More and Better - Innovative tips and tricks for production and purification of unstable proteins

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Introduction

In some cases, production of recombinant proteins for therapeutic development or for *in vitro* studies is a real challenge and requires a lot of investment in terms of time and cost in order to obtain high yields of pure and active proteins.

Our aim is to provide a **guide** on how to **easily and rapidly** determine in which **production and purification conditions** the proteins will be the happiest, and how certain innovative solutions can really be **helpful** in this quest. Our optimization strategies are based on **3 axes** (Fig. 1): the optimization of expression systems, purification processes and buffer composition. *E. Coli* and HEK 293 are the most widely used protein production systems for pharmaceutical target studies. They respectively present considerable advantages.

Indeed, bacterial protein production is cheap and is able to rapidly provide non-mammalian proteins. Conversely, HEK 293 cells are able to produce human proteins closest to *in vivo* with the same glycosylation profiles, well folded and active.

First of all, we have focused on how to enhance the **transfection efficiency** of HEK 293 EBNA cells and the **quantity** of protein produced in our both prokaryote and eukaryote production systems. In a second time, we have studied how we could **stabilize and solubilize unstable proteins** during the purification step.

This study is focused on **three proteins**, one viral and two human candidates which are known to be hard to produce and unstable (Fig. 2). The **viral protein** production is optimized in **bacterial expression system**; not only by testing several tags and bacteria strains, but also by using culture media additives and customized media. The **2 human proteins**, an interleukin (approx. 16 kDa), involved in the inflammatory process, and an enzyme (approx. 80 kDa), which plays a role in cell morphology, are both produced in **HEK 293 EBNA** (Yves Durocher, Biotechnology Research Institute of Montreal). Transfection and production steps have been improved for the 2 proteins by using **innovative additives**. After precise Design of Experiment (**DoE**) establishment, various purification conditions are tested including the use of different specific and customizable cutting-edge chromatography technics, several novel osmolytes, ionic and alpha-helix stabilizers. Data extrapolation is done and best production and purification conditions are identified in a very short time.

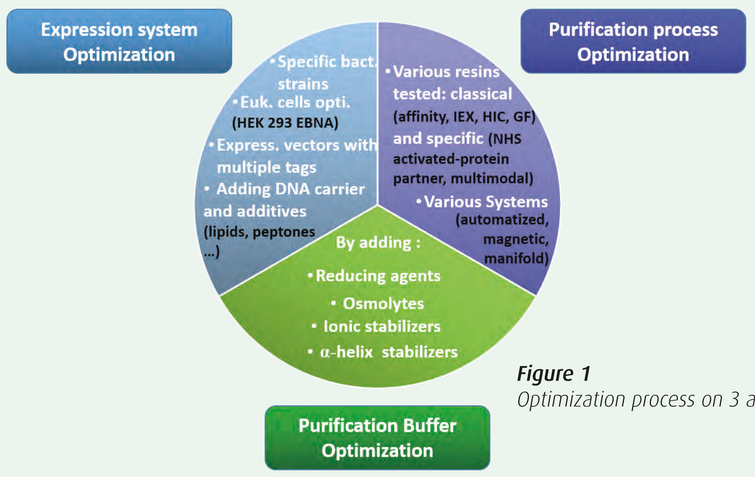

Figure 1
Optimization process on 3 axes.

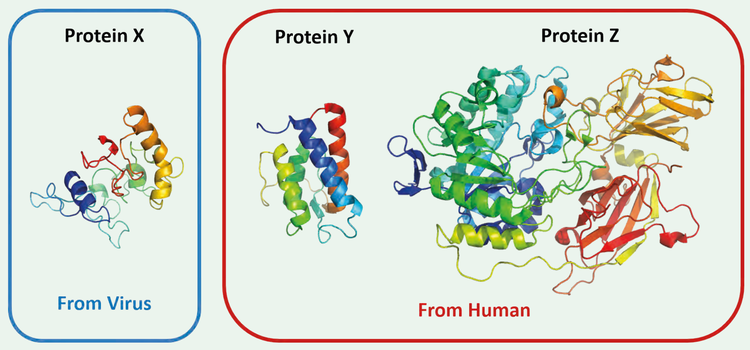

Figure 2 *- 3D structures of proteins X (Virus), Y and Z (Human).*

Material and Methods

The aim of this following optimization strategy is to determine a **holistic process for the production and purification of problematic proteins** from Bioinformatic analysis to getting pure proteins in microtubes by following precise DoE based strategies and by performing molecular biology, expression and purification studies (Fig.3).

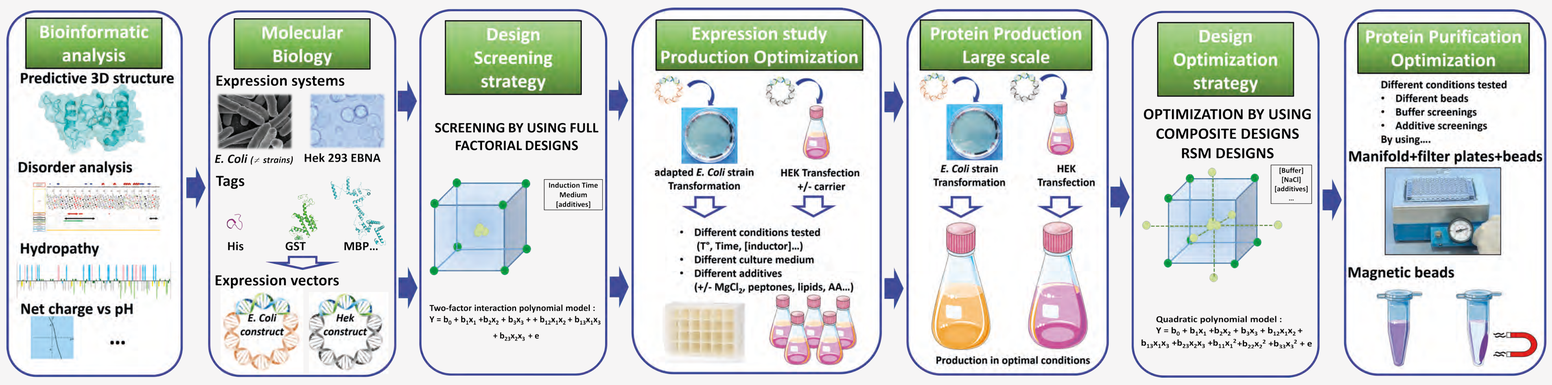

Figure 3 *- Overview of an internal holistic optimization process.*

Optimization of the protein production process

In bacterial strain system (*E. Coli*)

To enhance the quantity and the quality of proteins produced in *E. Coli*, several strategies with innovative approaches have been developed over the last decades. Various tools are available on the market such as a plethora of **bacterial strains** with specific characteristics for the production of toxic or membrane proteins, or for proteins with rare codons, or with di-sulfide bonds... Moreover, several **tags** can be used to improve the protein solubility and the purification efficiency. Incidentally, identified **interacting sequences** of partners may also be used to help get the right protein folding which has a direct impact on the **protein conformation, solubilization and activity**.
These solutions are not the only ones used within the laboratory. Indeed, an action can also be carried out at the level of the **culture media composition** and the **additives** used. The experiments corresponding to Fig. 4 were aimed at determining the bacterial growth medium composition (s) and the optimal conditions for the viral **protein X** production. Indeed, the quantity of Protein X produced was highly enhanced in **SuperBroth and Magic Media** in presence of a cocktail of **additives** compared to the amount produced in classical media (LB, 2YT, TB...).

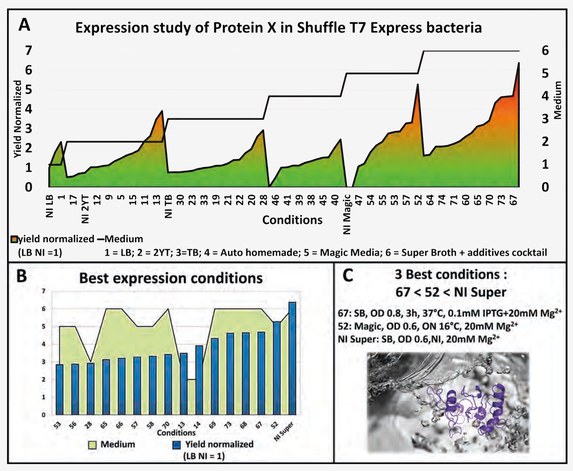

Figure 4 *- Expression study results for Protein X produced in 6 different Media and with different induction conditions ([inductor], time, temperature, additives...). 72 conditions tested by using the same DoE for each Media.*

In Human cell system (HEK293 EBNA cells)

As for protein production in *E. Coli*, the choice of an adapted **tag** and co-expression of **interacting partner peptides** can help. Nevertheless, other factors can also be taken into account like the **protein Y intracellular** expression **vs**. its **secretion** in the media (see Fig. 5), the effect of DNA carrier addition on the transient cell transfection efficiency with expression vector including the Protein Z DNA sequence (Fig. 6) and the effect of nature and concentration of innovative additives (peptones and/or lipids) added to the media on the quantity of the protein Y produced (Fig. 7).

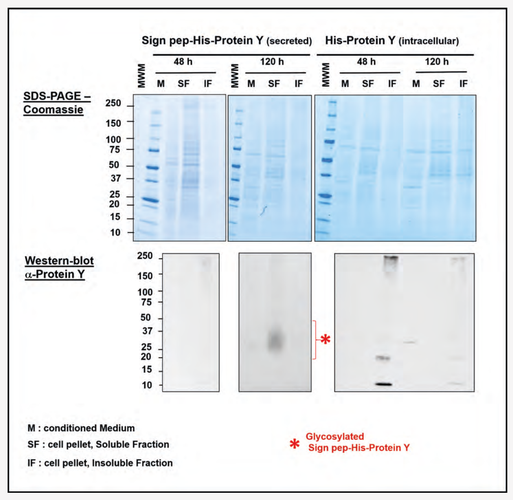

Figure 5 *- Comparison of Protein Y production in cell cytoplasm (intracellular) vs in medium (secreted).*

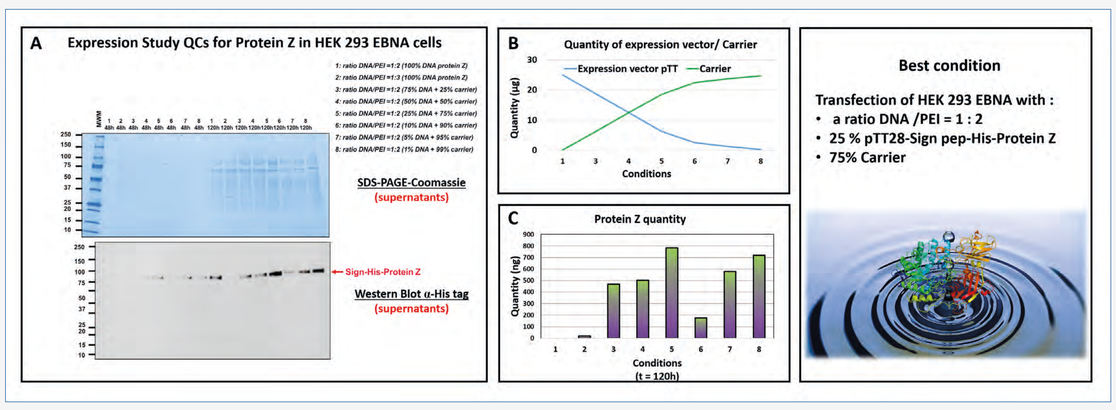

Figure 6 *- **Expression study results for Protein Z produced by transfecting human cells (HEK 293 EBNA) (A) with different DNA/ carrier ratios (B).** The quantities of Protein Z obtained are summarized in (C).*

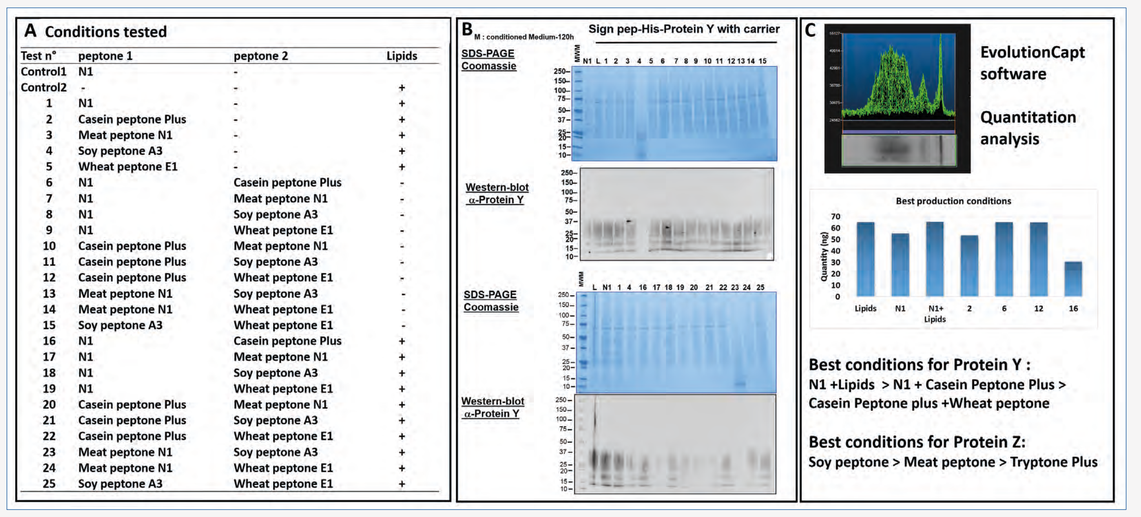

Figure 7 *- **Expression study results for Protein Y production in presence of peptones** (combined or not) +/- lipids (A-B). The best production conditions are identified and quantified with the EvolutionCapt software and are represented in (C).*

These studies have permitted to highlight that **Protein Y** production can be optimized by adding a **signal peptide** on the DNA sequence for the protein secretion, by using **25% DNA carrier** to enhance the cell transfection efficiency and by adding **combined or not peptones** (which are water-soluble protein hydrolysates containing peptides, amino acids, inorganic salts, lipids, vitamins and sugars from plants or animals), **with or without lipids**, to the media during the production step.

Moreover, the **positive effect of DNA carrier** addition during the cell transfection is **confirmed** with the experiments done during the **Protein Z** production optimization. Interestingly, the **peptones** identified for improving the productivity for this protein are **not the same** as for the **Protein Y**.

Optimization of the protein purification process

Different tests are performed to optimize the quantity and the stability of the **Protein Y** during the purification step. 2 types of beads were used: Ni magnetic beads and NHS activated beads linked with a specific antibody against Protein Y. The best results are obtained with the **Ni magnetic beads** in presence of **2mM β–Mercaptoethanol + detergents** (0,05 % Triton X114 or Tween 20 or 11mM OGP) combined with 25 mM **sucrose monolaurate** or **D-Glucose**. Addition of amino acids and TMAO seems to have also a small positive effect.
Concerning the NHS activated beads, new assays need to be performed to optimize, in particular, the binding step.

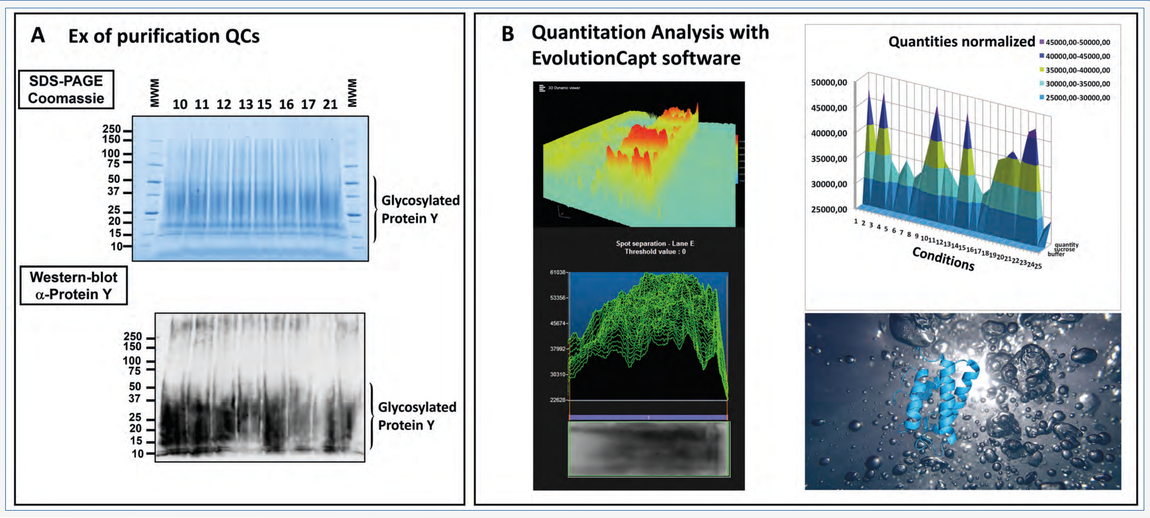

Figure 8 - Purification tests with Ni magnetic beads for Protein Y. 25 conditions tested with different buffers *(25-100 mM Tris HCl, PBS 1x, Tricine),* ***osmolytes*** *(glycerol, detergents (0.02%- 0.05% Tween 20, Triton X114, OGP, polyethyleneimine...) and sugars (several concentration tested of sucrose, D(+)-glucose, sucrose monolaurate)),* ***ionic stabilizers*** *(different concentrations of salts), α-helix stabilizers (TMAO) and* ***amino acids in presence or not of reducing agents.***

Conclusion

In conclusion, each protein is a particular case. Nevertheless, taking into account their specific physico-chemistry, performing an efficient DoE, implementing new strategies and/or using innovative reagents, and above all taking some time to properly interpret data and screening results make the quest less difficult.
This study has permitted to define an efficient, holistic process that can be applied for each protein as a first intention screening. The benefits of our strategy in terms of time and cost are very impressive and of great value, and are accessible to researchers through our protein production platform.