STREAMLINING RECOMBINANT PROTEIN PRODUCTION

The pharmaceutical industry is undergoing a deep transformation from small molecule drugs to biologics. Over the last decade, the percentage share of biologic-based drugs -- including vaccines, therapeutic proteins and monoclonal antibodies -- has seen a steady growth of 13.3 percent per year, compared to a 4.2 percent decrease for other drugs. Biologics now account for 55 percent of new drug approvals and 64 percent of pipeline drugs.[1] By 2016, biologics are projected to account for approximately 17 percent of total global spending on medicines, reaching an overall market value of up to $210 billion.[2]

Use of recombinant proteins as therapeutics has become an attractive strategy for altering the biology of disease progression and offers significant commercial opportunities. However, bringing a recombinant protein to market requires a substantial investment of time and resources, and the process is generally complex and subject to technical pitfalls. Because the synthesis and purification processes are technically complicated and vary with each protein, prospectively estimating resources and timelines is difficult.

There is opportunity to augment platforms for the development of therapeutic biologics. This paper summarized some of the challenges of recombinant protein production, and presents an efficient, simplified alternative to conventional platforms. By eliminating multiple steps in the production of complex proteins, the novel Corynex® Recombinant Protein System reduces potential difficulties, resources and costs and can accelerate the time to market.

Protein Synthesis and Purification: Microbial Versus Mammalian Systems

Most biologics, with the vast majority being monoclonal antibodies, are produced in mammalian culture; however, 40% of therapeutic biologics in development use a microbial production platform.[3] Recombinant protein production in microbial systems (mainly bacteria for non-antibodies) has significant advantages over mammalian systems. The robust cellular structure of bacteria makes them amenable to culturing. They also tend to be more affordable and fast-growing than mammalian systems.

In mammalian systems, since cells are not adapted to survive outside the body, they are sensitive to external conditions such as shear stress and osmotic shock. While bacteria replicate every 20 minutes to two hours, mammalian cells do so every 24 to 48 hours. Unlike mammalian cells, which require complex, often proprietary media with amino acids, bacterial cell medium often consists solely of simple carbon and nitrogen sources and inorganic salts. Further, the cost for bacterial cell media is 90 percent lower

than for mammalian Chinese hamster ovary (CHO) media. Fermentation generally takes 24 to 96 hours for bacteria or yeast compared to 14 days or longer for CHO cells. Microbial systems can generate very high titers of the recombinant proteins of interest, compared to mammalian cells, which grow to lower density and take longer to grow and accumulate product.

These advantages of microbial upstream processes do not necessarily translate into increased yield, cost or time savings as the purification process is much more complicated compared to the fermentation process itself or purification from mammalian systems.

Proteins synthesized by host cells ultimately need to be recovered in a functionally active state. Mammalian cell lines have inherent mechanisms for secreting properly folded, active proteins in culture medium. In *Escherichia coli* (*E. coli*), synthesized protein accumulates internally and cell lysis is required to isolate the target protein. Lysis releases intracellular contents such as proteases or endotoxins, which can decrease yield and complicate purification. Overexpressed protein often accumulates as inclusion body aggregates, which require purification, solubilization and subsequent refolding of the protein. Other disadvantages are protein aggregation or poor final bioactivity resulting from difficulties transitioning from a denatured state into a properly folded state. Each step in the process requires time and resources, and every step of recovery and purification results in loss of yield.

As will be shown in this white paper, the Corynex® Recombinant Protein System takes advantage of the best parts of both mammalian and microbial protein production platforms. The bacteria can be grown robustly and cheaply, and the recombinant protein products are secreted as properly folded, active proteins into the culture medium.

Steps in Microbial Protein Manufacturing Processes

While every protein production process is unique to the specific protein, certain steps are followed in every microbial protein process: fermentation is followed by harvest, then primary recovery, purification, and bulk formulation.

There are three main paths to purification. In the simplest, soluble protein is secreted into the supernatant, and chromatography steps are used to purify the protein. This is the case for yeast-derived products. More commonly used in manufacturing, bacterial cells produce inclusion bodies or intracellular, soluble protein. In both cases, after cells are harvested, they are resuspended and lysed. Soluble protein is then recovered by chromatography steps. In the case of inclusion bodies, these aggregates are liberated from cells during lysis and are washed. The denatured protein is released by solubilization and is then refolded and further purified through chromatography.

The potentially high costs for development of an inclusion body process are highlighted by myriad parameters that must be optimized. For the inclusion body wash, solubilization, and refold parameter screening, a wide variety of agents in boundless combinations under a wide variety of conditions can be used for each of these steps. This process is commonly not robust, and the yields can easily be less than 20%.

Test Parameters:

Inclusion Body wash

- Buffer composition
  - Chaotropic agents
  - Detergents
  - Salts
- w/v ratio
- Cycles of centrifugation

Solubilization

- Buffer composition
  - Chaotropic agents
  - Detergents
  - Reducing agents
- Duration
- Protein concentration

Refold

- Buffer composition
  - Chaotropic agents
  - Redox agents
  - Amino Acids
  - Detergents
  - Sugars
  - Salts
- Duration
- Protein concentration
- pH
- w/v ratio

Coronyx®: A Streamlined System for Improved Productivity

With all the challenges of producing recombinant proteins, there is still significant opportunity for development. Surveys over the past few years have shown a shift in the industry's focus areas for improvement, with an increased interest in improving productivity and lessening concern about cost-cutting.[3]

Traditionally, overcoming the challenges of complex recombinant protein production has meant refining individual steps in the process, which further escalates process cost and time, or increasing scale to meet quantity requirements to support clinical trials. To speed and simplify the process, the Corynex® Recombinant Protein Expression System was developed, producing properly folded protein and requiring fewer purification steps, thus improving overall yield.

Corynex® is a microbial system that incorporates the best aspects of microbial and mammalian platforms -- the speed and ease of handling of upstream processes with microbial platforms and the straightforward purification of mammalian platforms. The system uses a gram-positive bacterium, *Corynebacterium glutamicum*, which was discovered in Japan in 1956 and has been used extensively to produce amino acids for the food industry. Generally Regarded as Safe (GRAS), the bacterium has also been used in the pharmaceutical industry to produce raw materials. *C. glutamicum* is non-endotoxic, non-sporulating, non-pathogenic and genome-sequenced.

To develop a protein secretion system, scientists studied the extracellular proteins of *C. glutamicum*, and found substantial accumulation a cell surface protein (CspB). Taking

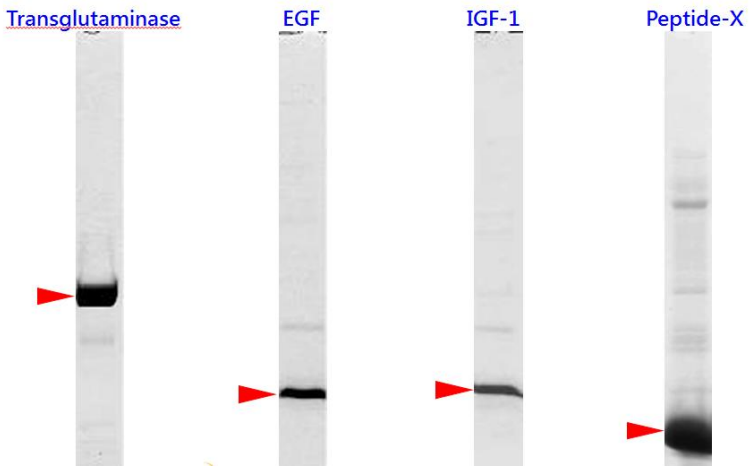
advantage of this cellular machinery, the CspB promoter and signal peptide of *C. glutamicum* can be used to synthesize and secrete soluble, properly folded, biologically active recombinant proteins directly into the growth medium with high purity. Since the two secretory pathways were elucidated, about 154 signal peptides have been screened. Since the best signal peptide for secretion is target-dependent and some proteins show a preference for one path over the other, both pathways are commonly screened during process development.

In the Corynex® system, proteins can be targeted for secretion by either the well known Secretory (Sec) or more recently identified Twin Arginine Translocase (Tat) pathway. Secretion of properly folded recombinant proteins has been achieved with both pathways, including those with disulfide bonded and dimerized structures. The system has also been successfully used to express some of the most difficult to produce proteins.

The Corynex® system provides a scalable, high-cell-density fermentation process offering ample productivity for scale-up and manufacture of commercially valuable proteins. Importantly, the system reduces downstream processing and purification steps in that there is no need for cell disruption, inclusion body purification, protein refolding or chromatography steps dedicated to removal of certain significant contaminants like endotoxin or host cell DNA. Because there are many fewer steps than required with other bacterial platforms, there is less loss of protein during purification and overall yield is increased. The result is reduced costs and time in the development of protein therapeutics.

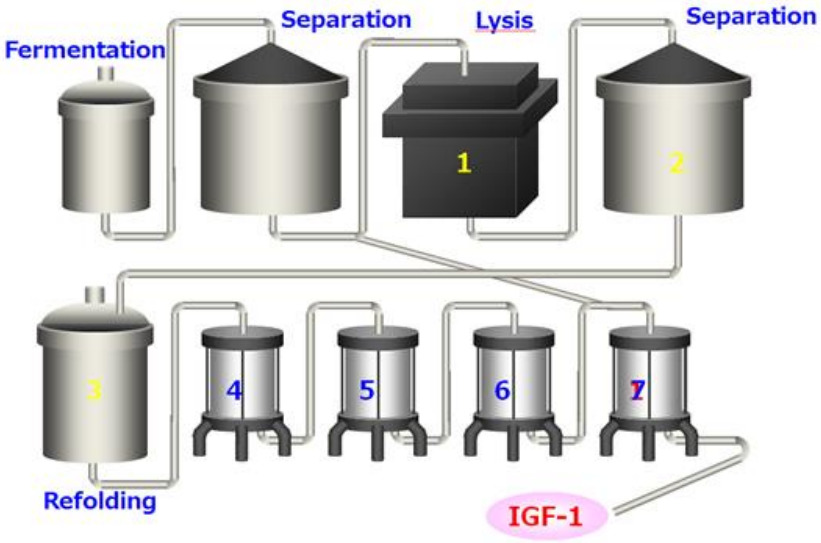
System Features: Simple. Pure. Active.

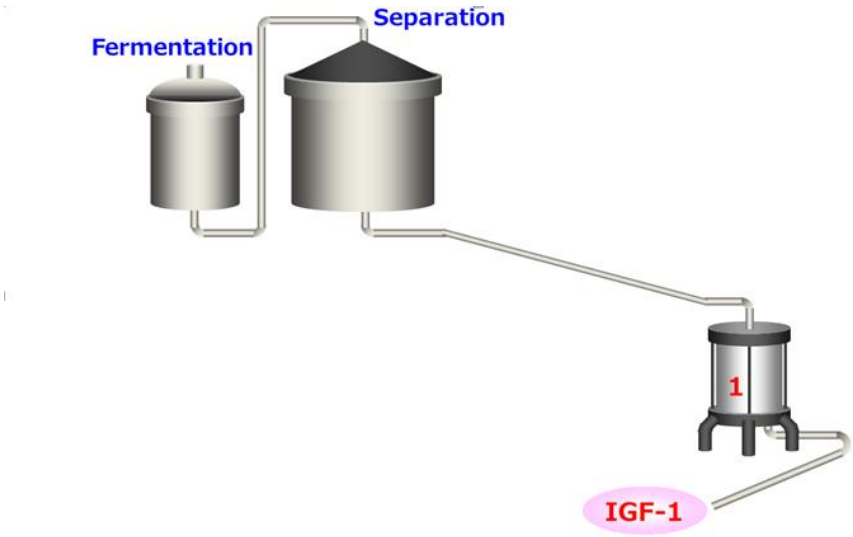
Corynex® can be tuned to the particular characteristics of the recombinant protein of interest and is tolerant to a wide range of conditions, such as pH and osmolarity. This flexibility creates conditions for high productivity.



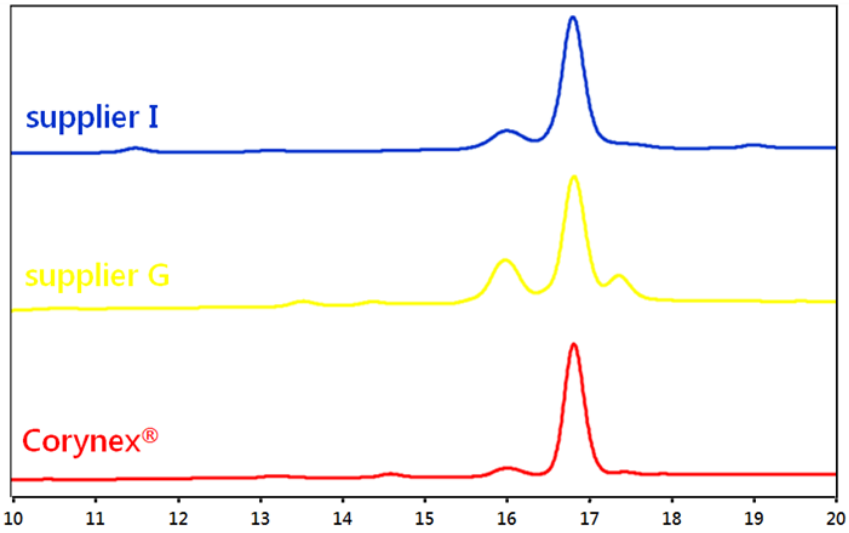
In addition, the protein of interest is the dominant protein in the cell culture medium. Importantly, there are no degradative enzymes secreted, such that prior to purification, there is no protease degradation of the product. The lack of endotoxins, the main contaminant that must be removed during *E. coli* manufacturing, greatly simplifies the purification burden. Real-world manufacturing of IGF-1 illustrates this point as the purification process costs 80% less using Corynex® compared to *E. coli* and the final product has higher purity.

Process Simplification:



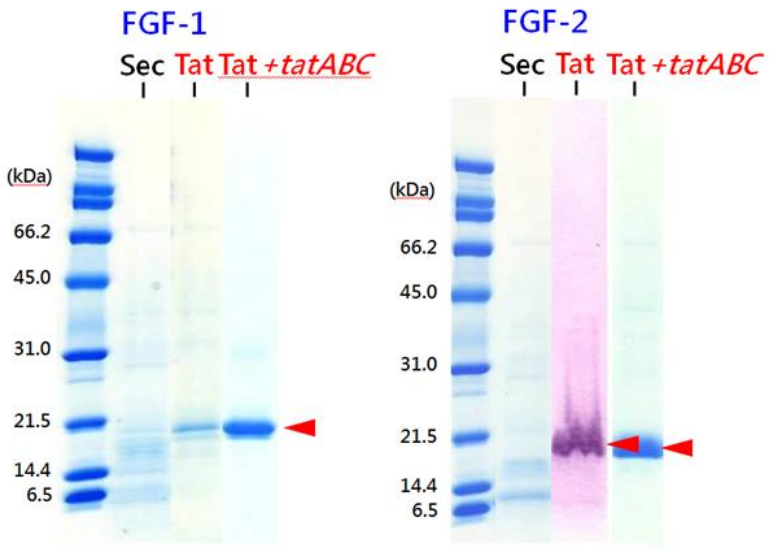


Corynex®: IGF-1 Quality



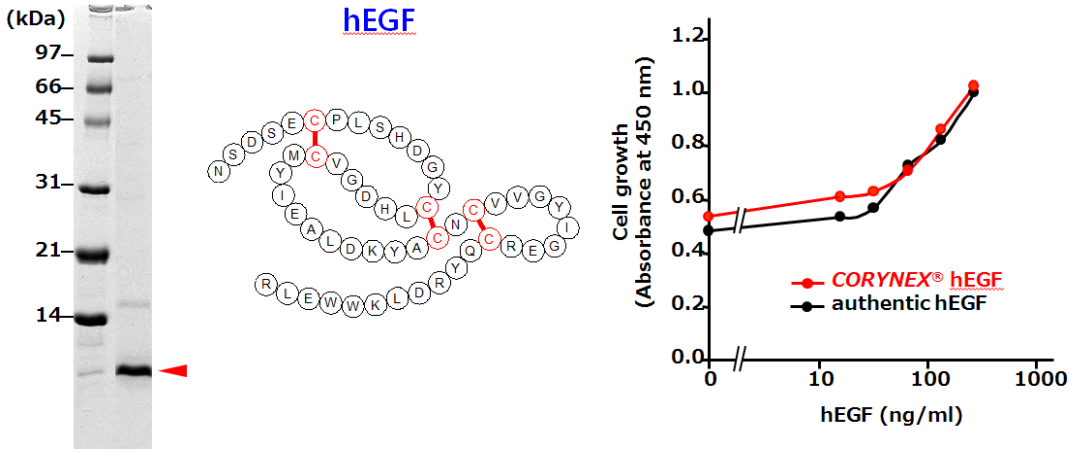
IGF-1 produced by Corynex® has fewer impurities than IGF-1 produced by others using *E. coli*, in spite of using only a single-step chromatography.

As an example of pathway preference and flexibility, the Tat, but not the Sec, pathway was able to secrete two fibroblast growth factor (FGF) proteins. Moreover, the number of Tat complexes at the cell surface were found to be rate limiting. With tatABC overexpression, the accumulation levels of both FGFs dramatically increased and reached several grams per liter in jar fermenters.



- The number of Tat complexes at the cell surface can be rate limiting.
- With *tatABC* overexpression, the accumulation levels of both FGFs dramatically increased and reached several g/L in jar fermenters.
- The Tat pathway has great potential in industrial-scale protein production.

Proteins secreted using Corynex® are in a properly active state. For example, human epidermal growth factor (hEGF), secreted through the Sec pathway, is properly folded, has disulfide bonds, and is functional, with the same biological level of activity as that of endogenous EGF.



Conclusion

While the path to producing a functionally active, meaningful recombinant pharmaceutical can lead to great commercial opportunity, it can also be a long, costly process fraught with technical pitfalls and failures. The Corynex® Recombinant Protein System provides a solution to many of these problems, overcoming difficulties with low production yields or inefficient downstream processing. Compared to other microbial expression systems, this novel patented system offers a higher purification yield with no protein degradation during accumulation, properly folded protein, and biologically active protein. These benefits combined with the elimination of additional costly purification steps and refolding ultimately translate into lower downstream costs and faster time to market.

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