Recombinant Protein Production using a Mammalian Unconventional Secretory Pathway

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Abstract

Recombinant proteins are widely used throughout biomedical research, and the expansive and diversified usage of such reagents requires continued development of novel production processes. Unconventional protein secretion might offer a unique solution to some of the niche problems in traditional protein production systems, such as enabling the heterogeneous expression and secretion of functional bacterial proteins in mammalian cells. To investigate the feasibility of such a system, a panel of unconventionally secreted proteins was identified and used to compare their ability to induce secretion of GFP. Afterwards, IL-1β, an unconventionally-secreted pro-inflammatory cytokine, was identified and used to produce *Streptococcus agalacticae* Ig-degrading enzyme in HEK293 cells. The resulting protein was characterized and compared to a conventionally-secreted variant, where it exhibited increased activity.

Dedication

This work is dedicated to all the people who make the world just a little bit better.

Acknowledgments

This work would not have been possible without the help of my thesis director, Dr. Bing Gong, whose patience and guidance throughout this past year were indispensable to the completion of this work.

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Chapter I.

Introduction

The development of recombinant DNA technologies in the early 1970s ushered in a new era of biomedical research that firmly laid the foundations for the then-nascent field of biotechnology (Pavlou & Reichert, 2004). Building upon earlier work in characterizing the function of bacterial restriction endonucleases (Arber & Linn, 1969) and in the construction of recombinant DNA molecules (Cohen et al., 1973; Jackson et al., 1972; Mertz & Davis, 1972), scientists at the University of California, San Francisco would establish what many consider to be the world's first biotechnology company, Genentech (South San Francisco, CA, USA). In collaboration with Eli Lilly (Indianapolis, IN, USA), Genentech would go on to develop and commercialize the world's first recombinant human protein, Humulin, in the 1980s (Pham, 2018). Since then, recombinant proteins have become a central pillar of the biotech industry as a whole – to date, they represent a multi-billion-dollar market spanning over 130 therapeutic products and more than a third of total biopharmaceutical sales (Table 1, Table 2, Figure 1) (Oliveira & Domingues, 2017).

Table 1. Examples of first-generation therapeutic recombinant proteins

Brand	Generic	Company	Therapeutic category	Indications
Humulin	Insulin	Eli Lilly	Diabetes	Diabetes
Hematrope	Recombinant somatropin	Eli Lilly	Hormones	Growth failure
Genotropin	Somatropin	Pfizer	Hormones	Growth failure
Saizen	Somatropin	Serono	Hormones	Growth failure
Nutropin/Protropin	Somatropin/Somatrem	Genetech	Hormones	Growth failure
Intron A	Interferon alpha-2b	Schering-Plough	Anti-infective	Viral infections
Avonex	Interferon beta-1a	Biogen Idec	Multiple sclerosis	Chronic inflammatory demyelinating polyneurophathy
Betaseron/Betaferon	Interferon beta-1b	Schering AG	Multiple sclerosis	Multiple sclerosis
Procrit/Eprex	Epoetin alpha	J&J	Blood modifier	Anemia
Epogen	Epoetin alpha	Amgen	Blood modifier	Anemia
NeoRecormon	Epoetin beta	Roche	Blood modifier	Anemia
Kogenate	Factor VIII	Bayer	Blood modifier	Hemophilia
NovoSeven	Factor VIIa	Novo Nordisk	Blood modifier	Hemophilia
Benefix	Factor IX	Wyeth	Blood modifier	Hemophilia
Fabrazyme	Agalsidase beta	Genzyme	Enzymes	Fabry disease
Replagal	Agalsidase alfa	TKT Europe	Enzymes	Fabry disease
Pulmozyme	Domase alpha	Genetech	Enzymes	Cystic fibrosis
Activase/Acitlyse	Alteplase	Genetech	Blood factor	Myocardial infarction

A list of several first-generation commercialized therapeutic recombinant proteins, including manufacturer and indication, as of 2018 (reproduced from Pham, 2018).

Table 2. Examples of second-generation therapeutic recombinant proteins

Brand	Generic	Company	Therapeutic category	Indications
Humalog/Liprolog	Insulin Lispro	Eli Lilly	Diabetes	Diabetes
Lantus	Glargine insulin	Sanofi-Aventis	Diabetes	Diabetes
Levemir	Detemir insulin	Novo Nordisk	Diabetes	Diabetes
Pegasys	Pegylated interferon alpha-2a	Roche	Interferon	Hepatitis C
Peg-Intron	Pegylated interferon alpha-2a	Schering Plough	Interferon	Hepatitis C
Aranesp	Darbepoetin alpha	Amgen	Blood modifier	Anemia
Neulasta	PEG-Filgrastim	Amgen	Blood modifier	Neutropenia
Refacto	Factor VIII	Wyeth	Blood modifier	Hemophilia
Amevive	Alefacept	Biogen Idec	Inflammation/Bone	Plaque psoriasis
Enbrel	Etanercept	Amgen	Anti-arthritic	Arthritis
Ontak	rIL-2-diptheria toxin	Ligand Pharmaceuticals	Cancer	Cancer

A list of several second-generation commercialized therapeutic recombinant proteins, including manufacturer and indication, as of 2018 (reproduced from Pham, 2018).

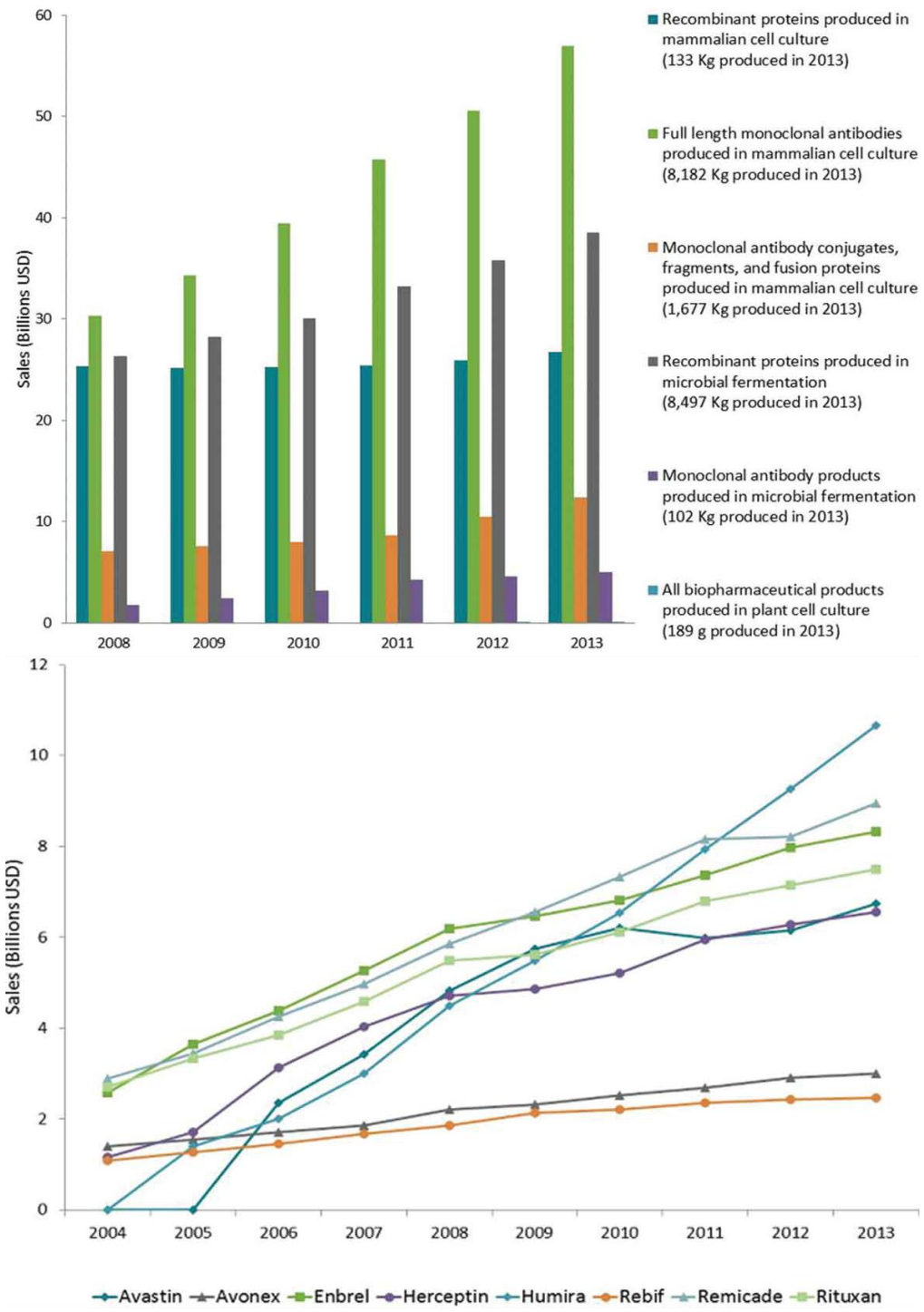

Figure 1. Growth in the monoclonal antibody market

Sales of recombinant protein therapeutics (particularly antibodies) drastically increased in the years leading up to 2015 (reproduced from Ecker et al., 2015).

There is a constant need to produce recombinant proteins given the scope of their usage in both clinical and pre-clinical research settings (Assenberg et al., 2013). They are often used in biophysical studies to determine the structural properties of novel proteins (Vedadi et al., 2010) and in functional assays to characterize the mechanisms underlying biological activity (Spoerry et al., 2016). Applications such as these are especially relevant in fields like immuno-oncology where scientists and clinicians often rely on recombinant proteins as a key part of their therapeutic approach. Examples of such proteins include recombinant monoclonal antibodies (mAbs) and their antigens used in epitope determination and functional assays, as well as recombinant enzymes used in antibody production, de-glycosylation, and fragmentation (Eskiocak et al., 2020). In order to support the increasingly high demand for and variable usage of secreted recombinant proteins, this work seeks to investigate the feasibility of utilizing unconventional secretory pathways in mammalian cells as a novel method of producing recombinant proteins.

Overview of Recombinant Protein Production

Many recombinant protein production processes generally follow similar approaches (Figure 2). First, recombinant DNA that expresses a protein of interest must be generated by molecular cloning (Green & Sambrook, 2012). This is a multi-step process that requires: 1) the isolation of the gene of interest, typically accomplished via polymerase chain reaction (PCR) or restriction digestion; 2) the identification of any necessary genetic regulatory elements such as promoters, replication origins, and selection markers required for the replicon, typically a plasmid expression vector; 3) the assembly of the gene of interest and regulatory elements into the replicon using one of a

suite of well-characterized cloning techniques, most commonly recombination-based, ligation-based, or ligation-independent; and 4) the amplification of sequence-confirmed clones for use in protein expression processes. Next, the resulting replicons are delivered into host cells using one of many possible gene transfer techniques, including but not limited to heat-shock transformation, electroporation, chemical transfection, lipofection, and physical injection (Wurm, 2004). After a sustained period in which the host cells over-express the protein of interest, the resulting crude material must be harvested and purified in a host-dependent manner (Structural Genomics Consortium et al., 2008).

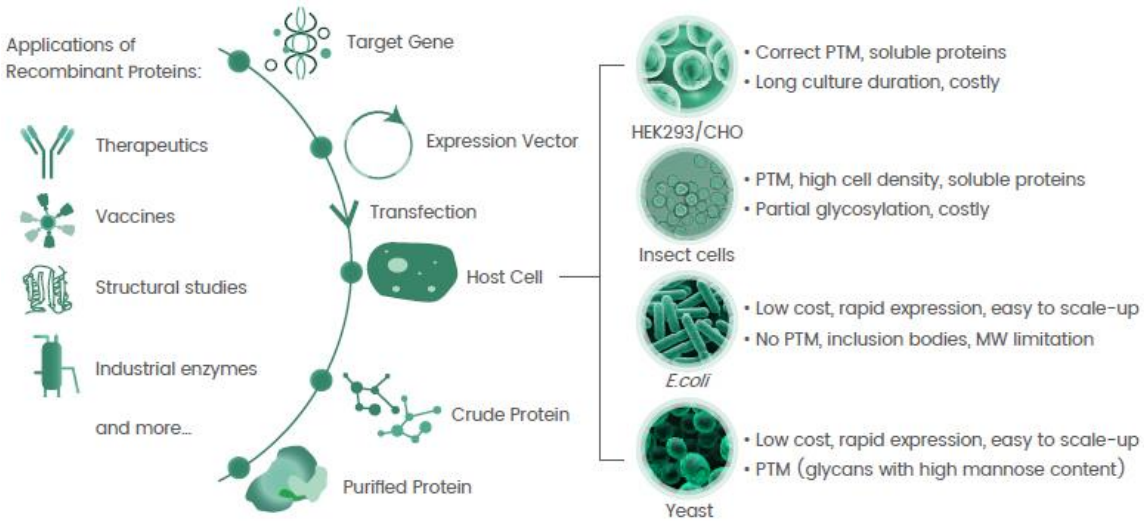

Figure 2. Recombinant protein production workflow

A general strategy for producing recombinant proteins involves the selection of a target gene, the cloning of the target gene into a plasmid expression vector, the delivery of the expression vector into a host cell, the expression and harvest of crude protein, and the purification of recombinant protein (reproduced from Sino Biological, 2023).

The purified protein obtained during the production process is typically characterized prior to application. The exact assays performed are dependent on the type of protein and its intended use but can broadly be categorized into biochemical/biophysical and functional assays (Figure 3) (Oliveira & Domingues, 2017; de Marco et al., 2021). The most basic biochemical/biophysical assays include assessments of protein purity, integrity, and homogeneity, typically accomplished using SDS-PAGE and SEC-HPLC. Additional in-depth studies can be performed to confirm sequence identity (e.g. mass spectrometry), protein identity (e.g. Western blot), and thermostability (e.g. DSF). Functional assays can be performed after completing basic quality assessments to characterize biological activity. The exact assays used again will again vary by protein but can include tests such as binding characterization for antibodies and proteolytic activity for enzymes (Spoerry et al., 2016).

Conventional Protein Expression Systems and their Limitations

A key issue that must be addressed during the production process is which organism to use as an expression system. The expression system used determines not only the appropriate host, but also the design of the recombinant DNA replicons and the subsequent harvest and purification strategies (Tripathi & Shrivastava, 2019). Each expression system comes with its own unique benefits and drawbacks; as a result, the optimal organism is heavily dependent on the protein to be expressed and its intended usage. Conventional protein expression systems typically fall into one of two categories – bacterial expression systems and mammalian expression systems.

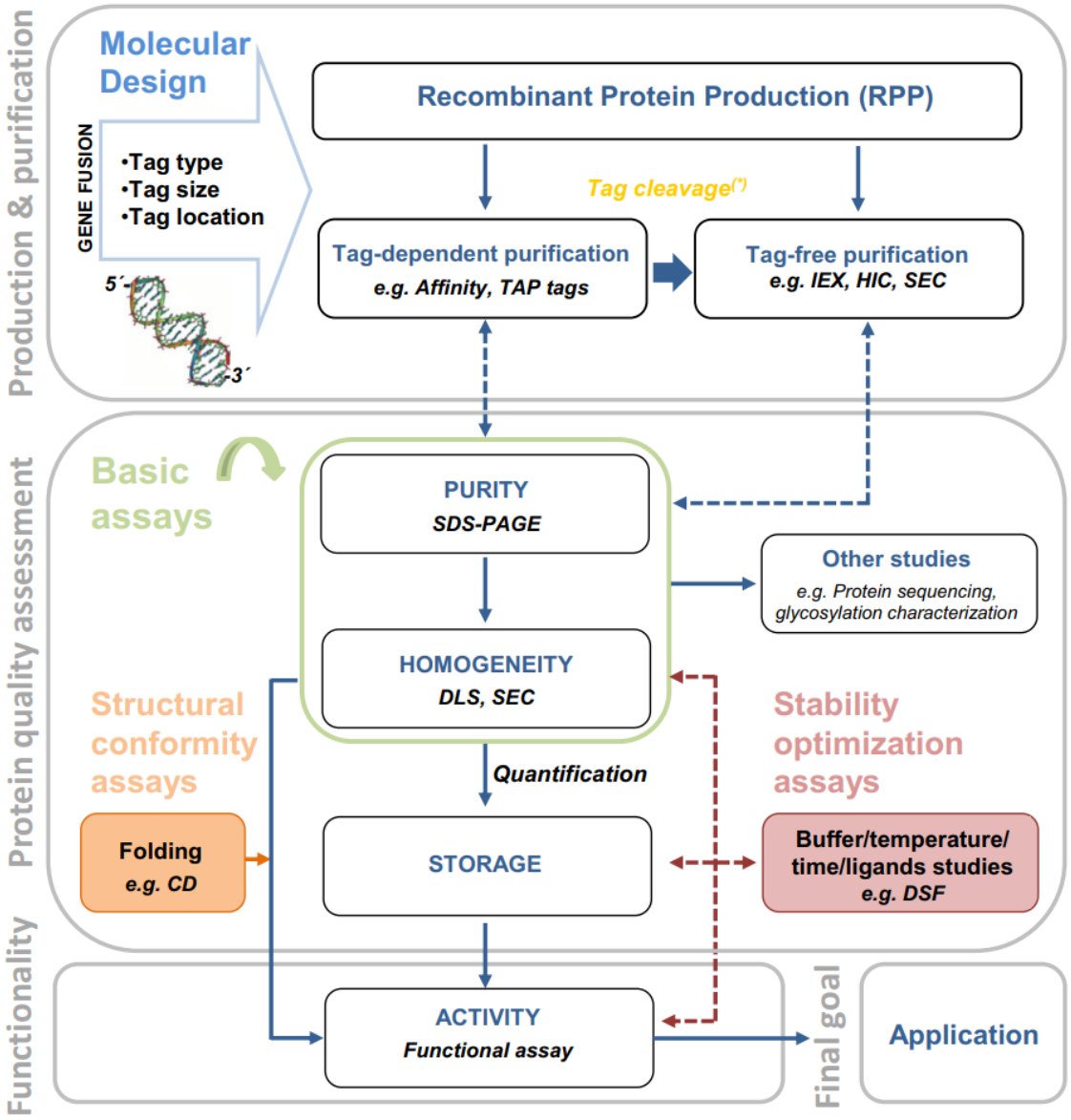

Figure 3. Protein quality assessment and characterization

Following recombinant protein purification, several assays should be performed to ensure quality and activity. These can range from basic protein quality assessments such as purity (SDS-PAGE) and homogeneity (HPLC-SEC) to detailed functional activity assays (reproduced from Oliveira & Domingues, 2017).

Bacterial expression systems such as *Escherichia coli* cells are inexpensive to culture and scale up, relatively straightforward to manipulate, and typically yield a large amount of protein, making them a popular host organism (Rosano & Ceccarelli, 2014). However, the resulting material usually requires extensive downstream processing to release the protein from the cells, solubilize inclusion body aggregates, refold the protein, and remove endotoxin. Many eukaryotic proteins also require native post-translational modifications (PTMs) or molecular chaperones to fold and function correctly, and some larger proteins may fail to fold at all, making heterologous expression of these proteins in prokaryotes difficult (Tripathi & Shrivastava, 2019). As a result, bacterial expression systems are typically utilized when dealing with small and relatively simple proteins with few to no eukaryotic PTMs.

Although mammalian expression systems like HEK293 and CHO cells suffer from higher costs of production and lower protein yields, they typically avoid the need for the complicated downstream processing associated with bacterial expression systems (Andersen & Krummen, 2002). Mammalian expression systems also secrete protein directly into the culture supernatant which obviates the need for cell lysis, protein solubilization, and refolding steps in the purification process. In addition, mammalian cells maintain abundant protein quality control mechanisms and lack some of the proteases and endotoxins that degrade and contaminate proteins from bacterial cells (Assenberg et al., 2013). As a result, mammalian expression systems are typically utilized when dealing with larger and more complex proteins that require mammalian PTMs and low bacterial endotoxin contamination – this is particularly important for proteins that are used in therapeutics such as antibodies or enzymes that modify those antibodies.

Alternative Approaches for Protein Expression

Various approaches have been developed to address the drawbacks of bacterial and mammalian expression systems. One of the most popular approaches involves the utilization of alternative eukaryotic expression systems (Assenberg et al., 2013). These typically aim to balance the advantages and disadvantages afforded by bacterial and mammalian expression systems. For example, yeast expression systems such as *Saccharomyces cerevisiae* cells benefit from the fast growth and low expense of bacterial cultures while simultaneously enabling eukaryotic PTMs – however, they still suffer from difficulties in downstream processing and do not fully recapitulate mammalian glycosylation PTMs (Tripathi & Shrivastava, 2019).

Other approaches involve the engineering of native protein production pathways either in vivo or in vitro. Previous work has demonstrated the feasibility of engineering some eukaryotic PTMs into E. coli such as disulfide bonds and glycosylation. In addition, researchers have introduced completely cell-free in vitro protein production methods using cell lysates or recombinant protein mixtures. Although promising, these approaches are not yet mature enough to fully assess their usage (Andersen & Krummen, 2002; Assenberg et al., 2013).

All the above methods utilize canonical protein production or secretory pathways in some manner to generate recombinant protein (Figure 4). In recent years, a number of unconventionally-secreted mammalian proteins have been characterized and their secretory pathways elucidated (Rabouille et al., 2012). Although researchers have investigated the role of these unconventional secretory pathways in various diseases states such as cancer and Alzheimer's, there has been little work in the literature

describing their utilization as a platform for recombinant protein production. Investigation and implementation of a mammalian unconventional secretory pathway could therefore represent a novel method of producing recombinant proteins.

Expression system	Most common application	Advantages	Challenges
Mammalian 	• Functional assays • Structural analysis • Antibody production • Expression of complex proteins • Protein interactions • Virus production	• Highest-level protein processing • Can produce proteins either transiently, or by stable expression • Robust optimized transient systems for rapid, ultrahigh-yield protein production	• Gram-per-liter yields only possible in suspension cultures • More demanding culture conditions
Insect 	• Functional assays • Structural analysis • Expression of intracellular proteins • Expression of protein complexes • Virus production	• Similar to mammalian protein processing • Can be used in static or suspension culture	• More demanding culture conditions than prokaryotic systems • Production of recombinant baculovirus vectors is time consuming
Yeast 	• Structural analysis • Antibody generation • Functional analysis • Protein interactions	• Eukaryotic protein processing • Scalable up to fermentation (grams per liter) • Simple media requirements	• Fermentation required for very high yields • Growth conditions may require optimization
Bacterial 	• Structural analysis • Antibody generation • Functional assays • Protein interactions	• Scalable • Low cost • Simple culture conditions	• Protein solubility • May require protein-specific optimization • May be difficult to express some mammalian proteins
Algal 	• Studying photosynthesis, plant biology, lipid metabolism • Genetic engineering • Biofuel production	• Genetic modification and expression systems for photosynthetic microalgae • Superb experimental control for biofuel, nutraceuticals, and specialty chemical production • Optimized system for robust selection and expression	• Nascent technology • Less developed compared to other host platforms
Cell-free 	• Toxic proteins • Incorporation of unnatural label or amino acids • Functional assays • Protein interactions • Translational inhibitor screening	• Open system; able to add unnatural components • Fast expression • Simple format	• Scaling above multimilligram quantities may not be costly

Figure 4. Comparison of protein expression systems

Applications, advantages, and challenges of several commonly-utilized recombinant protein expression systems (reproduced from Thermo Fisher Scientific, 2018)

Unconventional Secretory Pathways as a Novel Production Method

Conventional protein secretion (CPS) in mammalian cells is well characterized. Mammalian secretory proteins often contain a signal sequence that is recognized by a signal recognition particle (SRP) during ribosomal translation. This SRP directs the ribosomal complex towards the endoplasmic reticulum (ER) via the SRP receptor, whereby the nascent polypeptide chain is translocated into the ER lumen (Figure 5). From the ER, the polypeptide chain makes its way through the Golgi apparatus via COPII-coated vesicles before being secreted as a protein at the plasma membrane (Reid & Nicchitta, 2015). The ER and Golgi organelles are the primary sites of eukaryotic PTMs such as glycosylation and disulfide bond formation, and thus a secretory protein that bypasses one or more of these organelles is considered to be unconventionally secreted (Figure 6) (Rabouille et al., 2012).

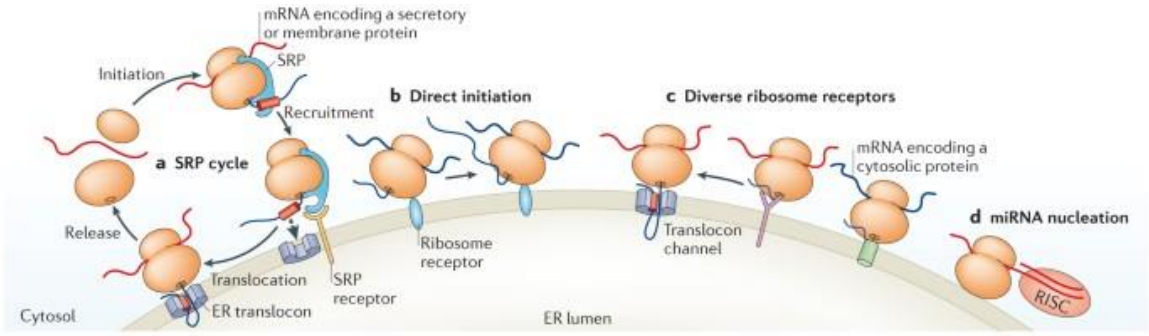

Figure 5. Conventional translation by ER-proximal ribosomes

Conventional protein secretion is directed by SRP-mediated interactions between proteins bearing signal sequence motifs and the ER (Reid & Nicchitta, 2015).

Unconventional protein secretion (UPS) encompasses multiple secretory routes that bypass the ER/Golgi and can be broadly categorized into Type I (direct protein translocation across the plasma membrane), Type II (ABC-mediated protein translocation across the plasma membrane), Type III (protein uptake and secretion by vesicular bodies), and Type IV (Golgi bypass of plasma membrane-resident proteins). Of these pathways, the Type II pathway is not particularly well characterized in mammals, and the Type IV pathway primarily impacts membrane-bound proteins. As a result, investigation of the Type I/III pathways will form the basis of this work (Rabouille, 2017).

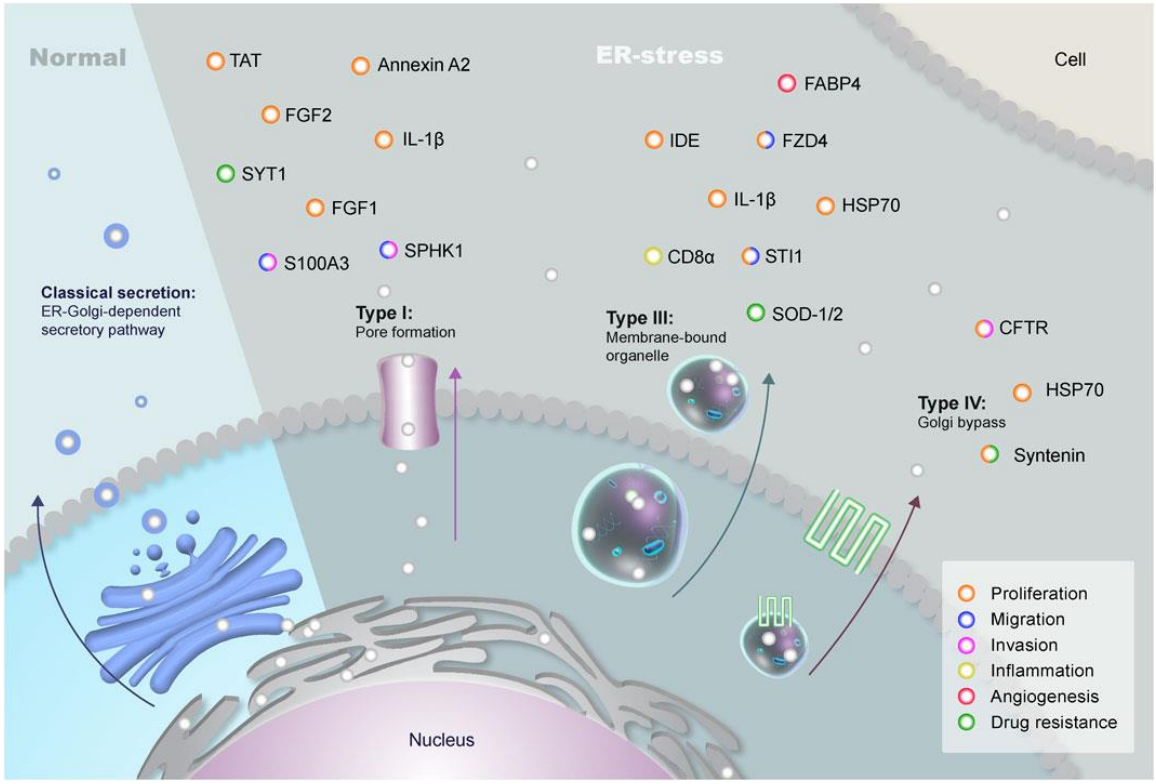

Figure 6. Conventional and unconventional protein secretory pathways

CPS pathways share an ER/Golgi-dependent secretory mechanism, while UPS pathways can utilize various alternative mechanisms to secrete proteins (Iglesia et al., 2022)

Type I UPS pathways describe the direct translocation of proteins from the cytosol to the extracellular space via plasma membrane pore formation (Rabouille, 2017). This is a tightly regulated process that encompasses both constitutively-expressed proteins (e.g. FGF2 and HIV-TAT) and stress-induced proteins (e.g. IL-1β). The exact mechanism by which these proteins are secreted is variable – for example, both FGF2 and HIV-TAT are dependent on plasma membrane localization via their interaction with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (Cohen et al., 2020). However, FGF2 undergoes self-oligomerization which drives membrane insertion and lipidic pore formation in the plasma membrane, after which it is trapped on the extracellular face with the assistance of heparan sulfate proteoglycans. On the other hand, HIV-TAT and other cell-penetrating peptides (CPPs) may be secreted via alternative mechanisms such as direct insertion and translocation without pore formation (Patel et al., 2019). In some cases, Type I pathways are even induced by inflammation and stress pathways – one mechanism by which the cytokine IL-1β is secreted involves pyroptotic pore formation via inflammasome signaling and caspase/gasdermin activation (Iglesia et al., 2022) (Figure 7).

Type III UPS pathways describe several mechanisms by which cytosolic proteins are trafficked to the extracellular environment via vesicular localization and trafficking (Rabouille, 2017). These mechanisms are typically associated with stress responses, including those initiated by starvation and other extracellular stresses (e.g. IL-1β), or as a result of protein misfolding and aggregation (e.g. CSPα) (Xu et al., 2018; Zhang et al., 2020). As with the Type I pathways, the organelles involved in Type III pathways are diverse and include late endosomes, autophagic vesicles, and multi-vesicular bodies

(Rabouille et al., 2017). For example, research by Zhang et al. describes how KFERQ-like motifs in IL-1β enable HSP90A-mediated protein unfolding and translocation via TMED10 into the intermembrane space of autophagic vesicles. This mechanism functions independently of the pyroptotic pathway and enables the constitutive secretion of IL-1β unconventionally. Similarly, research described by Xu et al. describes how aggregation-prone proteins such as tau or α-synuclein are unconventionally secreted by a misfolding-associated protein secretion (MAPS) pathway. This pathway involves the recruitment and enrichment of aggregated or misfolded proteins via USP19/HSC70, and subsequent translocation via CSPα into late endocytic vesicles.

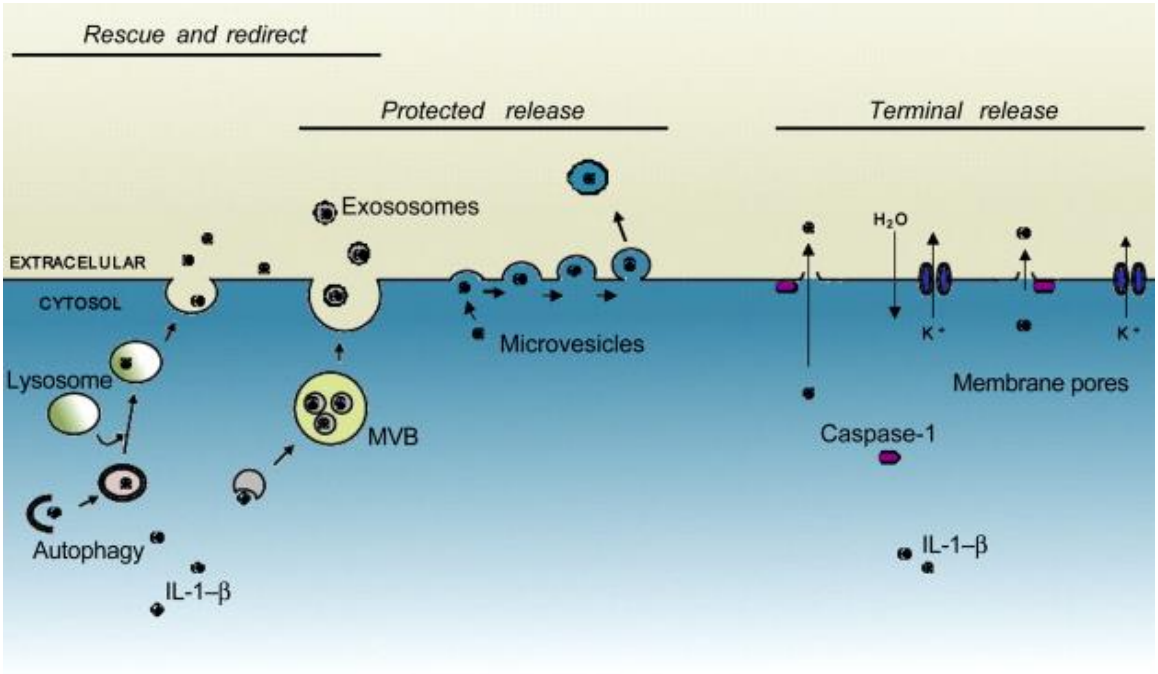

Figure 7. Unconventional mechanisms of IL-1β release

IL-1β is an archetypal unconventionally-secreted protein and participates in multiple unique UPS pathways including autophagic release, microvesicular shedding, and pyroptotic/apoptotic release (Lopez-Castejon & Brough, 2011).

Potential Applications

Utilizing the previously described unconventional secretory pathways can prove to be a novel mechanism for protein production. Previous research has suggested that tagging a cytosolic cargo protein with proteins associated with unconventional protein secretion results in the secretion of the tagged cargo protein (Xu et al., 2018; Zhang et al., 2020; Lee et al., 2022). While this has been demonstrated individually for a select few unconventional secretory pathways, there has been no research either in comparing the secretion efficacy between unconventional secretory pathways or in assessing the quality and function of the resulting recombinant protein.

Unconventional protein secretion may have some benefits over conventional protein secretion. For example, although half of all ribosomes are ER-bound, a large fraction of proteins translated code for cytosolic proteins. In HEK293 cells, mRNAs encoding membrane and secretory proteins only account for roughly 13% of all mRNAs, suggesting that the cytosolic protein reservoir may be a more capable protein production platform in terms of production capacity (Figure 8) (Reid & Nicchitta, 2015).

In addition, although many recombinant proteins require the expression and purification of only a single protein of interest (PoI), some production processes utilize additional recombinant proteins to facilitate modification and processing steps. However, utilization of such reagents can be hampered in some cases by process limitations. One example of such a limitation can be found in the preparation of Fab fragments from intact IgG. *Streptococcus agalacticae* IgDE is a cysteine protease that specifically cleaves one site above the hinge region of human IgG antibodies (Spoerry et al., 2016). For the purposes of Fab generation, this makes *S. agalacticae* IgDE superior to other enzymes

like papain that do not have the same level of substrate specificity. However, production of IgDE typically utilizes bacterial organisms and may be contaminated with endotoxin that is undesirable when incubating with antibodies intended for *in vivo* usage (Oliveira & Domingues, 2017).

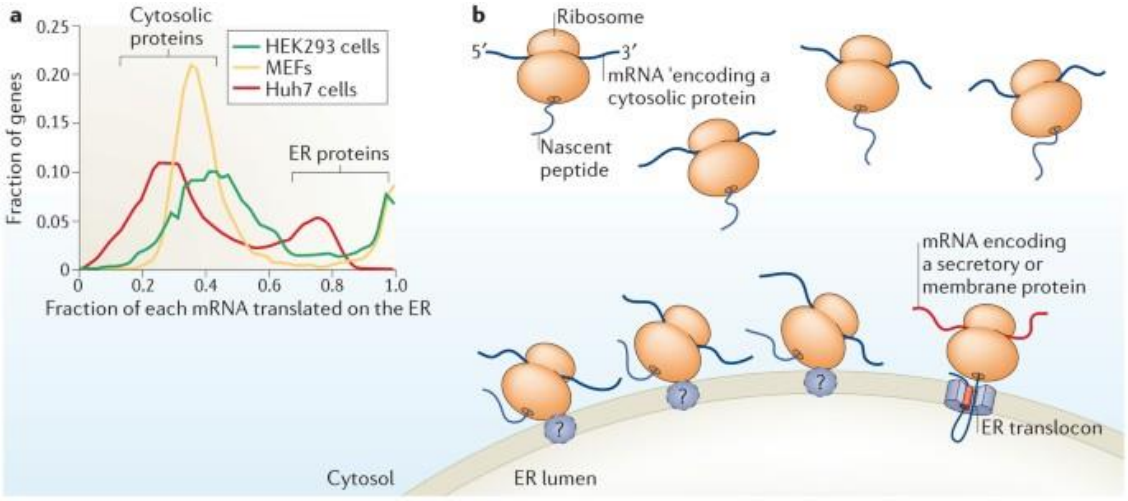

Figure 8. Distribution of ribosomal translation

ER and cytoplasmic-directed proteins show two distinct populations, with ER-enriched mRNAs composing a smaller fraction of genes across three different cell lines (Reid & Nicchitta, 2015).

While a typical workflow utilizing this protein might involve the expression and purification of an antibody, co-incubation of the antibody with the *S. agalacticae* IgDE, and subsequent re-purification and cleanup of the digested Fab fragment, consolidating these parallel processes into a single workflow using unconventional secretory pathways could provide some benefits. For many proteins, this could drastically reduce complexity, error, and cost of certain protein production processes, while simultaneously increasing

workflow flexibility. In such a case, production of both the antibody and the enzyme in the same mammalian host would prove beneficial. Although bacterial enzymes may be subjected to undesirable PTMs in the mammalian secretory pathway, there is potential in the usage of unconventional secretory pathways to both bypass eukaryotic PTMs and avoid resource competition for conventional protein production of mammalian cells.

Chapter II.

Materials and Methods

The following section outlines the materials and methods used in the design, production, screening, and characterization of several unconventionally-secreted recombinant proteins. Briefly, conventional molecular cloning techniques were used to generate plasmids coding for the protein of interest. These plasmids were subsequently expressed in mammalian cell cultures before being harvested and purified by affinity chromatography. A rapid screening assay was performed to rank the top construct designs, from which the top-ranked UPS tag was utilized for biophysical and functional characterization.

Construct Design

In order to identify a suitable candidate pathway for an unconventional recombinant protein production platform, a panel consisting of 17 amino acid sequences representing various Type I and III UPS pathway motifs was identified from the literature. In all construct designs, sequences functioned as UPS tags when paired with varying cargo proteins to investigate different aspects of the UPS pathway on key parameters of protein secretion including product titer, post-translational modifications, and quality.

Table 3. Selected UPS tags

UPS Tag	UPS Pathway	Rationale
IL-1b motif	UPS Type III	TMED10-mediated translocation into secretory vesicles (Zhang et al., 2020)
FGF2	UPS Type I	PI(4,5)P2-mediated oligomerization and pore-formation at plasma membrane, followed by heparan sulfate-mediated extracellular translocation (Rabouille, 2017)
IL-1b	UPS Type I/III	Variety of mechanisms, including Caspase-mediated pyroptotic release and translocation into secretory vesicles
CSPa	UPS Type III	CSPα-mediated enrichment and translocation into secretory vesicles (Xu et al., 2018)
TAT	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
cTAT	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
cR8	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
Transportan	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
cTransportan	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
Penetratin	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
cPenetratin	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
PenShuf	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
PenetraMax	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
MAP	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
IgV	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
CL	UPS Type I/III (CPP)	Cell penetrating peptide (CPP) (Patel et al., 2019)
GFP1-10	UPS Type III (MAPS)	Misfolding-associated protein secretion (MAPS) (Lee et al., 2022)

A list of selected UPS tags, their pathways, and mechanism of secretion.

All UPS tags and cargo proteins were ordered as synthetic gBlocks™ from Integrated DNA Technologies (Coralville, IA) with compatible 5’ and 3’ DNA overhangs to facilitate molecular cloning, as well as linkers containing unique restriction sites to facilitate molecular subcloning. All constructs were cloned into the multiple cloning site (MCS) of a pcDNA3.4™ plasmid vector (Cat# A14697, Thermo Fisher Scientific, Waltham, MA), and were assembled following standard isothermal DNA assembly or DNA ligation protocols. Briefly, pcDNA3.4™ or previously-cloned plasmid DNA was linearized via re-striction digestion of the MCS and purified by gel electrophoresis and extraction (Cat# D4002, Zymo Research, Irvine, CA). For isothermal assembly of UPS tags and cargo sequences into pcDNA3.4™, the linearized plasmid DNA was mixed with resuspended gBlock™ DNA in a 1:2 molar ratio and assembled using the NEBuilder® HiFi DNA Assembly Cloning Kit (Cat# E5520S, New England Biolabs, Ipswich, MA) following manufacturer protocols. For routine subcloning of UPS cargo across different plasmids, the linearized plasmid DNA was mixed with resuspended gBlock™ DNA in a 1:4 molar ratio and assembled using the Quick Ligation™ Kit (Cat# M2200L, New England Biolabs, Ipswich, MA) following manufacturer protocols.

All plasmid DNA constructs followed the same basic design consisting of a UPS tag, glycine-serine linker (G4S), UPS cargo protein, 2x glycine-serine linker (2xG4S), and a 6xHis-tag downstream the pcDNA3.4™ CMV promoter (Figure 9). All con-structs were transformed into chemically-competent DH5α E. coli cells (Cat# C2987U, New England Biolabs, Ipswich, MA) following manufacturer protocols and plated onto LB (Miller) agar plates containing 100µg/mL Carbenicillin (Cat# L1010, Teknova, Hollister, CA) to select for transformed clones. Single colonies were picked, cultured, and sent for

sequence validation by Sanger sequencing (Wyzer Biosciences, Cambridge, MA). Sequence-verified clones were subsequently cultured in LB (Miller) media containing 100µg/mL Carbenicillin (Cat# L8185, Teknova, Hollister, CA) overnight and maxi-prepped using the ZymoPURE II Plasmid Maxiprep Kit (Cat# D4203, Zymo Research, Irvine, CA) following manufacturer protocols to obtain transfection-quality DNA.

Figure 9. Plasmid map for experimental constructs

All constructs were designed for modular pairing of UPS tags and cargo proteins in the same backbone. The UPS tag represents either a CPS signal sequence (IL2ss) or a UPS tag from Table 3; G4S represents a linker comprised of four glycine and one serine residue; UPS cargo represents a potential cargo protein; (G4S)2 represents a linker comprised of two G4S linkers; 6xHis represents a six-residue histidine affinity tag. Created with BioRender.com.

Construct Expression

Production of experimental constructs utilized a HEK293-based transient expression system. Expi293F™ suspension cells (Cat# A14527, Thermo Fisher Scientific, Waltham, MA) were routinely passaged in fresh FreeStyle™ F17 expression medium (Cat# A1383501, Thermo Fisher Scientific, Waltham, MA) supplemented with glutamine and a non-ionic surfactant to reduce shear stress. Cell density and viability

were determined via Trypan Blue staining on the Countess II Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA).

On the day of transfection, HEK293 cells were passaged to a density of 5e6 cells/mL in fresh F17 media. For every 1mL of culture, 1µg of plasmid DNA was mixed into 50µl fresh F17 media and 2µl of transfection-grade linear polyethylenimine (PEI, Cat# 24765-1, Polysciences, Warrington, PA) into 50ul of separate fresh F17 media. The DNA:media and PEI:media solutions were combined and allowed to complex at room temperature for 10 minutes. The complexed DNA:PEI solution was then gently mixed into the cell culture and incubated for five days in a 37°C incubator (8% CO2, 80% humidity, 150 RPM). One day after transfection, cells were fed with a proprietary feed supplemented with 50uM N-azidoacetylmannosamine-tetraacetylated (Ac4ManNAz, Cat# 900917-50MG, MilliporeSigma, Burlington, MA) to metabolically label glycans on secreted proteins.

After five days, cell culture supernatants were harvested and clarified. Cells were pelleted in a benchtop centrifuge for 30 minutes at 3000rcf, and the supernatants carefully decanted into separate tubes. The harvested supernatants were then filtered through a 0.2µm polyethersulfone (PES) filter (Cat# 97066-212, VWR, Radnor, PA) to clarify. The clarified supernatants were stored at 4°C until further use.

UPS Screening Assay

A screening assay was designed to compare the secreted protein titers of a UPS-tagged cargo protein. In order to easily quantify relative protein secretion, GFP was used as the cargo protein, enabling rapid fluorescence measurements on a plate reader. Comparator constructs were included in the assay design – GFP without any secretion

tags served as a nonspecific secretory control, while GFP with an IL2ss substituting a UPS tag served as a conventional secretory pathway control. A mock transfection using herring sperm DNA (Cat# 15634017, Thermo Fisher Scientific, Waltham, MA) was also included to serve as a blank.

Following construct design and expression, all clarified supernatants were serially diluted in fresh F17 media and loaded onto an opaque, flat-bottom 96-well plate (Cat# CLS3922-100EA, MilliporeSigma, Burlington, MA) at 100µl per well. Fluorescence was measured on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) using top read at 488nm/507nm excitation/emission. All samples were tested in replicates and final rankings were calculated based on normalized and averaged data.

Protein Purification

Experimental proteins were purified using affinity chromatography in either batch or automated mode. His-tagged UPS constructs were purified in batch mode using the Capturem™ His-Tagged Purification Maxiprep Kit (Cat# 635713, Takara Bio, Kusatsu, Japan) following manufacturer protocols. His-tagged UPS constructs were purified in automated mode using the ÄKTA pure™ automated fast-protein liquid chromatography system (Cytiva, Marlborough, MA). Samples were loaded onto a 5mL HisPur™ Cobalt prepacked chromatography column (Cat# 90094, Thermo Fisher Scientific, Waltham, MA) and washed/eluted following manufacturer protocols. Purified proteins were subsequently concentrated and buffer-exchanged into PBS pH7.2 (Cat# 20012027, Thermo Fisher Scientific, Waltham, MA) using 10kDa Amicon® Ultra-15 centrifugal filter units (Cat# UFC901096, MilliporeSigma, Burlington, MA) following manufacturer protocols. Concentrated proteins were stored at 4°C until further use.

Antibodies were purified using the ÄKTA pure™ automated fast-protein liquid chromatography system. Samples were loaded onto a 5mL HiTrap MabSelect SuRe prepacked chromatography column (Cat# 11003495, Cytiva, Marlborough, MA) and washed/eluted following manufacturer protocols. Purified proteins were subsequently concentrated and buffer-exchanged into PBS pH7.2 using 10kDa Amicon® Ultra-15 centrifugal filter units and stored at 4°C until further use.

Biophysical Characterization of *S. agalacticae* IgDE

S. agalacticae IgDE was selected as a cargo protein to characterize the effects of secretion from the top UPS tag. Two separate comparator constructs were included – IgDE with an IL2 signal sequence (IL2ss) enabling conventional secretion served as an internal reference, while FabALACTICA®, a commercial IgDE purified from *E. coli*, served as an external reference (Cat# A0-AG1-020, Genovis, Cambridge, MA).

Protein Expression (Western Blot)

Protein expression was characterized by Western Blot. Clarified supernatants were mixed with 4x Laemmli sample buffer (Cat# 1610747, Bio-Rad, Hercules, CA) (non-reducing) and incubated at 90°C for 10 minutes to denature. Samples were loaded onto Bolt™ 4 to 12% Bis-Tris gels (Cat# NW04120BOX, Thermo Fisher Scientific, Waltham, MA) along with Precision Plus Protein Dual Color Standards (Cat# 1610374, Bio-Rad, Hercules, CA). Gels were run at 200V constant voltage for 30 minutes in Bolt™ MES running buffer (Cat# B0002, Thermo Fisher Scientific, Waltham, MA). After electrophoresis, gels were transferred onto nitrocellulose membranes using the iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific, Waltham, MA). The transferred

membranes were probed on the iBind™ Western Device (Thermo Fisher Scientific, Waltham, MA) using HRP-conjugated rabbit polyclonal antibody to 6xHis-tag (Cat# ab1187, Abcam, Cam-bridge, United Kingdom).

Protein Yield (NanoDrop)

Sample yield was measured using UV/Vis spectrophotometry. Sample absorption at 280nm was measured on a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Measurements were blanked on sample buffer, PBS pH7.2. Concentrations were calculated using molecular weights and extinction coefficients from the ProtParam tool hosted on ExPASy.

Protein Purity (SDS-PAGE/Western Blot)

Protein purity was characterized by SDS-PAGE and Western Blot. Purified proteins were mixed with 4x Laemmli sample buffer with β-mercaptoethanol (reducing) (Cat# M6250, MilliporeSigma, Burlington, MA) or without (non-reducing) and incubated at 90°C for 10 minutes to denature. Approximately 3µg of protein was loaded onto Bolt™ 4 to 12% Bis-Tris gels along with Precision Plus Protein Dual Color Standards. Gels were run at 200V constant voltage for 30 minutes in Bolt™ MES running buffer. After electrophoresis, gels were either stained with ReadyBlue protein gel stain (Cat# RSB-1L, MilliporeSigma, Burlington, MA) or were transferred onto nitrocellulose membranes using the iBlot™ 2 Gel Transfer Device. The transferred membranes were probed on the iBind™ Western Device using HRP-conjugated rabbit polyclonal antibody to 6xHis-tag.

Protein Glycosylation (SDS-PAGE/Western Blot)

Protein glycosylation was characterized by SDS-PAGE and Western Blot. Purified proteins were diluted into PBS pH7.2 and incubated with molar excess sulfo-dibenzocyclooctyne-biotin conjugate (sulfo-DBCO-biotin, Cat# 760706, MilliporeSigma, Burlington, MA) for 2 hours to selectively tag Ac4ManNAz-labelled glycans. After incubation, the biotin-tagged proteins were buffer-exchanged into PBS pH7.2 to remove excess sulfo-DBCO-biotin using 10kDa Amicon® Ultra-15 centrifugal filter units. Biotin-tagged proteins were mixed with 4x Laemmli sample buffer (non-reducing) and incubated at 90°C for 10 minutes to denature. Approximately 3µg of protein was loaded onto Bolt™ 4 to 12% Bis-Tris gels along with Precision Plus Protein Dual Color Standards. Gels were run at 200V constant voltage for 30 minutes in Bolt™ MES running buffer. After electrophoresis, gels were either stained with ReadyBlue protein gel stain or were transferred onto nitrocellulose membranes using the iBlot™ 2 Gel Transfer Device. The transferred membranes were probed on the iBind™ Western Device using HRP-conjugated streptavidin (Cat# ab7403, Abcam, Cambridge, United Kingdom).

Functional Characterization of *S. agalacticae* IgDE

S. agalacticae IgDE was functionally characterized by either co-incubation or co-expression with recombinant monoclonal antibodies. Conventionally-secreted IgDE served as a control in both models, while FabALACTICA® served as a commercial comparator in the co-incubation model only. HEK293 cells co-transfected with antibody and herring sperm DNA served as a negative control in the co-expression model.

IgDE Co-Incubation (SDS-PAGE)

Sample activity was first functionally characterized by co-incubation with an internal human IgG1 recombinant monoclonal antibody. Purified IgDE was co-incubated with 20µg IgG1 at 2-fold unit excess for 4, 8, and 16 hours. Digestion products were mixed with 4x Laemmli sample buffer with β-mercaptoethanol (reducing) (Cat# M6250, MilliporeSigma, Burlington, MA) or without (non-reducing) and incubated at 90°C for 10 minutes to denature. Approximately 3µg of protein was loaded onto Bolt™ 4 to 12% Bis-Tris gels along with Precision Plus Protein Dual Color Standards. Gels were run at 200V constant voltage for 30 minutes in Bolt™ MES running buffer. After electrophoresis, gels were stained with ReadyBlue protein gel stain and imaged.

IgDE Co-Expression (SDS-PAGE/Western Blot)

In the second experiment, IgDE was co-transfected with an internal human IgG1 recombinant monoclonal antibody for the duration of the expression period, and antibody/antibody fragments were purified from the supernatant using Protein A. Digestion products were mixed with 4x Laemmli sample buffer with β-mercaptoethanol (reducing) (Cat# M6250, MilliporeSigma, Burlington, MA) or without (non-reducing) and incubated at 90°C for 10 minutes to denature. Approximately 3µg of protein was loaded onto Bolt™ 4 to 12% Bis-Tris gels along with Precision Plus Protein Dual Color Standards. Gels were run at 200V constant voltage for 30 minutes in Bolt™ MES running buffer. After electrophoresis, gels were either stained with ReadyBlue protein gel stain or were transferred onto nitrocellulose membranes using the iBlot™ 2 Gel Transfer Device. The transferred membranes were probed on the iBind™ Western Device using HRP-conjugated anti-human IgG (Cat# ab6858, Abcam, Cambridge, UK).

Chapter III.

Results

The focus of this research was to assess the feasibility of utilizing an unconventional secretory pathway as a method of recombinant protein production. To accomplish this, a screening assay was developed to assess the secretion efficiency of GFP when paired with varying UPS tags. The top-ranked construct was subsequently used to produce and characterize a recombinant enzyme, *Streptococcus agalacticae* Ig-degrading enzyme (IgDE).

Preliminary Assessment of UPS Constructs

In order to rank the suitability of unconventional secretory pathways for protein secretion, a screening assay was designed using GFP and glycosylated GFP (glyco-GFP) as cargo proteins. Previous research into the mechanisms underlying CPS utilized GFP as a cargo protein, and it was selected in this work as a cargo protein to enable facile assessment of secreted protein titers on a plate reader (Zhang et al., 2020). Additional literature also suggested that a point mutation introducing a glycosylation motif into GFP can decrease fluorescence in a PTM-dependent manner (Losfeld et al., 2012). Thus, glyco-GFP has the potential to function as a glycosylation-sensitive reporter for protein secretion, and was also selected as a cargo protein to simultaneously enable facile assessment of PTMs.

To assess the feasibility of such a strategy, several mammalian expression vectors were cloned using synthetic gene fragments (Table 4). Both GFP and glyco-GFP were tagged with the IL2ss for conventional secretion (UPS-3 and 4), as well as a known IL-1β motif for unconventional secretion (UPS-7 and 8) (Zhang et al., 2020). To account for non-specific leakage and GFP-specific unconventional secretory mechanisms, untagged GFP and glyco-GFP were also generated (UPS-1 and 2). Finally, to confirm that changes in secretion were driven by the UPS tag and not by alternative mechanisms, UPS-tagged GFP and glyco-GFP were also tagged with IL2ss for conventional secretion (UPS-5 and 6). Single clones of each construct were sequenced and maxiprepped before expression.

Table 4. Cloned UPS constructs

ID	Description	Description
UPS-1	G4S_EGFP_2xG4S_6xHis	NS control
UPS-2	G4S_glyco-EGFP_2xG4S_6xHis	NS control
UPS-3	IL2ss_G4S_EGFP_2xG4S_6xHis	CPS control
UPS-4	IL2ss_G4S_glyco-EGFP_2xG4S_6xHis	CPS control
UPS-5	IL2ss_IL-1β_G4S_EGFP_2xG4S_6xHis	CPS control
UPS-6	IL2ss_IL-1β_G4S_glyco-EGFP_2xG4S_6xHis	CPS control
UPS-7	IL-1β motif_G4S_EGFP_2xG4S_6xHis	UPS Type III
UPS-8	IL-1β motif_G4S_glyco-EGFP_2xG4S_6xHis	UPS Type III
UPS-9	FGF2_G4S_EGFP_2xG4S_6xHis	UPS Type I
UPS-10	IL-1β_G4S_EGFP_2xG4S_6xHis	UPS Type I/III
UPS-11	CSPα_G4S_EGFP_2xG4S_6xHis	UPS Type III
UPS-12	TAT_G4S_EGFP_2xG4S_6xHis	CPP
UPS-13	cTAT_G4S_EGFP_2xG4S_6xHis	CPP
UPS-14	cR8_G4S_EGFP_2xG4S_6xHis	CPP
UPS-15	Transportan_G4S_EGFP_2xG4S_6xHis	CPP
UPS-16	cTransportan_G4S_EGFP_2xG4S_6xHis	CPP
UPS-17	Penetratin_G4S_EGFP_2xG4S_6xHis	CPP
UPS-18	cPenetratin_G4S_EGFP_2xG4S_6xHis	CPP
UPS-19	PenShuf_G4S_EGFP_2xG4S_6xHis	CPP
UPS-20	PenetraMax_G4S_EGFP_2xG4S_6xHis	CPP
UPS-21	MAP_G4S_EGFP_2xG4S_6xHis	CPP
UPS-22	IgV_G4S_EGFP_2xG4S_6xHis	CPP
UPS-23	CL_G4S_EGFP_2xG4S_6xHis	CPP
UPS-24	GFP1-10_G4S_EGFP_2xG4S_6xHis	MAP
UPS-25	IL2ss_G4S_saIgDE_2xG4S_6xHis	CPS control
UPS-26	IL-1β_G4S_saIgDE_2xG4S_6xHis	UPS Type I/III
UPS-27	IL-1β_G4S_VH3-30/VK1-39-scFv_2xG4S_6xHis	UPS Type I/III
UPS-28	IL-1β_G4S_I21-R33-scFv_2xG4S_6xHis	UPS Type I/III
UPS-29	IL-1β_G4S_HRAS_2xG4S_6xHis	UPS Type I/III

A list of UPS-tagged constructs and controls used throughout the experiments.

The preliminary GFP/glyco-GFP screening assay was validated using harvested supernatants from HEK293 cells transiently transfected with the maxipreps of UPS-1 through 8 (Figure 10). Following a 5-day expression period, the cells were spun down and the supernatants were harvested and measured on a plate reader. The relative fluorescence of each construct was calculated by subtracting the background (determined from the fluorescence of supernatants from HEK293 cells mock-transfected with herring sperm DNA) and normalizing against fluorescence from supernatants of nonspecific GFP secretion (UPS-1). Notably, all the glyco-GFP constructs (UPS-2, 4, 6, and 8) exhibited a lower relative fluorescence compared to their GFP counterparts (UPS-1, 3, 5, and 7). This decrease was observed regardless of whether the cargo was secreted via nonspecific leakage (UPS 2), conventional secretion (UPS-4 and 6), or unconventional secretion (UPS-8). Although glyco-GFP is expected to exhibit variable fluorescence depending on its glycosylation state, the low relative fluorescence of these constructs across varying secretory pathways makes it difficult to accurately compare changes in fluorescence between different tagged proteins. This low fluorescence may perhaps be due to altered protein folding or stability following the introduction of the glycosylation motif. As a result, it was determined that glycosylated GFP would not be utilized to assess PTMs.

From the preliminary GFP/glyco-GFP screening assay, it was observed that supernatants containing the unconventionally-secreted GFP (UPS-7) exhibited a 1.4-fold increase in relative fluorescence compared to the nonspecific control. Interestingly, conventional secretion of GFP (UPS-3) resulted in a lower relative fluorescence compared to nonspecific leakage of GFP into the supernatant (UPS-1). This could be due to intrinsically poor expression or secretion of GFP through the secretory pathway either

from codon usage or signal peptide pairing, which is supported by the poor secretion of the ER-routed UPS-tagged GFP (UPS-5). Nevertheless, the observation that supernatant containing unconventionally-secreted GFP exhibits increased relative fluorescence over the nonspecific leakage/release of GFP supports the investigation of a wider array of UPS tags.

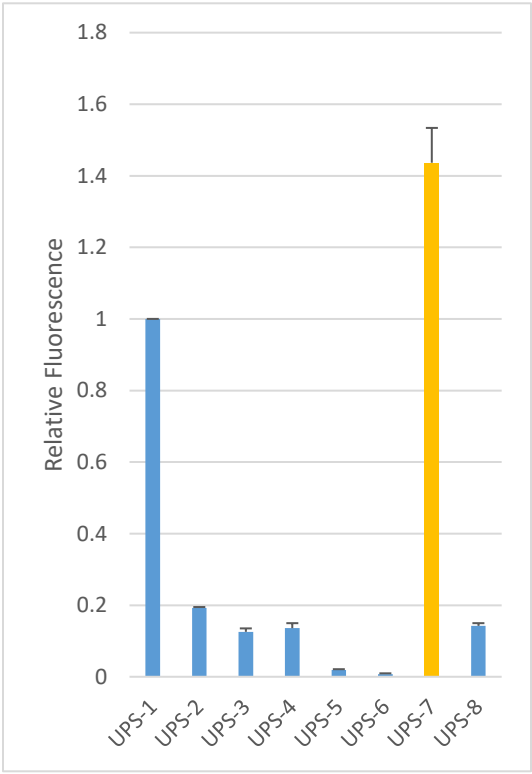

Figure 10.Glyco-GFP secretion efficiency across multiple secretory pathways

Relative fluorescence of supernatants from HEK293 cultures transiently transfected with UPS-tagged and control GFP and glycol-GFP constructs across multiple secretory pathways. Glyco-GFP constructs show lower expression/secretion across all pathways.

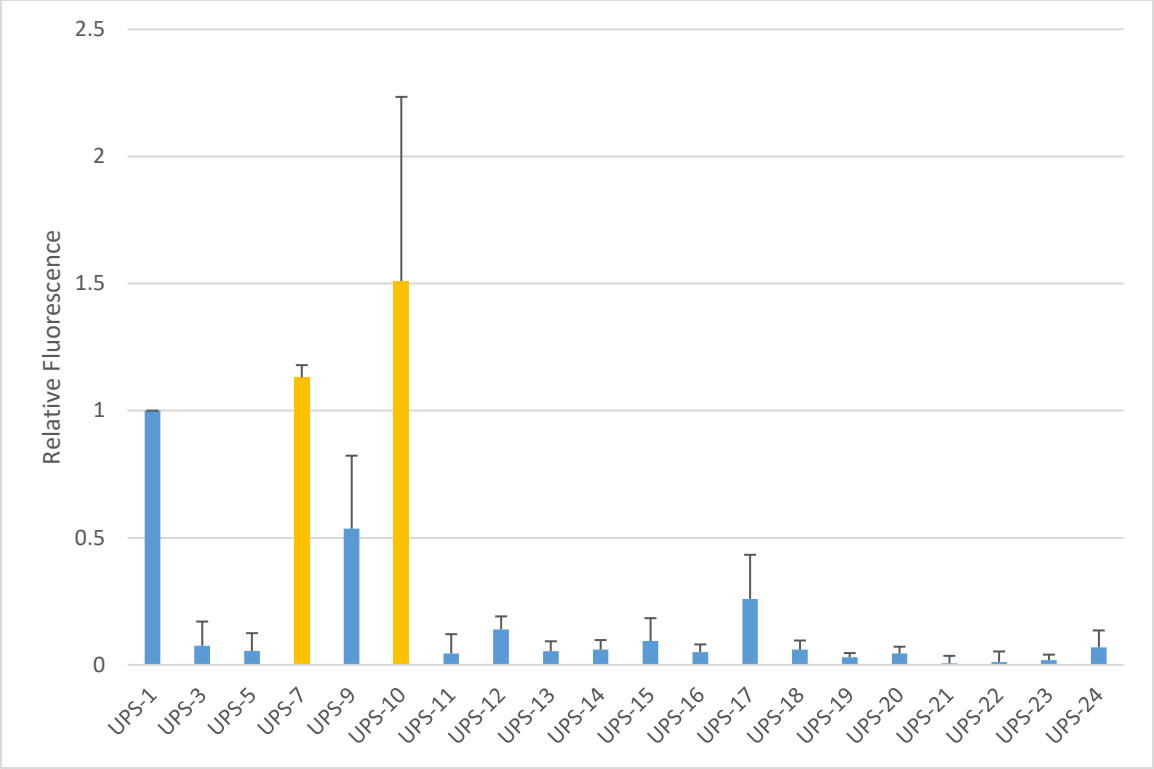

Figure 11.GFP secretion efficiency across multiple unconventional secretory pathways

Relative fluorescence of supernatants from HEK293 cultures transiently transfected with UPS-tagged and control GFP constructs across multiple unconventional secretory pathways. IL-1β motif (UPS-7) and full-length IL-1β (UPS-10) exhibit higher relative fluorescence compared to baseline (approximately 1.2 and 1.5-fold, respectively).

The validated screening assay was used to assess the secretion of GFP tagged with multiple different UPS tags (Figure 11). Based on the results, only two constructs produced supernatants that exhibited an increased relative fluorescence over the nonspecific control (UPS-7 and 10). Both of these constructs utilized a UPS-tag derived from IL-1β, and the GFP tagged with the full-length IL-1β (UPS-10) exhibited a higher relative fluorescence than the GFP tagged with a UPS motif derived from IL-1β (UPS-7). This discrepancy may be driven by the other alternative unconventional secretory

mechanisms of full-length IL-1β, allowing UPS-10 to take advantage of multiple UPS pathways simultaneously. Again, both the conventionally-secreted constructs (UPS-3 and 5) appear to exhibit low relative fluorescence, and many of the other unconventionally-secreted proteins similarly exhibited low relative fluorescence. Given that UPS-7 is a derivative of UPS-10, the latter was selected for further characterization.

Expression and Characterization of UPS-tagged *S. agalacticae* IgDE

To further assess the feasibility of IL-1β as secretion tag, several constructs were generated using this tag, with *S. agalacticae* IgDE (saIgDE) selected for in-depth characterization (Table 4). Conventionally-secreted saIgDE (UPS-25) and unconventionally-secreted saIgDE (UPS-26) were transiently expressed in HEK293 cells. Following a 5-day expression period, the cells were spun down and Western blots were run on clarified supernatants to confirm protein expression (Figure 12). Both conventionally-secreted saIgDE and unconventionally-secreted saIgDE appeared to express well, with the former exhibiting a broader, more intense band at its predicted MW (approximately 70 kDa) and the latter exhibiting a more compact band at the predicted MW (approximately 80 kDa), perhaps suggestive of variance in glycosylation patterns.

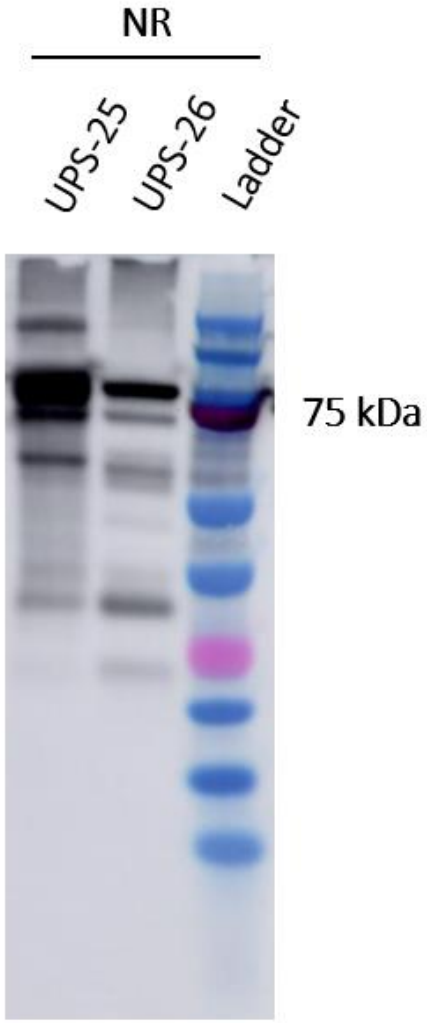

Figure 12.Expression levels of unconventionally-secreted saIgDE

Clarified supernatants from saIgDE-transfected cultures exhibit the presence of both conventionally and unconventionally-secreted saIgDE. Lane 1: UPS-25, non-reduced. Lane 2: UPS-26, non-reduced. Lane 3: ladder.

Since there appeared to be a significant amount of secreted material, both proteins were purified and quantified for further characterization (Table 5). Approximately 0.7mg of UPS-25 and 0.4mg of UPS-26 were purified from 60mL transient HEK293 culture, demonstrating the feasibility of IL-1β as a secretory tag. To further validate the efficacy

of IL-1β as a secretory tag, several other proteins (including VH3-30/VK1-39 germline scFv, I21-R33 scFv, and human HRAS) were cloned and expressed as IL-1β-fusion constructs, yielding titers >10 mg/L. Although the unconventionally-secreted saIgDE had relatively low titers at almost half that of conventionally-secreted saIgDE, there may be further room for expression optimization given that the transfection and expression protocols were optimized for conventional secretion.

Table 5. Secreted protein titers

Sample ID	Conc. (mg/mL)	Buffer	Volume (mL)	Amount (mg)	Titer (mg/L)
UPS-25	0.467	in His EB	1.500	0.700	11.663
UPS-26	0.281	in His EB	1.500	0.422	7.025
UPS-27	0.713	in His EB	1.500	1.070	17.825
UPS-28	0.694	in His EB	1.500	1.041	17.350
UPS-29	0.860	in His EB	1.500	1.290	21.500

Several unconventionally-secreted proteins transiently expressed in HEK293 cells show favorable titers. Conventionally-secreted saIgDE (UPS-25) exhibits 1.75-fold higher protein titer than unconventionally-secreted saIgDE (UPS-26).

To assess the purity of the purified saIgDE constructs, SDS-PAGE and Western Blot were performed on the purified samples. Approximately 100 units of commercial IgDE purified from E. coli (Fabalactica®), along with 2ug UPS-25 and UPS-26 were loaded and run on a gel. All proteins were observed at their expected molecular weights. UPS-25 and UPS-26 appeared to have a higher proportion of intact species, possibly due to the proteolytic digestion of Fabalactica® IgDE in *E. coli* (Figure 13, 14). In addition, UPS-25 appeared to have a wider band compared to Fabalactica® and UPS-26, possibly suggesting the presence of heterogeneous eukaryotic glycosylation in the conventionally-secreted protein.

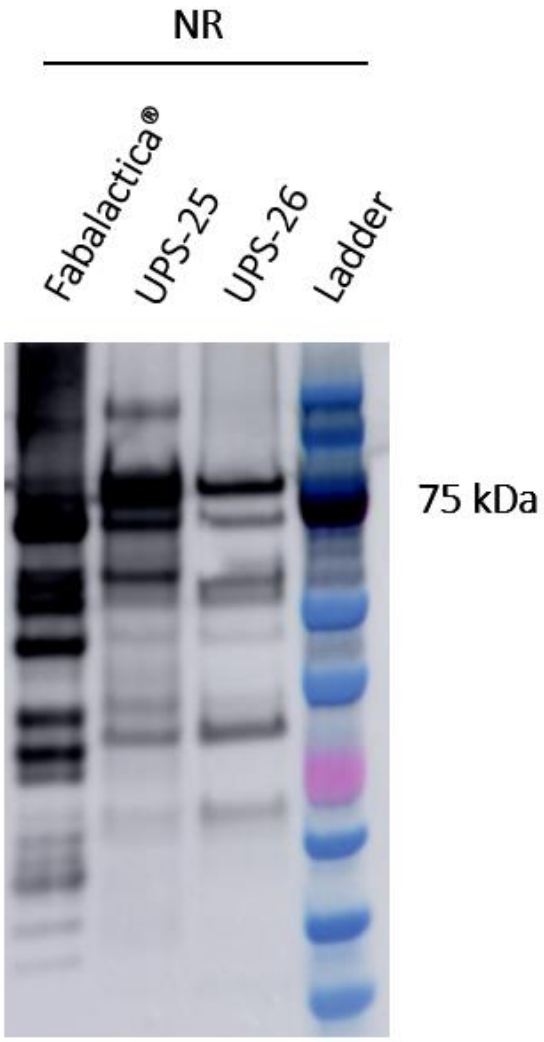

Figure 13.saIgDE purity following single-step IMAC

Both conventionally and unconventionally-secreted saIgDE show primary species around the expected MW band on Western Blot. Lane 1: Fabalactica®, non-reduced. Lane 2: UPS-25, non-reduced. Lane 3: UPS-26, non-reduced. Lane 4: ladder.

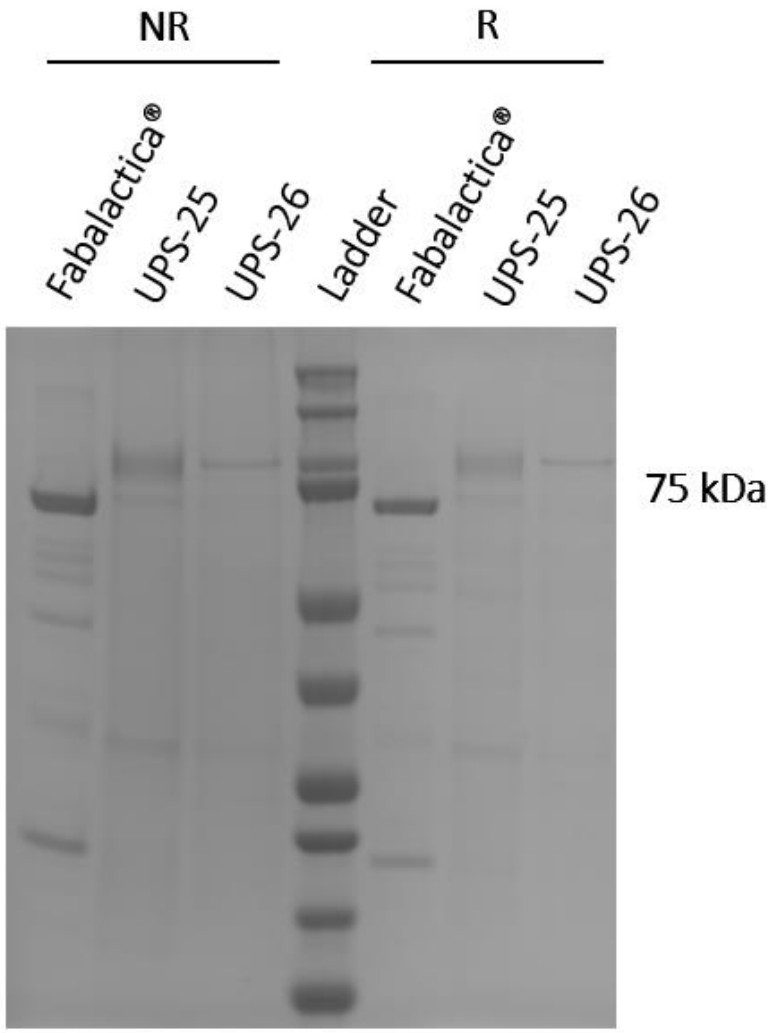

Figure 14.saIgDE purity following single-step IMAC

Both conventionally and unconventionally-secreted saIgDE show primary species around the expected MW band on SDS-PAGE. Lane 1: Fabalactica®, non-reduced. Lane 2: UPS-25, non-reduced. Lane 3: UPS-26, non-reduced. Lane 4: ladder. Lane 5: Fabalactica®, reduced. Lane 6: UPS-25, reduced. Lane 7: UPS-26, reduced.

A Western Blot was run to confirm the absence of glycosylation on the unconventionally-secreted saIgDE. During expression, cells were cultured in media supplemented with Ac4ManNAz to metabolically label glycans on secreted proteins.

After purification, the purified saIgDE was incubated with molar excess DBCO-biotin to biotin-label proteins via click chemistry. HRP-labeled streptavidin was then used to selectively detect biotin-labeled proteins – the intensity of the resulting bands on a Western blot could therefore be used to compare the relative abundance of glycans on the protein (Figure 15, 16). Samples were loaded and ran on SDS-PAGE and Western Blot. The UPS-25 construct exhibited a much wider band on the SDS-PAGE gel compared to the UPS-26 construct, as observed previously. After blotting and probing with streptavidin-HRP, an intense band was observed for the UPS-25 sample but not the UPS-26 sample. This suggests that conventionally-secreted IgDE is heavily glycosylated while unconventionally-secreted IgDE lacks glycosylation.

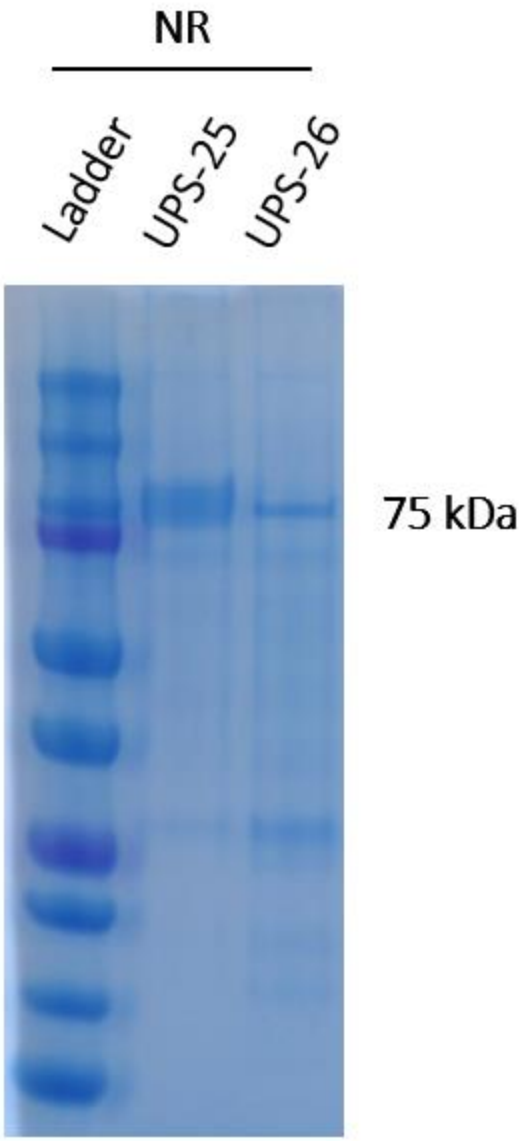

Figure 15.Glycosylation profile of unconventionally-secreted saIgDE

Unconventionally-secreted saIgDE shows a tighter band compared to conventionally-secreted saIgDE around the expected MW. Lane 1: Ladder, non-reduced. Lane 2: UPS-25, non-reduced. Lane 3: UPS-26, non-reduced.

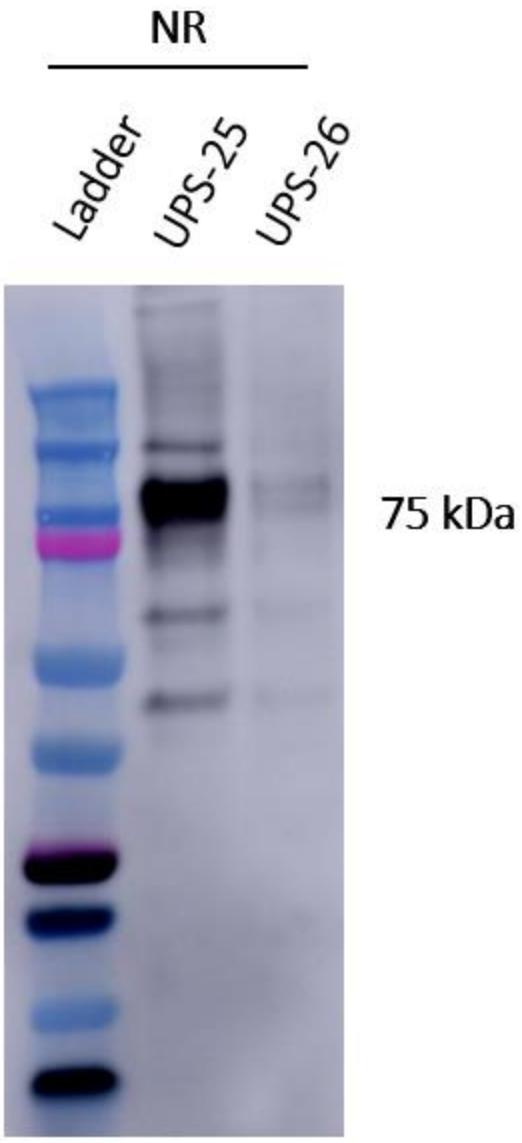

Figure 16.Glycosylation profile of unconventionally-secreted saIgDE

Unconventionally-secreted saIgDE shows markedly reduced levels of glycosylation compared to conventionally-secreted saIgDE. Lane 1: Ladder, non-reduced. Lane 2: UPS-25, non-reduced. Lane 3: UPS-26, non-reduced.

In order to assess the functional activity of the protein, Fabalactica®, UPS-25, and UPS-26 were co-incubated with an internal monoclonal human IgG1 antibody (Figure 17). Based on the presence of lower MW species, there was no observable digestion in any of the treated samples. As a result, an alternative strategy of co-transfection and co-incubation was attempted to assess the efficacy of saIgDE digestion.

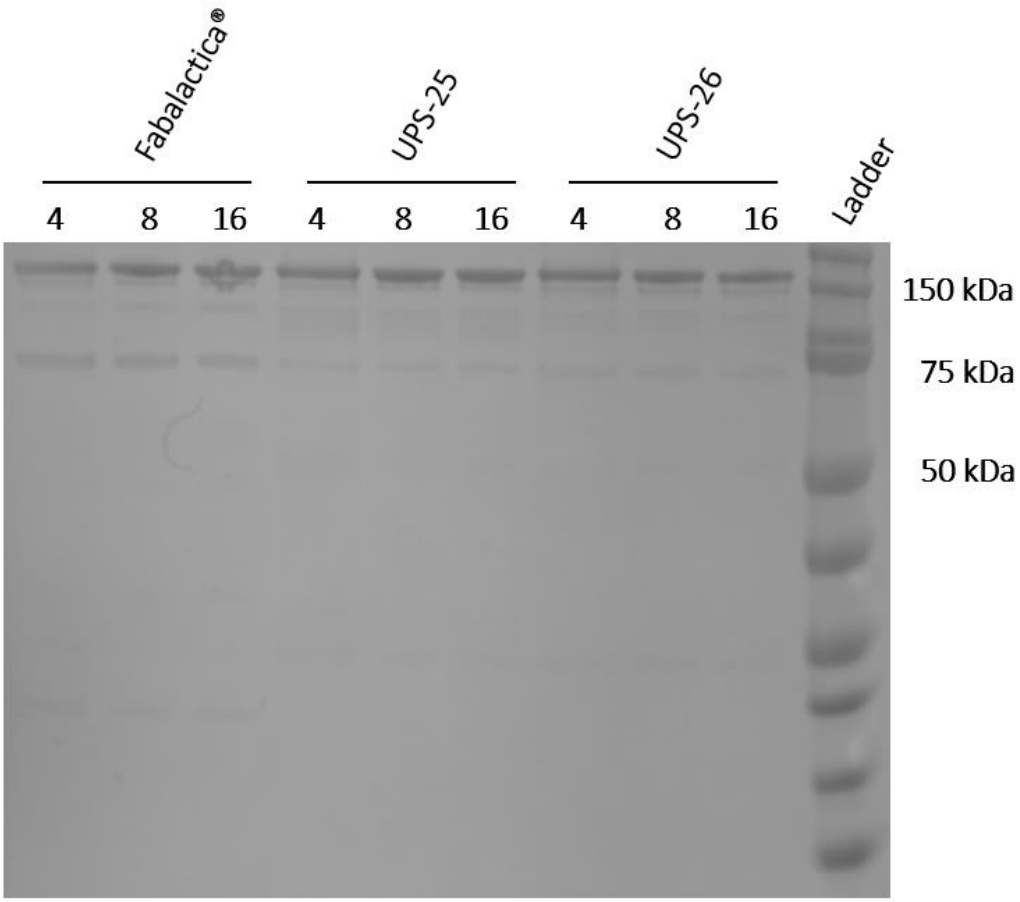

Figure 17: Digestion of IgG.

No digestion of IgG is observed for Fabalactica®, UPS-25, and UPS-26-treated samples. Lane 1-3 is Fabalactica® incubated with IgG, 4hr, 8hr, and 16hr. Lane 4-6 is IL2ss-IgDE incubated with IgG, 4hr, 8hr, 16hr. Lane 7-9 is UPS-IgDE incubated with IgG, 4hr, 8hr, 16hr.

An alternative co-transfection strategy was adapted to test whether recombinant antibody could be co-expressed with the UPS constructs for in-process digestion. Plasmids for UPS-25 and UPS-26 were co-transfected into HEK293 cells along with plasmids for the monoclonal antibody. Following expression, clarified supernatant was run on a gel to observe protein expression (Figure 18). There was a noticeable difference in the bands observed in the UPS-25 co-transfected samples versus the UPS-26 co-transfected samples, with the latter exhibiting a much lighter band at the expected MW of full-length, intact mAb (~150kDa). Following Protein A purification to isolate fragments containing the Fc domain, the samples were re-run on a gel to observe the presence of digestion products (Figure 19). Again, there was a noticeable difference in the bands observed, with UPS-26-digested samples exhibiting significantly more digestion products than UPS-25-digested samples.

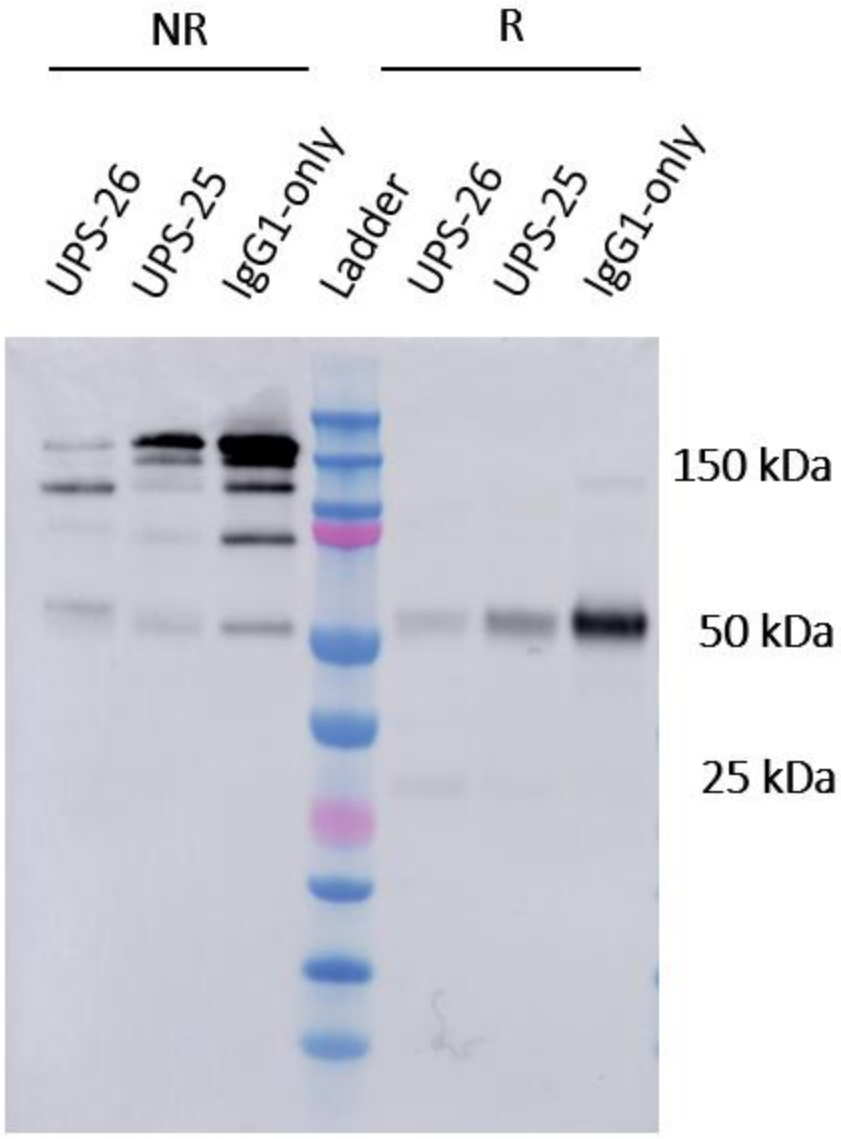

Figure 18.IgG digestion from supernatant

IgG samples co-transfected and co-incubated with UPS-26 exhibit greater digestion. Lane 1 is UPS-26 co-incubated with IgG, non-reduced. Lane 2 is UPS-25 co-incubated with IgG, non-reduced. Lane 3 is IgG only, non-reduced. Lane 4 is UPS-26 co-incubated with IgG, reduced. Lane 5 is UPS-25 co-incubated with IgG, reduced. Lane 6 is IgG only, reduced.

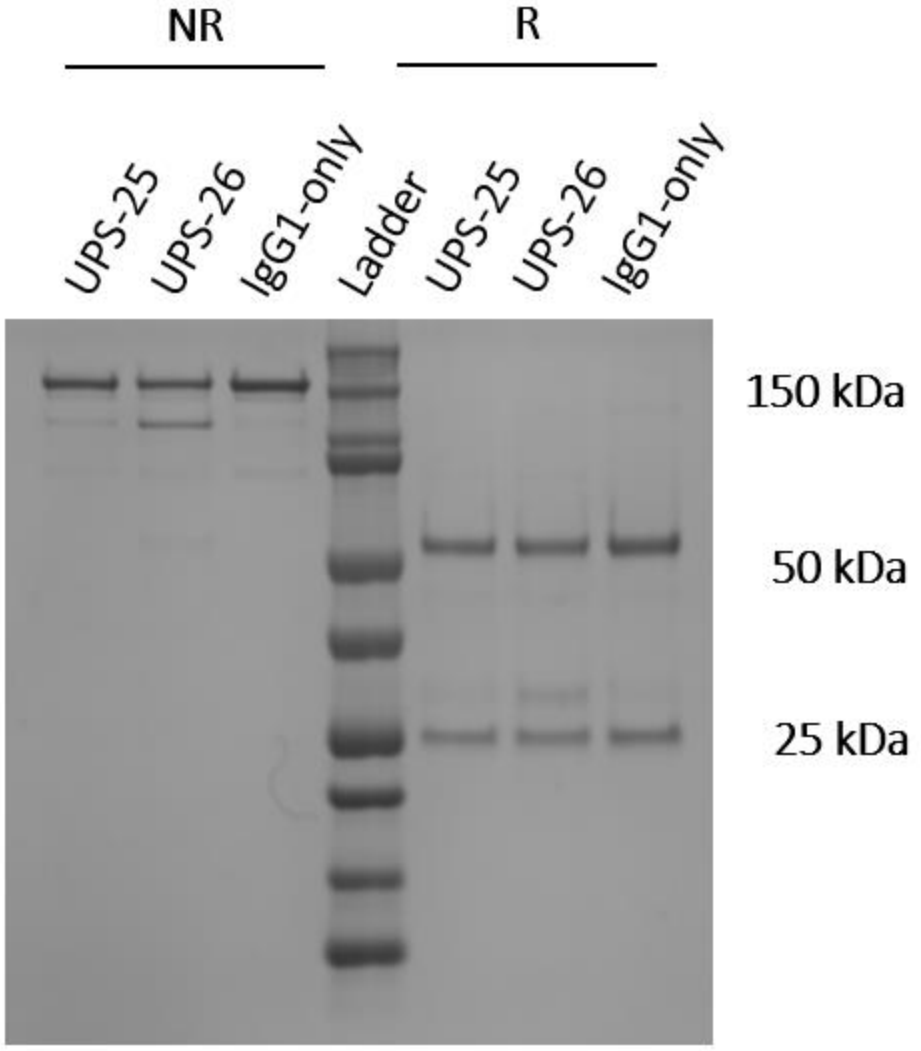

Figure 19.IgG digestion purified

IgG samples co-transfected and co-incubated with UPS-26 exhibit greater digestion. Lane 1 is UPS-25 co-incubated with IgG, non-reduced. Lane 2 is UPS-26 co-incubated with IgG, non-reduced. Lane 3 is IgG only, non-reduced. Lane 4 is UPS-25 co-incubated with IgG, reduced. Lane 5 is UPS-26 co-incubated with IgG, reduced. Lane 6 is IgG only, reduced.

Chapter IV.

Discussion

The aim of this study was to investigate the feasibility of using UPS pathways to produce recombinant proteins. Given the ever-expanding and diversified usage of recombinant proteins, such expression pathways could provide unique alternatives to conventional protein production systems. Although previous research has demonstrated the unconventional secretion of endogenous proteins and fluorescently-labeled versions of such proteins, no work has directly compared the use of these pathways in protein production (Zhang et al., 2020; Lee et al., 2022).

Notably, there are several unique mechanisms that can drive unconventional secretion of proteins, the most well-characterized of which is probably the unconventional secretion of the pro-inflammatory cytokine IL-1β (Rabouille, 2017). Assessments of a panel of UPS tags identified this protein as the most promising in inducing secretion of GFP over baseline. Interestingly, the full-length IL-1β protein induced greater secretion of GFP than a UPS-motif derived from IL-1β. This may be due to the multiple alternative UPS pathways that the full-length IL-1β protein might be capable of traversing rather than the defined TMED-10-mediated translocatory UPS pathway afforded by the IL-1b UPS-motif (Zhang et al., 2020). However, it is also possible that variations in protein expression or stability induced by the shorter motif might play a role in the expression. Although the IL-1β-derived UPS constructs appeared most effective in this format, other UPS constructs cannot be ruled out, including those related to secretion of proteins like α-synuclein or tau (Xu et al., 2018). Nevertheless,

none of the other UPS tags exhibited GFP secretion higher than baseline, including the conventionally-secreted GFP. It is possible that the pairing of the IL2ss with GFP is not an efficient combination, or that the direction of GFP towards the ER/Golgi secretory route drastically reduces its overall expression. To account for this discrepancy, saIgDE was selected as an alternative cargo protein for further characterization of the UPS production system.

Antibody-specific proteases are desirable due to the various applications antibody and antibody-like molecules have in research (Spoerry et al., 2016). However, internal research shows that saIgDE, an IgG-specific protease that cuts below the hinge region, has relatively poor activity when expressed in mammalian cells. One possible explanation might be the altered folding and PTM patterns introduced during heterologous expression in the mammalian ER/Golgi-secretory pathway. Thus, saIgDE was of particular interest in developing a UPS-based production system. When compared to conventionally-secreted saIgDE, unconventionally-secreted IgDE has a lower titer but similar banding pattern on Western Blot and SDS-PAGE. However, given that the expression process is not optimized for unconventional secretion, it is promising that saIgDE still generated a modest amount of material (>5 mg/L). It is possible that HEK293 cells are not sufficiently geared towards cytosolic protein production, and alternative mammalian cell lines, transfection strategies, and culture conditions should be investigated (Reid & Nicchitta, 2015).

Surprisingly, the activity of saIgDE is greater when expressed via an unconventional secretory pathway compared to a conventional secretory pathway. After co-expression of human IgG1 with saIgDE, IL-1β-tagged saIgDE induced greater

proteolytic cleavage despite expressing lower amounts of enzyme. To assess whether mammalian PTMs played a role in the differential function of this activity, protein glycosylation was assessed and found to be significantly higher in the conventionally-secreted saIgDE compared to the unconventionally-secreted saIgDE. Future tests confirming the relationship between PTMs like glycosylation and function for mammalian-expressed saIgDE (as well as other heterologous proteins) could provide additional insight into the protein's function, and additional cargo proteins should be evaluated to confirm the efficacy of IL-1β and other unconventionally-secreted proteins as tags for UPS-mediated protein production pathways. However, preliminary results demonstrate that IL-1β may be an effective alternative protein production strategy in niche cases.

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