final extract, and at the same time recover the purified product in a buffer adequate for its later use. The elimination of tags and other elements used in chromatography (but without any use in the purified product) are also included in this stage, deeply contributing to the procurement of the desired level of purity.

Chapter 11. How to purify recombinant proteins through magnetic particles

It is well known that most purification processes of recombinant proteins are conducted through chromatographies of different types, mainly in column chromatography. Whether it's by affinity chromatography, gel filtration or ion exchange chromatography, these already established methods are not exempt from limitations. Research in recent years has allowed us to develop a series of alternatives to chromatography that allow us to avoid many of these limitations.

One of the main restricting factors in the conventional process is the clarification step, which is necessary because particulates cannot be injected into a chromatography column. This is about eliminating all of the cell debris and organelles that can interfere in the column, but it's always a process that involves additional time and dedication.

The sample volume is also critical, since purifying small volumes is not a huge problem, but when it comes to several liters of culture (as in the case of proteins secreted in the medium) several complications appear. Recurring solutions are concentrating the protein or injecting this volume through the column. This process already involves the use of more equipment and time, aside from monitoring the input pressure and flow in order to avoid the resin of the column getting clogged and, therefore, becoming disabled.

A great alternative to chromatography that solves these limitations is the purification of recombinant proteins through magnetic particles. They contain a core with magnetic properties, a key element that allows a fast and much simpler purification than chromatography. These small size spherical particles (in the nano- and micro- range in diameter) are coated with an appropriate ligand (as those used in chromatography procedures).

Magnetic particles allow selective separation and purification of molecules suspended in raw culture mediums. This is possible by coating them with any ligand or structure that specifically capture the protein of interest, such as nickel ions, in order to purify proteins with histidine tags.

Simply, we need to add the adequate magnetic particles in the medium where the molecule of interest is found, allow an incubation time to produce the capture and apply a magnetic field to attract the particle-protein complexes towards the walls of the recipient.

Supernatant containing unbound molecules are removed. Then, magnetic field is removed and a wash buffer is applied to eliminate non-specific binding. Finally, we capture the particles once again (still bound to the protein of interest) and we apply an elution buffer to separate the protein from the magnetic particles.

Cells and the remaining cell fractions (such as organelles or membrane fragments) do not have magnetic properties; therefore, they will not hinder the separation of the protein. This allows us to eliminate the clarification step, which is needed in chromatography in order to avoid clotting of the column.

At the same time, concentration is greatly simplified. Even though at the beginning we might have several liters of the sample, after the capture we can resuspend the particles in a smaller volume with which we can work much more comfortably.
On the other hand, the purification time is reduced, since passing several liters of the medium with a peristaltic pump in chromatography can take several hours, with a constant control of the process. With magnetic particles, capture and separation steps can occur within a few minutes.

Also, the required machinery and infrastructure notably decrease with the use of magnetic particles. When we work with chromatography, even though the column is small and simple, the necessary equipment is cumbersome and requires high maintenance and technical knowledge. However, when working with magnetic particles we only need the particles and a device capable of creating a magnetic field, such as, for example, an adequate magnet.

As we can see, the use of magnetic particles in the purification of proteins brings many advantages when compared to the traditionally used chromatography procedures. The separation is quick, specific and scalable, allowing an easy automation. Now, due to its limited capture capacity, it allows the purification of reduced amounts of protein; therefore, its industrial application is currently not feasible and its use is restricted to the research labs.

Chapter 12. The 3 most important considerations in designing magnetic particles

It is well known that most recombinant protein purifications are mainly done through different types of chromatography. However, the use of magnetic particles is a very interesting alternative to these techniques, providing great advantages and simplifying the process in many aspects. The necessary equipment for purification with magnetic particles is simple: we need a magnet or any device capable of creating a magnetic field, and the particles themselves.

Each magnetic particle contains a nucleus with magnetic properties, which constitutes the base of the structure. The nucleus is generally formed by magnetite (Fe_3O_4), which has superparamagnetic properties, or by maghemite (Fe_2O_3), with ferromagnetic properties.

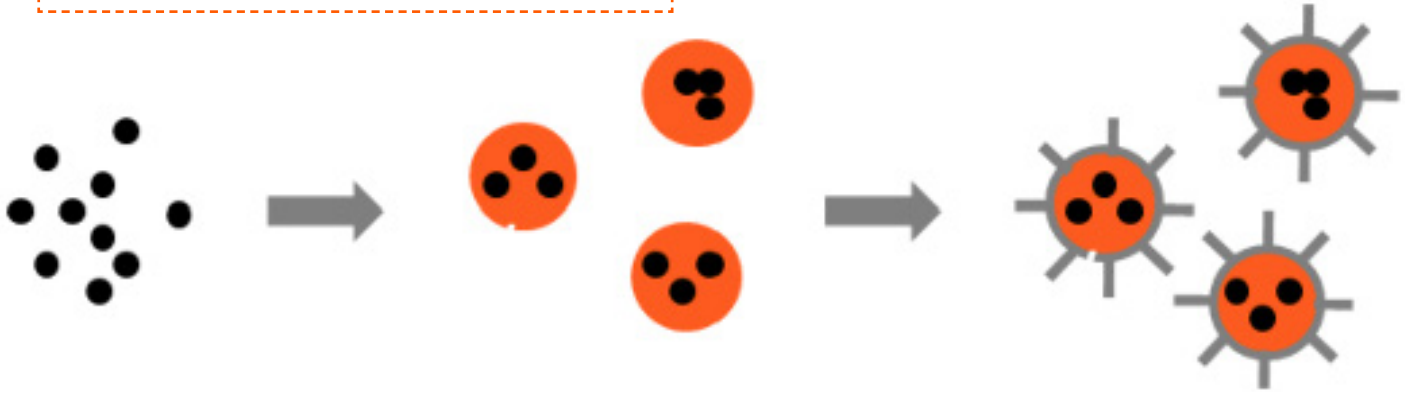
The advantages of superparamagnetic particles are their easy resuspension, large surface area, slow sedimentation and even distribution in the medium. When they become magnetized, they behave as small permanent magnets, forming aggregates or reticles due to the magnetic interactions.

On the other hand, ferromagnetism confers the particles a permanent magnetic moment, which exceeds the fluctuation caused by the thermal excitement. This means that the ferromagnetic particles have strong magnetic properties, allowing an easy separation when an external field is applied, even in a viscous medium.

Aside from having a magnetic nucleus and the polymeric layer, magnetic particles have a coating that has to be designed to specifically bind the protein to be purified. There are magnetic particles designed to capture proteins with histidine tags, and they are covered by cobalt or nickel ions for this purpose. By adding the particles in the medium, the histidine tag of our protein will interact with the ions on the particles, allowing for an easy, fast capture and separation.

Other particles have chemical radicals on their surface to decorate them as needed (for example, a specific antibody to our target protein can be coated onto the particle surface).

Dedicated companies sell magnetic particles already functionalized (they have a specific name for each one), but they also sell blank particles without functional groups (for example, a coated particle with –COOH or –NH_2 group). These last ones can be coated by using such functional groups through easy chemical reactions. These groups allow ligand binding and decorate the particle. Examples of them are antibodies, enzyme substrates, streptavidin, G or A proteins (that specifically bind IgG) or amylose (for proteins that are Maltose Binding Protein, MBP).



Therefore, another great advantage of the magnetic particles is their great versatility derived from the possibility to personalize them.

Researching in order to find the optimal design of the magnetic particle allows carrying out the separation in optimal conditions. The inclusion of specific antibodies or selective ions for histidine residues provides an increased level of purity in a relatively simple procedure in comparison to conventional chromatography. However, we need to remember that due to its low binding capacity to the ligand, magnetic particles are focused on the purification of small amounts of proteins, reserving the uses at an industrial scale for the chromatography techniques.

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Follow us on:

SEPMAG SYSTEMS
contact@sepmag.eu
+34 935 820 161

Parc Tecnològic del Vallès
E-08290 Cerdanyola del Valles. Barcelona