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Ion Metallic Affinity Chromatography and Purification of Bacterial Toxin

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1. Introduction

The history of the scientific development of the Chromatography as a chemical separation tool cannot be considered ancient, as the proper establishment of Chemistry as a science is considered modern when compared to the other sciences developed before it. The first literary description of the Chromatography separation technique development is attributed to the works or Mikhail Tswet in 1890 by some authors, where the chemical separation of plant extracts was conducted inside laboratory and first established, permitting the chemical separation of plant chemical substances according to their chemical characteristics of polarity and chemical affinity to the stationary phase. At that time, due to the color attributed to the plant extracts separated experimentally, the technique was named as "chromatography", where chromato means color and graphy means registering or writing. Although the many chemical substances and bio molecules that can be separated by this technique have no color, the name was kept and the technique recognized as one of the most efficient and applicable for laboratory purification and analytical processes.

The same way Chemistry is a modern and new science, the chromatography techniques also are, as over the years chromatography has proven to be an important tool for chemical substances separation. During this processes of development of the many techniques of chemical separations promoted by chromatography, one of them was structured as an affinity chromatography technique of immobilized ions, also known as Immobilized Metallic Ion Affinity Chromatography (IMAC), which is the main focus of this book and chapter.

The first description of this technique apply was related by the methods discovered and developed Cuatrecasas et al. (1968), by which Pedro Cuatrecasas and Mier Wilchek were awarded the wolf prize in medicine in 1987, who demonstrated that affinity chromatography is a really recent technique, where the immobilization of metallic ions was first established, permitting the chemical separation of chemical substances presenting affinity to this stationary phase.

In fact, this affinity technique works on separating proteins presenting amino acids like cysteine and histidine, which have chemical affinity to metallic ions. In general manner, there are many areas where IMAC can be applied and the practice has shown it to be a very useful tool when applied to proteins presenting such amino acids expressed on their

surfaces. One good example of this is the purification of the Human Immunodeficiency Virus (HIV) reverse transcriptase by the use of IMAC.

The development on bacteria toxins purification and their characterization is one of the focus and goals of our developed works and IMAC has proven to be an excellent tool in separating toxins presenting this characteristic of ion metallic affinity, mainly by nickel (Ni^{++}) and cooper (Cu^{++}). Basically, as will be described afterwards, IMAC separates proteins by specific amino acids affinity, being selective to proteins presenting such amino acids on the surfaces of their tertiary or quaternary structures.

2. Applicability of the IMAC technique to molecular biology

Nowadays, there are many examples of the applicability of the IMAC associated to Molecular Biology on trying to purify bacteria proteins.

The Molecular Biology plays its important role in selecting the genes of the bacteria coding portions of interest proteins and toxins, where these portions are frequently selected and extracted by the action of restriction enzymes like Eco R1, among others.

After obtaining the desired restriction fragments, these ones are incorporated by complementary binding into commercial disposable bacteria plasmids, treated with the same restriction enzymes, in order to promote the correct complementary junctions.

More than the correct complementarities, these Molecular Biology tools give the assurance of a protein being synthesized with a poly histidine tag, generally introduced into the amino terminal of the protein structure to be transcribed and translated, which can be determined by the plasmids structure and the specific site of action of the restriction enzyme, promoting the target DNA (fragments obtained form bacteria) incorporation by nucleotide complementation of restricted DNA fragments.

It seems to be an easy process to be explained, but in practice, this has proven to be one of the most difficult parts of the work to be defined and conducted inside the laboratory, side by side to the problems of protein expression and purification, considering the many problems and fails that may occur in these steps. After a well succeed incorporation, it is necessary to insert these transformed plasmids into competent bacteria and verify the presence of the desired proteins expression, without forgetting that many fails during the steps of the plasmids insertion may also occur in a kind of work that may take a generous amount of time to be established in the case of unknown or not described proteins.

As a general manner, these plasmids carry a coding of an antibiotic resistance product. The target transformed bacteria can be checked by the exposition and growth in the presence of the specific antibiotic, showing the insertion step was well succeed when compared to the target bacteria not submitted to the transformation, which must be necessarily and naturally not resistant to the applied antibiotic, what gives the certainty, in the end of this step, that the bacteria submitted to the transformation process received or not the genetically inserted plasmids. Actually, nowadays these target plasmids, the restriction enzymes and the competent bacteria can be purchased in specific kits, which have the purpose of conducting and making possible this kind of work, in a manner and trying of making the process a little easier.

The fact of having the target protein or toxin being synthesized with a poly histidine tag improves the IMAC processes by the affinity of these matrixes to this amino acid.

There are also many examples of well succeed separations by this combination of Molecular Biology tools and IMAC (Catani et al., 2004), what gives the many possibilities of studying these proteins structures due to the many quantities of target protein produced by the bacteria, as the plasmids are structured and designed with a synthesis promoter portion, in a open reading frame (ORF) generally associated to the presence of a determined carbohydrate like lactose, galactose or mannose for instance.

After the protein purification steps, the products are submitted to a final specific enzymes action, which promote the poly histidine tag cleavage. Overall a final purification step is associated to this process, such as a gel filtration (molecular exclusion) chromatography, for example, to separate the target protein and the poly histidine tag fragments, or any other technique like a second dimensional electrophoresis (2D Gel Electrophoresis).

These techniques have permitted, in practice, many researches to reveal the third dimensional structures of many structural proteins, toxins and enzymes, associating the IMAC tool to other kind of analyses like Mass Spectrometry, Crystallography, Nuclear Magnetic Resonance, Nuclear Magnetic Resonance Spectroscopy, among the examples of possibilities of analytical processes offered by the recent Chemistry analysis developed technologies.

3. Why does IMAC Chromatography works on toxin purification of bacteria not genetically modified?

The concept of IMAC chromatography has been attributed nowadays as a chromatography technique applied to the areas where Molecular Biology have been also applied, where proteins are designed to be synthesised with poly histidine tags, making ion metallic affinity possible by its interaction. In fact this first explanation of applicability to this kind chromatography is the one which is the mostly used and scattered in the scientific world, but what has to be reminded is that these molecular tools are applied and adjusted to the proteins that do not present these characteristics of natural ion metallic affinity. Though, reminding also the history of the development of the technique, it must not be forgotten that there are many proteins, from the most different possible origins, presenting natural ion metallic affinity, attributed to the presence of histidine and cysteine in their structures, which can be purified by the applying of this technique as well.

By having this general overview, its easier to understand why sometimes it is naturally possible to have contaminants and interferences when conducting IMAC purification processes of products of genetically transformed microorganisms. Our practice in purifying bacteria toxins has proven that there are naturally microorganisms products that present also affinity to metallic ions, which may not only interfere in the purification processes, but must be separated from the target protein in order not to prejudice the following analyses steps, when the focus of studying a determined protein has or presents sample standards for structure determinations, for three dimensional shape determination, Crystallography and others.

The purification process is so important in these cases that its success must be followed in each step made, in order to verify the purity of the target protein before it is submitted to the following characterization sequential steps.

These verification stages generally are followed by gel electrophoresis specific for protein analyses like first and bi dimensional (2D) PAGE or SDS-PAGE, including also other verification assays when the target protein has an specific biological activity, for instance, if the target protein is an enzyme, its biological activity can be detected by assays offering its

substrate and measuring the formation of the enzymatic product. When the focus is a toxin, it must be applied to a target of its toxicity, for example, haemolysins can be applied to haemolytic assays, enterotoxins can be applied to enterotoxicity assays, cytotoxins can be applied to cytotoxic *in vitro* assays, or proteases can be applied to target protein extracts. This step of verification not only defines if the target protein is pure enough and prepared to the next steps of chemical characterizations, but also defines and shows how drastic the purification processes have been to its integrity and maintenance of the biological activity, as chromatographic processes are always associated to minor or greater losses. This way, the verification processes are very useful for the losses data calculation and comparison of the best choice of chromatographic technique to be applied.

In general, IMAC is an excellent technique for protein purification due to its characteristics of not abolishing drastically the protein biological activities or promoting denature. That is why this technique was associated to the modern tools of Molecular Biology, where, with the help and knowledge of Microbiology in manipulating and transforming microorganisms, the applicability of this chromatography is such a respected success.

So, considering the many possibilities in the applicability of IMAC and the fact it is a kind of chromatography which is able to promote a good performance to the separation of raw and crude extracts, it is perfectly applicable to the purification of untransformed bacteria product proteins or peptides that present histidine and cysteine able to interact with the IMAC stationary phase.

4. Needs of association to other chromatography techniques

Nowadays it is known that the progress of proteomics depends on the development of protein separation techniques advances and technologies, being the IMAC a really robust method to be used for proteins and peptides or bacterium origin or not.

The immobilized metal affinity chromatography can be widely applied as a pre fractionation method as so as to the analytical separations, where it increases the resolution in protein separation.

Although being a robust technique, sometimes practice shows that it is necessary to combine IMAC to other protein analytical technologies. In fact this occurs also to genetically modified and expressed proteins the same way to the native proteins presenting natural ion affinity because of the presence of specific amino acids like histidine on their surfaces. So the natural occurrence of other proteins considered undesired or contaminants may easily occur and it is necessary to keep in mind the possible needs of applying a next step to the bacteria toxins purification processes to promote a high quality chemical separation. These chromatographic and chemical separation technologies can eliminate the undesired contaminants from the target protein or peptide and our observations have proven that the use of IMAC as a fractionation and analytical technique can be followed by any other separation step. Actually it depends most on the chemical characteristic of the target protein being purified and attention must be given to its chemical nature.

In our routine, many techniques have been applied after IMAC step, most of them proving to be really well succeed, like ion exchange, gel filtration, reverse phase, hydrophobic interaction, among other simple methods, like salting out or molecular weight ultra filtration separation techniques, which can be easily used and applied. The only thing to be considered while choosing the next step is which are the important conditions for keeping

the protein or peptide structures because, depending on the separation technique chosen, denature may occur and depending also on the objective of the separation and purification work, it is not interesting to have a denatured product in the final of the processes, even if this final product is extremely pure in the end, because of the necessity of keeping the biological activity. That is why the needs of knowing the denaturizing agents and processes aligned to the knowledge of the chemical structure is important while making choose of the next purification steps and mainly choosing the right next step to be applied after IMAC fractionation.

Its also important to remember that IMAC is a common place technique in the modern protein purification and many other technologies are being developed worldwide in order to associate this technique to other chromatographic principles, and one good example of this is the effective function of IMAC supported on cationic exchangers, where the chromatographic separation permits two modes of purification in a single column. This proves that many efforts are being done in this area and that new technologies can be expected in the nearly future, making these needs of associating IMAC to a second chromatography technique easier to native or genetically cloned and expressed proteins.

5. Examples of IMAC applied in our work

In our laboratory (Bacterium Virulence Factors Laboratory of the Biology Institute of the State University of Campinas, São Paulo, Brazil), we work focused on the studies of human enteropathogenicity mechanisms of bacterium origin. Having this focus, with no doubt, purifying these enterotoxins is substantial to characterize biologically and chemically these toxins. Actually, during our trajectory, some bacterial toxins were purified experimentally in our laboratory, like some virulence factors of human pathogens like *Escherichia coli, Plesiomonas shigelloides*, *Aeromonas hydrophila, Aeromonas sobria, Aeromonas veronii biovar sobria* and others.

Among these purified toxins there are some examples of purification successes obtained by the use of IMAC when purifying native or genetically cloned bacterium toxins. Some of these examples include the works on the cloning, expression, and purification of the virulence-associated protein D from *Xylella fastidiosa* (Catani et al., 2004), a very important citric fruit pathogen associated to orange plantation production and financial harm in Brazil, where the cloning technology was applied while characterizing the virulence factors associated to the orange trees and fruit diseases and also the purification of the Vacuolating Cytotoxic Factor (VCF) of *Aeromonas veronii* biovar *sobria* (Martins et al., 2007), an enterotoxic cytotoxin associated to cases of human enteric diseases. IMAC showed to be extremely important to the development of these scientific works as in the first case we described proteins structured to present poly histidine tags and in the second case we presented a toxin having natural ion metallic affinity to cooper. In the Figure 1 there is an example of the typical chromatogram obtained by the IMAC process of purification of the VCF of *Aeromonas veronii* biovar *sobria*. It is important to notice that the chromatographic process to this specific bacterium culture crude extracts reveals that IMAC is able to remove most of the undesired contaminant materials from the target sample, being only necessary to make a second chromatographic step in a molecular weight exclusion column to finally obtain a toxin product of highly pure degree, what was confirmed by bi dimensional (2D) SDS-PAGE analysis which revealed the presence of a single spot protein of 50KDa.

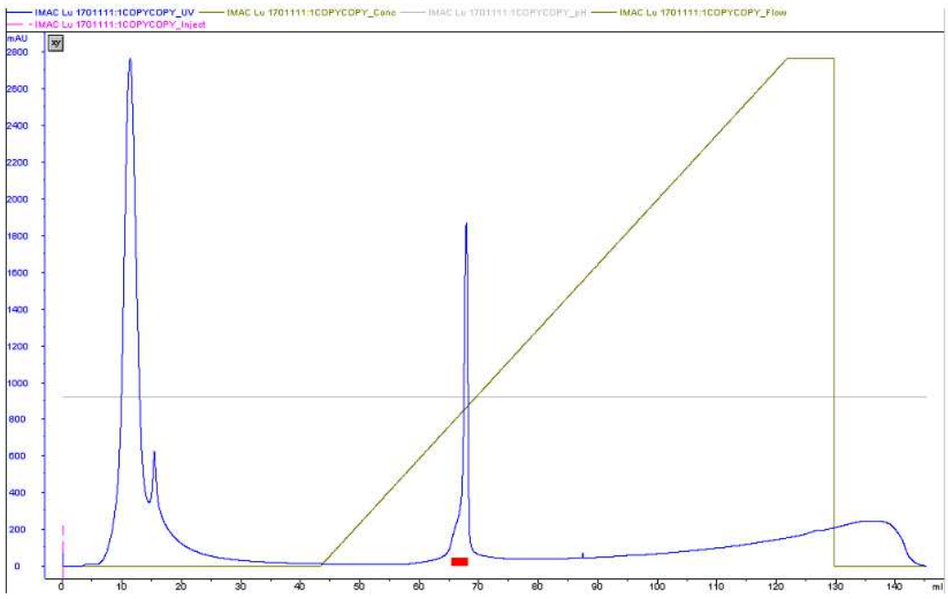

Fig. 1. Chromatographic profile observed while conducting the purification process of *Aeromonas veronii* biovar *sobria* VCF from crude extracts by IMAC charged with cooper (Cu^{++}). Assays were conducted by applying of linear Imidazole gradient elution (0 – 50mM) in Tris-HCl pH 8,0. The represented red line (▬) under the chromatogram is attributed to the portion which was adsorbed and eluted from the IMAC stationary phase and presented positive vacuolating cytotoxic activity over VERO (green kidney monkey) cells and positive enterotoxic activity over mice and rabbits.

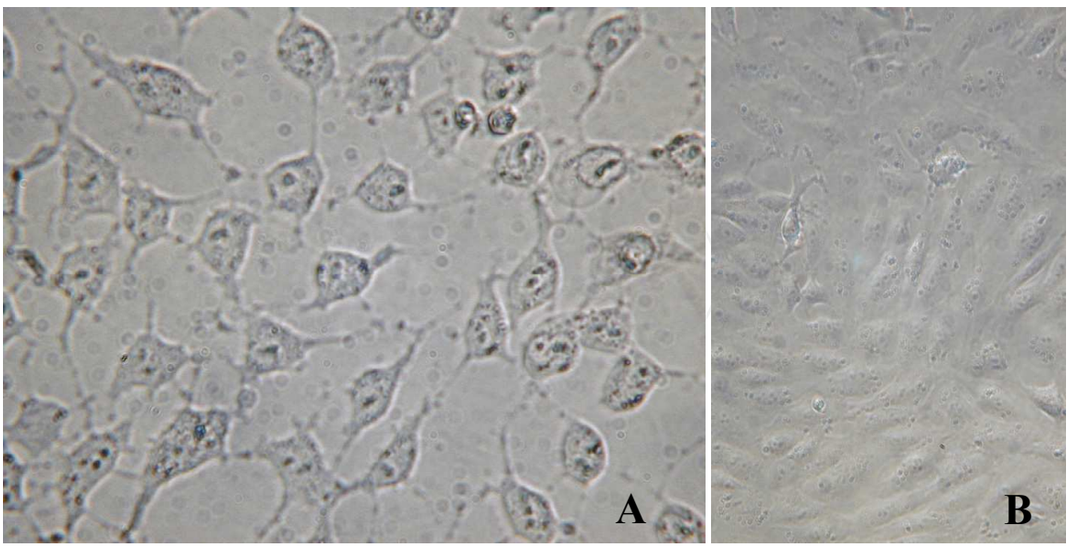

Fig. 2. Aspects of the cytotoxic effects of *A. caviae* thermo stable enterotoxic toxin (Ent-Ac) purified by IMAC charged with cooper ions over cultured Vero (green monkey kidney) cells. Fig A- Cells treated with the chromatographic eluted portion, presenting rounding, cell to cell leakage showing the loss of cellular membrane junctions and nuclear condensation. Fig. B- Cellular control. Magnifications 430X.

It is interesting to notice that among the studies that are being conducted in the moment in our laboratory, there are two low molecular weight bacterium toxins produced respectively by *Escherichia coli* and by *Aeromonas caviae*, both with enterotoxic and cytotoxic activities presenting strong cooper affinity. These toxins are under publication process and the aspects of ion metallic desorption is so strong that cooper is extracted from the stationary phase and carried out with the toxins, that, during the linkage to cooper, loose their biological activities.

Our findings reveal that only an effective treatment of these toxins with EDTA (Ethylenediamine tetraacetic acid) solutions after the chromatographic process is able to recover their biological activities by the chelating activity of this agent, unmaking this ions chemical linking, probably permitting the toxins to recover their active third dimensional structures configuration. As the stationary phase of IMAC support structure looses the immobilized ions, it is necessary to recover its structures with cooper solutions, preparing the stationary phases to subsequent chromatographic processes.

Our observations show that the immobilized metal affinity chromatography can be widely applied to experimental analyses of bacterium toxins, both for the pre fractionation or analytical separations, where it has shown a great resolution in protein separation.

6. Disadvantages or technical inconveniences

The selectivity or the adsorption capability of IMAC stationary phases depend not only on the chelating immobilized in the chromatographic matrixes, but also on the composition of the mobile phase. As mentioned, the retention of the adsorbed proteins into the IMAC stationary phase occurs due to the contribution of many physic-chemical interactions that can be intensified or minimized depending on the constitution of the mobile prepared phase. That is why it is of extreme importance to choose the right composition of mobile phases before starting IMAC separations to the best recovers of the target proteins and consequent obtaining of good results.

Basically, most of the traditional buffers applied to liquid chromatography of bio molecules may be applied as mobile phases to IMAC separations, where the ones possessing highly affinity to metallic ions, such as the ones presenting tricine and citrate in their composition, must be avoided. When applied, these kinds of buffers generally remove the metallic ions from the support of the stationary phase, prejudicing the chromatographic processes. The most applied mobile phases to IMAC processes are sodium phosphates, sodium acetates and the zwiterionics (considered as good buffers) as the examples of MOPS or 3-(N morfoline) propane sulfonic acid, MES 2-(N-moroline etanossulfonic acid and HEPES N-(2 hidrozietil) piperazine-N (2-etane sulfonic acid) (Winzerling et al., 1992).

The elution conditions to adsorbed bio molecules into the chromatographic matrixes of IMAC can be conducted by the use of different conditions, among them the use of pH gradual changes, the use of imidazole gradients and the most drastic of them, the apply EDTA (Ethylenediamine tetraacetic acid) solutions, when it is not possible to recover the target protein of the adsorption effect form the stationary phase. This last described method carries out all of the immobilized ions form the stationary phase support, what makes necessary the charging of the matrix with new ion metallic solutions, to the reconstitution of the stationary phases and extra work with the target protein. This process can be also used when a kind of metallic ion from the stationary phase is supposed to be substituted by another inside the

laboratory ambient, for instance, when a stationary phase containing nickel (Ni^{++}) needs to be substituted by cooper (Cu^{++}), accordingly to the methodologies needs.

7. Conclusion

The ion metallic affinity chromatography is a trustable, reproducible and robust technique for fractionation and analysis, not only to genetically modified cloned and expressed proteins but also to natural proteins presenting ion metallic affinity.

Hopes on the development of this chromatographic tool technology in the future are integrated with the proteomic methods where it will greatly continue on contributing to the revolution of expression, cell mapping and structural proteomics.

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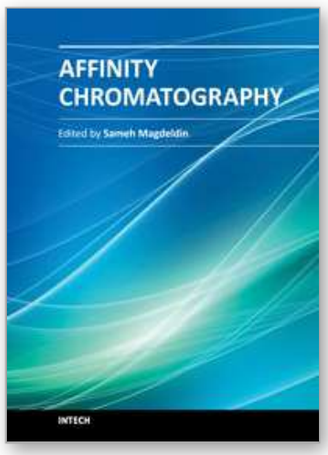

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Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

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