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Production of human heme-binding proteins in plants for potential pharmaceutical and nutritional uses

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Cover:

Top: Purified hemoglobin (fHbF) produced in *N. benthamiana* (photo Magnus Carlsson, edited by Simon Jeppson).

Left: Human mutant myoglobin structure (pdb: 3RGK, www.rcsb.org), visualized using YASARA software (www.yasara.org).

Right: Young *N. benthamiana* plant (photo Magnus Carlsson, edited by Simon Jeppson).

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Abstract

Plants have a long history as a source of nutrition and medication. In the last few decades, interest has also emerged in using plants as a production system for protein pharmaceuticals, in part due to possible scale-up- and safety-benefits with this method of production. One class of protein pharmaceuticals for which the use of a plant production system might be particularly interesting is hemoglobin-based oxygen carriers (HBOC's), a type of pharmaceutical intended to function as an alternative to donated blood in clinical situations. HBOC's face many safety and production challenges, that have so far prevented widespread use, and production in plants could perhaps help towards solving these problems. In this thesis, we aimed to express and characterize proteins of interest with the goal of using them in oxygen therapeutics, using transient expression in *Nicotiana benthamiana.* The expressed recombinant proteins were based on human myoglobin (Mb), fetal hemoglobin (HbF) and α_1-microglobulin (A1M). The proteins were successfully expressed in tobacco leaves and the purified proteins displayed their respective functional activity. The plant expression system thus showed its efficacy, and was capable of supplying the critical heme for Mb and Hb production, something that may also have implications for using plants as a source of nutritional iron. While more work is needed, the results help to show the feasibility of using heme-binding proteins produced in plants for pharmaceutical uses, something that was also underlined by animal- and cell-tests.

Keywords: Molecular farming, heterologous expression, transient expression, oxygen therapeutic, HBOC, hemoglobin, myoglobin, α_1-microglobulin, A1M, XTEN.

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Produktion av mänskliga heme-bindande protein i växter, för möjliga farmaceutiska och nutritionella applikationer

Abstract

Växter har en lång historia som en källa till läkemedel och näring. Under de senaste decennierna intresse också dykt upp för att använda växter som ett produktionssystem för proteinläkemedel, delvis på grund av möjliga uppskalnings och säkerhetsfördelar med denna produktionsmetod. En klass av proteinläkemedel för vilket produktion i växter kan vara särskilt intressent är hemoglobinbaserade syrebärare (HBOC), en typ av läkemedel som är avsedd att fungera som ett alternativ till donerat blod i kliniska situationer. HBOC:s står inför många säkerhets- och produktionsutmaningar, som hittills har förhindrat utbredd användning, och produktion i växter kan kanske bidra till att lösa dessa problem. I detta projekt syftade vi till att uttrycka och karakterisera proteiner av intresse i en HBOC kontext, genom transient uttryck i *Nicotiana benthamiana*. De uttryckta rekombinanta proteinerna baserades på humant myoglobin (Mb), fetalt hemoglobin (HbF) och α_1-mikroglobulin. Dessa proteiner uttrycktes framgångsrikt i växten och de renade proteinerna visade sina respektive funktionella aktiviteter. Den naturliga biosyntesen i växten visade således sin effektivitet och kunde leverera det kritiska hemet för Mb- och Hb-produktion, något som också kan ha konsekvenser i järnnutritionssammanhang. Även om mer arbete behövs så så visar resultaten på möjligheten av att använda hembindande proteiner som produceras i växter för farmaceutiskt bruk, något som också underströks av djur- och celltester.

Keywords: Molekylärt jordbruk, hetrolog expression, transient expression, läkemedel för syrgastransport, HBOC, hemoglobin, myoglobin, α_1-microglobulin, A1M, XTEN

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Dedication

To my family and friends.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Magnus L. R. Carlsson, Selvaraju Kanagarajan, Leif Bülow & Li-Hua Zhu (2020). Plant based production of myoglobin - a novel source of the muscle heme-protein. *Sci Rep*, 10 (920). doi.org/10.1038/s41598-020-57565-y

II. Selvaraju Kanagarajan, Magnus L. R. Carlsson, Sandeep Chakane, Emanuel Smeds, Karin Kettisen, Ranjeet Kumar, Magnus Gram, Bo Åkerström, Leif Bülow & Li-Hua Zhu (2020). Production of human fetal hemoglobin in Nicotiana benthamiana leaves by transient expression – an innovative method for producing hemoglobin-based oxygen carriers. Communications Biology, (*under revision)*

III. Magnus L. R. Carlsson, Amanda Kristiansson, Jesper Bergwik, Selvaraju Kanagarajan, Leif Bülow, Bo Åkerstöm, Li-Hua Zhu. Expression, purification and initial characterization of functional α_1-microglobulin (A1M) in *N. benthamiana*. (*manuscript*)

IV. Magnus L. R. Carlsson, Sandeep Chakane, Selvaraju Kanagarajan, Karin Kettisen, Leif Bülow and Li-Hua Zhu. Transient expression of XTENylated fusion human fetal hemoglobin in *Nicotiana benthamiana.* (*manuscript*)

The contribution of Magnus Carlsson to the papers included in this thesis was as follows:

I. Magnus L. R. Carlsson performed the experimental and analytical work, with some input from Selvaraju Kanagarajan, and was the main contributor to the writing of the manuscript.

II. Magnus L. R. Carlsson assisted with expression, extraction and purification, SDS-PAGE analyses, and in the manuscript writing.

III. Magnus L. R. Carlsson performed the protein production and most of the protein purification. Magnus L.R. Carlsson performed a large part of the protein characterization and analysis and wrote the manuscript with input from coauthors.

IV. Magnus L. R. Carlsson performed the majority of the protein production, purification, experimental and analytical work, and wrote the manuscript with input from coauthors.

Abbreviations

A1M	α_1-Microglobulin
ACR	Albumin to creatinine ratio
BUN	Blood urea nitrogen
CaMV	Cauliflower mosaic virus
CPMV	Cow pea mosaic virus
DPG	Diphosphoglycerate
E. coli	*Escherichia coli*
FC1	Ferrochelatase 1
FC2	Ferrochelatase 2
FDA	Food and drug administration (USA)
fHbF	Fusion-HbF
GMO	Genetically modified organism
Hb	Hemoglobin
HbA	Adult hemoglobin
HbF	Fetal hemoglobin
HBOC	Hemoglobin based oxygen carrier
HIV	Human immunodeficiency virus
LAL	Limulus amebocyte lysate
Mb	Myoglobin
N. benthamiana	*Nicotiana benthamiana*
N. tabacum	*Nicotiana tabacum*
PEG	Polyethylene glycol
PFCOC	Perfluorocarbon based oxygen carrier
RBC	Red blood cell
rfHbF	Recombinant fusion-HbF
ROS	Reactive oxygen species

SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
T-DNA	Transferred DNA
TMV	Tobacco mosaic virus

1. Aim and Objectives

The principal aim of this thesis work was to explore the possibility of using plants as a production system for heme binding proteins useful for oxygen therapeutics, thereby contributing to the overall objective of taking this class of compounds from a promising concept to a therapeutic reality. The hope is also to help meet other nutritional and clinical needs for which heme proteins produced in plants could be part of a future solution.

The specific objectives of this thesis were:

- To transiently express the proteins of interest, myoglobin (Mb), α-γ fusion fetal hemoglobin (fHbF), α_1-microglobulin (A1M) and XTENylated fusion fetal hemoglobin (XTEN-fHbF) in *N. benthamiana* for plant-based production of these proteins.
- To purify and characterize the chemical and physical properties of the proteins of interest, with an emphasis on functions and properties relevant to pharmaceutical use in an oxygen therapeutic context.

2. Introduction

2.1 Molecular farming – plants as a factory for protein pharmaceuticals

The use of plants for medical applications most likely predates civilization, with the earliest written evidence found on ancient Sumerian clay tablets (Petrovska, 2012). Even in modern times the metabolites of plants have been a good source of leads for drug development (Dias *et al.*, 2012), with a relatively recent example being artemisinin for use against malaria (Su & Miller, 2015). The advent of molecular medicine and recombinant technology opened new possibilities however and plants came to be of interest as a production platform for recombinant proteins for pharmaceutical and other applications, with early examples of this starting to emerge in the late 80's and early 90's (Sijmons *et al.*, 1990; Hiatt *et al.*, 1989; Barta *et al.*, 1986). The concept of harnessing agriculture and plants to produce protein based molecular products like pharmaceuticals in large quantities became known as molecular farming (Fischer *et al.*, 1999a), and it has long attracted interest due to the promise of large-scale and low-cost production of these important products (Buyel, 2019). Molecular farming has since been used for the production of pharmaceutical candidates (Yao *et al.*, 2015), and has had some success for the production of proteins for use as industrial and research reagents, cosmetics and for veterinary products (Tschofen *et al.*, 2016). Approval of plant-produced therapeutics has been limited however and in a recent review the commercial history of molecular farming was described in terms of a "Gartner hype cycle". That is, as an initial hype following the emergence of the new platform, followed by disillusionment and an eventual renewal of interest during the last decade as the technology advances (Fischer & Buyel, 2020).

When compared to other expression systems, the relatively low production cost, high scalability and low risk of contamination are commonly held up as the primary advantages of the plant expression systems, in particular compared to high cost mammalian expression systems typically relying on cell culture. Compared to low cost microbial systems, i.e. bacteria and yeast, plants are often considered to have the edge when it comes to product quality (Tripathi & Shrivastava, 2019; Yao *et al.*, 2015; Ma *et al.*, 2003). Being advanced eukaryotic organisms, plants are considered better capable of correctly folding and assembling complex proteins than bacteria (Yao *et al.*, 2015) and are capable of eukaryotic post-translational modifications, such as glycosylation (Strasser, 2016; Yao *et al.*, 2015). The plant N-glycosylation is in fact relatively mammalian-like, compared to that seen in yeast (Strasser, 2016), and is amenable to genetic engineering to further increase the similarity (Schoberer & Strasser, 2018; Castilho & Steinkellner, 2012). The low contamination risk and safety aspect of plant-based production comes from the lack of bacterial endotoxins and inability of human pathogens such as viruses to replicate (Buyel, 2019; Tripathi & Shrivastava, 2019; Sabalza *et al.*, 2014). An interesting possibility is also raised by molecular farming in edible crops: edible vaccines, which would result from recombinant expression of antigens in edible plants (Haq *et al.*, 1995; Mason *et al.*, 1992).

However, several challenges have also been encountered. The frequently occurring low yields of expressed protein resulting from nuclear transformation of plants has been pointed out as a cause that has held back the industry and prevented a wider adoption of molecular farming (Moon *et al.*, 2019; Schillberg *et al.*, 2019). In addition, the protein purification and isolation can be challenging, as plant extracts tend to have a high particle burden and large amounts of host cell proteins, and metabolites, such as phenolics. Overall, the downstream processing can amount to up to 80% of the production cost (Buyel, 2019). It has been argued that some of the challenges of plant protein purification primarily stem from the system being less commonly used, and hence less developed than microbial and animal cell production (Yao *et al.*, 2015). Indeed, techniques for effective extraction and clarification, for example using screw-presses, heating and diafiltration, have been shown to be effective in mitigating these issues (Buyel, 2019). As for edible vaccines, the effectiveness of this type of product has been

demonstrated, but it has also become clear that controlling the delivered dose in a reliable way can be difficult (Moon *et al.*, 2019). The debate over GMO has been an issue, due to concerns over the possibility of contamination of food crops with transgenic material used for molecular farming, which has presented obstacles to using food crops for molecular farming (Yao *et al.*, 2015; Paul & Ma, 2011). Biocontainment has also been an issue and is an important consideration for molecular farming (Clark & Maselko, 2020).

Over the years, a large number of techniques have been developed for plant protein expression, and various species have been considered as production systems (Moon *et al.*, 2019; Paul & Ma, 2011; Twyman *et al.*, 2003). Further considerations include for example the type of tissues used for the production, i.e. cell, root, leaf or seed, and culture conditions, which could be open-field, or closed culture, in turn based on either greenhouses, bioreactors or hydroponics (Moon *et al.*, 2019). These variations could be considered a strength of the plant expression systems, as there are many options to choose from to fit a given application. Plants that have been used for molecular farming purposes include for example potato (Sijmons *et al.*, 1990), tobacco (Hiatt *et al.*, 1989), maize (Hood *et al.*, 1999), rice, safflower (Paul & Ma, 2011), tomato and lettuce (Moon *et al.*, 2019). Carrot cell culture was used for production of recombinant glucocerebrosidase, which became the first plant produced pharmaceutical to be approved by the FDA (Yao *et al.*, 2015). Factors such as yield, storage stability and containment vary depending on the crop system (Stoger *et al.*, 2002). The type of tissue for accumulation of a protein of interest can also be important, targeting the expression to seeds for example, could improve storage stability (Yao *et al.*, 2015).

Strategies for heterologous expression of proteins in plants include nuclear transformation, chloroplast transformation and transient expression (Paul & Ma, 2011). Nuclear transformation of plants is a common approach for molecular farming and often relies on *Agrobacterium*-mediated transformation or particle bombardment (Ma *et al.*, 2003). Following introduction of the gene of interest into the plant genome, plants with the gene of interest can be generated and selected for, and transgenic seeds can be retrieved for future use (Chen *et al.*, 2013). Chloroplast transformation is an interesting alternative to nuclear transformation due to the high expression

levels that are often achievable (Ahmad *et al.*, 2016; Daniell, 2002; Bock, 2001), with levels up to 70% of total soluble protein reported (Oey *et al.*, 2009). It also offers a degree of biocontainment, as the plastid-incorporated sequence is rarely transmitted by pollen (Ahmad *et al.*, 2016). However, the post-translational modifications are more limited than for nuclear transformation, with no access to glycosylation. The range of species is also more limited than for nuclear transformation (Ahmad *et al.*, 2016). Transient expression techniques, using either *Agrobacterium tumefaciens* or virus vectors (Gleba *et al.*, 2007; Fischer & Emans, 2000) are capable of very high protein yields, (Chen *et al.*, 2013) and are faster than stable transformation techniques (Moon *et al.*, 2019). The high yields compared to stable transformation are believed to be linked to the lack of positional effects when not inserting the gene of interest into the plant genome, as it carries the risk of insertion into a chromosomal region with low transcriptional activity (Chen *et al.*, 2013). The downside is primarily a more limited scalability, as the gene is not stably integrated in the plants, which instead needs to be treated to induce expression, rather than grown from transgenic seeds, which increases labor input. The advantages of the viral vectors and *Agrobacterium*-mediated transient expression have also been combined in the use of deconstructed viruses delivered into the plant cell by *Agrobacterium* (Komarova *et al.*, 2010; Gleba *et al.*, 2007). This strategy, which was the focus in this thesis, will be described in more detail in section 2.3.

2.2 Oxygen therapeutics – a long sought goal

In the 17th century, the first well-documented transfusions of blood into humans took place, in an attempt to treat mental illness. The blood used came from calves and lambs, and the procedure had soon earned a bad reputation. It was only in the early 20th century, with a more thorough understanding of biology in general and the discovery of anticoagulants and blood groups in particular (not to mention species differences) that blood transfusions could become the staple of modern medicine that it is today (Giangrande, 2000). Even with the knowledge we have today donated blood is not simple to use for as a medication, however. It requires cross-matching between the patient and donor, a steady supply of healthy donors and can only be stored for a limited time. It can be logistically challenging to meet variations in demand

due to emergencies, and it can transmit infectious disease, for which expensive testing is required (Mozzarelli *et al.*, 2010). There are also occasional complications following treatments (Chen *et al.*, 2009). The HIV epidemic of the 1980s highlighted the risks of disease spread in particular (Kresie, 2001). These issues have motivated the search for alternatives to donated blood for transfusion use, intended to provide the oxygen delivery capability normally provided by red blood cells (RBC's) which have been termed "blood substitutes", or oxygen therapeutics (Alayash, 2004).

Different concepts have been considered to fulfill this role. These include RBC's grown from stem cells in vitro (Giarratana *et al.*, 2011), artificial RBC's, where Hb is encapsulated in artificial structures (Chang, 2006), such as liposomes (Phillips *et al.*, 1999) or polymer membranes (Sen Gupta, 2019), perfluorocarbon based oxygen carriers (PFCOC), which rely on the biologically inert perfluorocarbon to dissolve oxygen (Castro & Briceno, 2010). Finally there is also hemoglobin based oxygen carriers (HBOC's), which rely on, generally for the purpose chemically or genetically altered, Hb protein (Alayash, 2004). All these oxygen therapeutic strategies have had challenges of their own. (Bouhassira, 2012; Castro & Briceno, 2010; Alayash, 2004). PFCOC's are for example are not miscible with water and therefore needs emulsifiers to be added (Castro & Briceno, 2010), they have been known to affect to the reticuloendothelial system, and cause adverse effects like stroke and the only PFCOC to be approved for human use by the FDA (Fluosol-DA 20) was discontinued in 1994 (Henkel-Honke & Oleck, 2007).

HBOC development has faced similar challenges. Hemoglobin (Hb) is a heterotetrameric heme-protein (Park *et al.*, 2006; Hill *et al.*, 1962) that is normally contained inside RBCs. Human Hb has evolved to be an effective oxygen transporter, but presents a danger, when not confined in its natural environment in the RBCs (Quaye, 2015). Many of the downsides and challenges of using donated blood, such as the need to match blood groups and the short storage time, are due to the RBCs rather than the Hb protein itself, however, and a key challenge for the HBOC pharmaceutical concept is to safely use Hb when not contained in the RBC (Alayash, 2004). This is not trivial however, one test that illustrates the danger of free Hb was performed by the US navy in the 1950s, where infusions of Hb administered

to anemic or febrile sailors resulted in complications including hypertension and signs of renal damage (Chen *et al.*, 2009). Three main types of mechanisms have been proposed to explain the toxicity of free Hb, which involve nitric oxide scavenging, oversupply of oxygen and oxidative reactions mediated by the heme (Alayash, 2019). Nitric oxide acts as a vasodilator, and its scavenging by Hb can lead to blood vessel constriction (Alayash, 2019). When not contained in the Fe^{2+} oxidation state by antioxidants and enzymes in the RBC, Hb can rapidly undergo autoxidation reactions, which results in generation of reactive oxygen species (ROS), and can lead to Hb unfolding and the release of free heme (Alayash, 2019), which is in turn also toxic (Kumar & Bandyopadhyay, 2005). Free Hb also tends to dissociate into its dimers, which are cleared quickly by renal filtration (Bunn *et al.*, 1969), causing kidney damage (Gladwin *et al.*, 2012). The small size also allows for extraversion of Hb from the blood vessels, where it spreads into other tissues causing damage (Schaer *et al.*, 2013). To prevent damage associated with free Hb and heme, there are defense mechanisms, such as the Hb scavenger haptoglobin, and heme scavengers, such as hemopexin (Schaer *et al.*, 2013).

For the abovementioned reasons, the production of a safe and effective HBOC necessitates modifications of the Hb molecule chemically or by genetic engineering. Strategies that have been tried in clinical tests in humans include PEGylation (Sanguinate, MP), chemically- (HemAssist) or biologically- (Optro) cross-linked Hb and Hb polymerization (Polyheme, Hemopure, Hemolink) which have primarily aimed to stabilize Hb, preventing dissociation into dimers and to improve the ability of the molecule to transport oxygen (Alayash, 2019). The oxygen affinity and other factors vary between these HBOCs (Alayash, 2019), and various safety issues have been encountered during clinical (Silverman & Weiskopf, 2009) or preclinical testing (Buehler & Alayash, 2004) of HBOC candidates. Development continues however with new emerging concepts (Alayash, 2019; Ferenz & Steinbicker, 2019). Recombinant technology in particular could offer many possibilities for further protein engineering of Hb, allowing for more control over properties such as stability, oxidation, and NO scavenging (Varnado *et al.*, 2013). Recombinant production, using low cost expression systems such as *E. coli* is challenging however, with issues such as heme-supply, heme-orientational disorder and misfolding (Varnado *et al.*, 2013). Production of HBOC molecules in plants with recombinant

techniques could thus be an interesting option to explore, which has been previously attempted with stable expression, though with a very low reported yield (Dieryck et al., 1997).

2.3 Transient expression of heme-binding proteins in *N. benthamiana*

2.3.1 Production of heme proteins in plants

As discussed in section 2.1, plants have several advantages for the production of pharmaceutical proteins in plants. The heme synthesis capacity of plants provide a further benefit for recombinant heme-protein production. In plants, heme is primarily synthesized in the chloroplast, from where it can be exported to the cytosol, and shares most of its biosynthetic pathway with chlorophyll (Brzezowski *et al.*, 2015). Plants also produce monomeric plant Hbs (phytoglobins), involved in for example NO regulation and the oxygen supply of nitrogen fixing root nodules (Hoy & Hargrove, 2008). For this reason, plants could be expected to be capable of supplying heme for incorporation into heterologously expressed heme-proteins without the external addition of heme. Apart from pharmaceutical applications, recombinant expression of heme-proteins in plants could perhaps also have nutritional applications, due to the high bioavailability of heme iron (Hurrell & Egli, 2010).

2.3.2 Proteins of interest

In this thesis, we have focused on a few heme-binding proteins that we considered of particular interest for expression in plants. In this section, these proteins will be briefly introduced.

Myoglobin

Myoglobin (Mb) is mainly present in the muscle tissues of vertebrates and has important functions in the oxygenation of tissues (Wittenberg & Wittenberg, 1987) and in regulating the nitric oxide signaling (Wittenberg & Wittenberg, 2003). Animal Mb it is also an important nutritional source of bioavailable heme-iron (Hurrell & Egli, 2010). As a simple monomeric heme-protein, Mb is a good candidate for testing the plant production systems' capability and capacity for recombinant heme-proteins. While Hb folding in RBCs is dependent on chaperones to stabilize the α-chain (Kihm

et al., 2002), Mb folding is not known to be dependent on such additional factors. As plants are also capable of producing similar monomeric Hbs, Mb production should be within its capability. In addition to helping to show the capability of the production system, recombinant Mb production in plants could be interesting for iron nutrition purposes or for the study of heme biosynthesis. Mb might also be of interest for oxygen therapeutic purposes in the future, perhaps in combination with other heme proteins.

Fetal hemoglobin

Hemoglobin (Hb) has a long history as a focus in the development of oxygen therapeutics (Chen *et al.*, 2009). In addition to the capabilities shared with simpler heme proteins, i.e. binding and release of gaseous-ligands to its heme (Antonini & Brunori, 1971), Hb´s tetrameric structure allows for a variable oxygen affinity. This is due to a responsiveness to pH and to the ligand binding status of the other subunits of the holo-protein, i.e. cooperative binding (Eaton *et al.*, 1999; Bohr *et al.*, 1904). Fetal Hb (HbF) is a variant of Hb that is expressed primarily in the fetus, and only at very low levels in adult humans, it has the same two α-subunits as the adult Hb, but features two γ-subunits instead of the β-subunits (Sankaran *et al.*, 2010). Another factor that affects the oxygen affinity of Hb is the concentration of diphosphoglycerate (DPG) (Tomita, 1981; Benesch *et al.*, 1968). HbF has a higher oxygen affinity *in vivo*, due to a lower affinity for DPG, which helps in the transfer of oxygen from the maternal blood stream to the fetus (Simons *et al.*, 2018). HbF has been suggested to have beneficial properties for HBOC production, such as a lower tetramer dissociation constant (Yagami *et al.*, 2002), and has been recently considered as an interesting alternative HBOC platform (Simons *et al.*, 2018; Ratanasopa *et al.*, 2016).

α_1-Microglobulin

α_1-Microglobulin (A1M) is a blood plasma protein believed to have multiple protective functions, acting as a heme scavenger, a radical scavenger and a reductase (Åkerström & Gram, 2014; Olsson *et al.*, 2012). For this reason, A1M has attracted interest as a potential protein pharmaceutical, as a possible treatment for preeclampsia and acute kidney injuries during heart surgery (Gunnarsson *et al.*, 2017). In conjunction with HBOC's, A1M or other protective proteins such as hemopexin and haptoglobin could be interesting additives in order to reduce toxicity (Alayash, 2014; Schaer *et al.*, 2013). As a heme scavenger, A1M could bind to the reactive free heme

molecules that can be released by degrading heme-proteins. As free heme is highly toxic (Belcher *et al.*, 2010; Kumar & Bandyopadhyay, 2005), and has been suggested to be a significant contributor to the toxic effects observed for HBOC's (Alayash, 2019; Alayash, 2014) co-administration with heme-scavengers could be a promising approach.

2.3.3 Transient expression in *N. benthamiana*

Tobacco (*Nicotiana tabacum*) and its Australian relative *Nicotiana benthamiana* are popular choices for plant molecular farming, and *N. benthamiana* in particular has been widely used for expressing recombinant proteins for vaccines or other pharmaceutical purposes using agroinfiltration (Chen *et al.*, 2013). The advantages of these plant species lie both in a relatively high biomass yield per acre (Buyel, 2019) and in the fact that they are not food crops, thus avoiding risks of potential contamination with recombinant material (Chen *et al.*, 2013). *N. benthamiana* has an additional advantage as it is very susceptible to plant virus propagation (Yang *et al.*, 2004), and has often been used as a model system for studying plant viruses (Goodin *et al.*, 2008).

The soil bacterium, *Agrobacterium tumefaciens* has the natural ability to insert its transfer DNA (T-DNA) on the tumor inducing (Ti) plasmid into the plant genome and it has long been harnessed for plant genetic engineering purposes (Gelvin, 2003) (Figure 1A). This ability has been further adapted to a biotechnological role by rational design, such as the division of the Ti-plasmid into two complementary plasmids (Hoekema *et al.*, 1983). The development of expression systems combining the advantages of *Agrobacterium* with those of plant viruses by incorporating a deconstructed virus, or viral elements, in the T-DNA of *Agrobacterium* for delivery has also been shown to be a valuable strategy (Peyret & Lomonossoff, 2015; Gleba *et al.*, 2014). *Agrobacterium* can be used both for stable transformation and for transient expression in plants (Chen *et al.*, 2013). *Agrobacterium*-mediated transient expression is a comparatively quick and convenient method to test expression of heterologous proteins in plants (Sheludko, 2008; Fischer *et al.*, 1999b), and is capable of high yields of recombinant protein (Chen *et al.*, 2013; Schellenberger *et al.*, 2009).

The viral vectors offer several advantages for transient expression and different types of such vectors have been developed, based on different types

of plant viruses, they are often engineered for delivery by *Agrobacterium* (Hefferon, 2017; Komarova *et al.*, 2010). Two examples of vectors designed for delivery by *Agrobacterium* that contain viral elements are the pJL-TRBO (Lindbo, 2007) and pEAQ-*HT* vectors (Sainsbury *et al.*, 2009). The pJL-TRBO vector is based on the tobacco mosaic virus (TMV). It carries a cauliflower mosaic virus (CaMV) 35S promoter for high expression. The TMV sequence has been further modified by deletion of the capsid protein gene, which both increases the agroinfection efficiency and improves biocontainment by preventing plant-to-plant spread. The vector still contains other TMV genes, for replication and cell-to-cell movement (Figure 1B) however, that allows the vector to spread throughout an infected leaf (Lindbo, 2007).

The pEAQ-*HT* vector was designed following the finding that vectors based on the cowpea mosaic virus (CPMV) RNA-2 allowed high expression of recombinant proteins in infected plants even without the presence of the RNA-1 component required for replication (Sainsbury *et al.*, 2009; Sainsbury & Lomonossoff, 2008). The observation implied that the untranslated regions of the CPMV RNA-2 enhanced the expression (Sainsbury *et al.*, 2009; Sainsbury & Lomonossoff, 2008), something that it has also recently been possible to improve upon by rational design (Peyret *et al.*, 2019). The pEAQ-*HT* vector retains only a modified version of the 5' and 3' non-translatable regions of the CPMV RNA-2, with a large part virus sequence removed, and other elements for high expression such as CaMV 35S promoters, and a P19 gene added. While the pEAQ-*HT* thus lacks the virus capabilities of replication and spread, the deletion allows it to avoid disadvantages with such systems, such as the possibility of introducing errors in the sequence due to the low fidelity of viral RNA dependent RNA-polymerase (Castro *et al.*, 2005) while retaining a high yield (Sainsbury *et al.*, 2009).

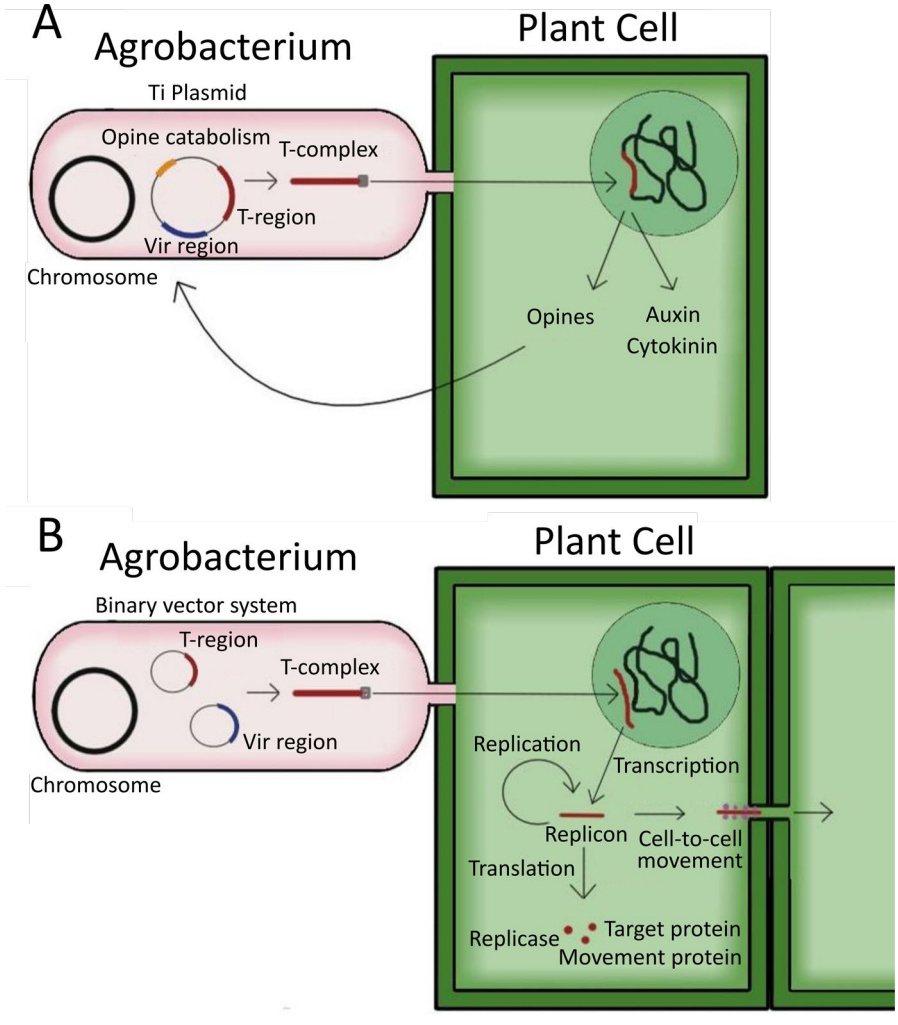

Figure 1. Schematic figure showing T-DNA transfer from an *Agrobacterium* to a plant cell. (**A**) Native *Agrobacterium* transfers the T-DNA complex for insertion of its genes into the plant genome, followed by production of opines for use as a nutrient by the *Agrobacterium*, and growth hormones auxin and cytokinin, for inducing tumor formation (Gelvin, 2003). (**B**) Genetically engineered *Agrobacterium* with a binary vector configuration, where separate plasmids carry the T-region and the virulence genes that help the transfer of the T-complex, transferring its T-DNA, containing viral elements, to a plant cell. The T-DNA here generates an RNA replicon, capable of

replication and cell-to-cell movement, and generation of the protein of interest through translation (based on the pJL-TRBO vector). (Illustration by Magnus Carlsson, A: design based on image from (www.universiteitleiden.nl), B: modified from A based on (Lindbo, 2007)).

2.3.4 Agroinfiltration and agrospray

Transfer of *Agrobacterium* harboring vectors and genes of interest into plant leaf tissues can be done using three different methods, agroinfiltration, vacuum infiltration and agrospray (Figure. 2). The most common used method, agroinfiltration relies on pushing an *Agrobacterium* suspension through the stomata of the leaves using a syringe without a needle (Chen *et al.*, 2013). While this is an effective method for testing expression and for small-scale production, it is labor intensive and thus cost-inefficient for larger scale production. Two methods, vacuum infiltration (Chen *et al.*, 2013) and agrospray, i.e. spray application of *Agrobacterium* (Hahn *et al.*, 2015; Azhakanandam *et al.*, 2007), have been developed to be better suited to large scale production. In contrast to agroinfiltration, which relies mainly on manual work, vacuum infiltration relies on rapid pressure change to force an *Agrobacterium* suspension into the leaf, and is more easily automated (Chen *et al.*, 2013). Agrospray is gentler than the first two methods as its entry is based on diffusion through the stomata in the presence of surfactants rather than force (Hahn *et al.*, 2015). Although a smaller amount of *Agrobacterium* suspension likely enters the leaf, the replication ability of viral vectors such as pJL-TRBO, is likely to compensate for this. Unlike vacuum infiltration, agrospray does not require machinery, although suitable facilities would still be necessary, and additional safety precautions are probably warranted due to the possibility of *Agrobacterium* suspension droplets being present in air during the spraying. For paper I and paper II the agrospray method was used to show the feasibility of this method for large-scale production in the future. Figure 2 illustrates the techniques discussed here.

Agroinfiltration

Agrospray

Vacuum infiltration

Figure 2. Photographs illustrating techniques for introducing *Agrobacterium* suspensions into leaves of *N. benthamiana*. Left: syringe agroinfiltration (photo: Per Snell). Top right: agrospray, application of *Agrobacterium* suspension using spray by a hand sprayer (photo: Magnus Carlsson). Bottom right: vacuum infiltration, where the aerial part of a tobacco plant has been submerged in *Agrobacterium* suspension inside a vacuum chamber (photo: Magnus Carlsson).

3. Methodology

3.1 Construct design considerations

3.1.1 Myoglobin (Paper I)

Two constructs of Mb were designed for expression in *N. benthamiana*, one for cytosol accumulation and one targeted to the chloroplast, by attachment of the *N. tabacum* rubisco small subunit chloroplast targeting peptide (Uniprot nr. P69249 (UniProt Consortium, 2019)). Targeting the expression to the chloroplast might be beneficial due to the organelle's involvement in heme biosynthesis (Brzezowski *et al.*, 2015), which may have implications for heme availability. It has been suggested, based on observations of tetrapyrrole synthesis regulatory mechanisms that the two variants of ferrochelatase responsible (FC1 and FC2) might produce separate pools of heme for use either in the chloroplast photosynthetic machinery (FC2) or for general use within the cell (FC1) (Woodson *et al.*, 2011). The response to high levels of heme-chelating Mb might therefore depend on the site of its accumulation.

3.1.2 Fetal Hemoglobin (Paper II, IV)

Two strategies were used in this thesis work to modify HbF to meet different challenges. One challenge of producing Hb is that the free Hb α-subunit is unstable and cytotoxic (Mollan *et al.*, 2010). Careful control to ensure equal amounts of α- and β-subunits is therefore required to avoid damage (Voon & Vadolas, 2008) and a dedicated chaperone, α-Hb stabilizing protein, helps with this in case of the native expression in red blood cells (Kihm *et al.*, 2002). To help ensure higher yields and equal expression of the polypeptide chains in the plant during expression, the α-chain was genetically fused with

a short flexible 12 amino acid linker (4xGGS) to the γ-chain. In addition to this, the larger minimum size may also be beneficial as a way to increase the molecules circulatory half-life, due to the decreased permeability of the glomerular membrane to larger molecules (Scott & Quaggin, 2015). This strategy is expected to ensure a very rapid combination of the two fused subunits into a dimer equivalent as it folds, stabilizing the α-chain. This approach has been shown to work better for expression of HbF in a bacterial expression system (Ratanasopa, 2015). This strategy was used for Paper II and IV.

A major design challenge for HBOCs is the toxicity and short half-life of Hb not contained in RBC's (Alayash, 2014), and various techniques have been used to attempt to adapt the native proteins properties, including chemical modifications such as PEGylation and genetic fusion strategies (Alayash, 2019). In an attempt to improve the physicochemical and pharmacokinetic properties of the plant-produced fHbF we tested fusing it to an XTEN peptide (Paper IV), something that has also shown promise when produced in *E. coli* (Chakane, 2017). XTEN is a hydrophilic and negatively charged peptide designed to mimic the properties of PEGylation of a protein or peptide pharmaceutical, and thus enhance its circulatory half-life (Podust *et al.*, 2016; Schellenberger *et al.*, 2009). Unlike PEGylation the genetic fusion of an XTEN sequence does not require additional processing steps (Jevsevar *et al.*, 2010), with resulting drawbacks when it comes to cost and yield. It has also been argued that XTEN could have an advantage when it comes to biodegradation (Podust *et al.*, 2016; Haeckel *et al.*, 2014), although the poor biodegradability of PEG is still debated (Ulbricht *et al.*, 2014; Jevsevar *et al.*, 2010). A cleavable histidine (his)-tag was also included in the N-terminal for some of the XTENylated sequences.

3.1.3 α1-Microglobulin (Paper III)

Recombinant production of α_1-microglobulin (A1M) is complicated by two N-glycosylation sites present on the native protein (Wester *et al.*, 2000), which may be important for the stability of the protein. In this study, three constructs were designed; one designed for cytosol accumulation, with two residues edited for enhanced stability (N17D and N96D) based on a previous study (Åkerström *et al.*, 2019). In addition to this non-glycosylated variant, two versions were designed with a targeting peptide (Uniprot nr. P08299 (UniProt Consortium, 2019)) for the secretory pathway, one of which also

carried a C-terminal ER-arrest peptide (KDEL) (Denecke *et al.*, 1992; Napier *et al.*, 1992), to prevent plant specific glycan motifs added downstream, in the Golgi apparatus (Strasser, 2016). All the constructs also carried a his-tag for purification and improved solubility (Åkerström *et al.*, 2019; Kwasek *et al.*, 2007).

3.2 Transient expression of the target proteins

In this thesis, *N. benthamiana* was used for expressing the proteins of interest through transient expression using virus derived vectors (viral vectors) delivered by *Agrobacterium*. Two vectors, pJL-TRBO (Lindbo, 2007) and pEAQ-*HT* (Sainsbury *et al.*, 2009), were used to harbor the genes of interest. Expression of the P19 RNA silencing suppressor gene is often used together with viral vectors for ensuring high transient expression levels (Sainsbury *et al.*, 2009; Lindbo, 2007). In this thesis, the P19 gene was either incorporated on the viral vectors used to express the target genes or expressed by a separate vector, which was co-infiltrated with the vector harboring the target genes. Standard molecular cloning techniques were used to insert the respective genes of interest into the viral vectors, which were then transformed into *Agrobacterium*. For transient expression, the *Agrobacterium* were delivered into plant cells mainly by agroinfiltration or agrospray, with the latter used primarily in Paper I and II, in order to show the feasibility of large-scale production. The plants used in the study were grown under controlled conditions at the biotron at SLU, Alnarp, and the plant materials used for the treatments were between 5-6 weeks old. For highest expression, and depending on type of proteins, the infiltrated tissue was harvested around 5-7 days following agroinfiltration, and around 9-14 days for agrospray with *Agrobacterium* harboring the pJL-TRBO vector, allowing for additional time to replicate.

3.3 Protein isolation and analysis

3.3.1 Protein isolation

Isolating proteins from plant material poses several challenges, including polyphenol oxidation, presence of high amounts of host cell proteins and a high particle load in the extracts, which contribute to making downstream

processes of isolation and purification a highly significant part of the total cost (Buyel, 2019). The large amounts of protein required for HBOC treatments would make it sensitive to costs, even more so if aiming at nutritional uses. We have thus put some efforts into developing efficient and scalable purification methodologies in this work. In Paper I, this is highlighted, and techniques including heat steps, ammonium sulfate precipitation and cross flow diafiltration were employed for the isolation of Mb. The excellent heat stability of Hb (Rieder, 1970) and Mb (Wan *et al.*, 1998) was relied upon in particular as extracts were heated to 60 °C while under CO gas to remove host cell proteins (Paper I, II, IV). Various chromatography-based techniques were then employed for obtaining final purity.

3.3.2 Protein analysis – heme and spectroscopy

While the analysis of the proteins of interest in Paper I-IV included many techniques, I will here focus on absorbance spectroscopy, as I found it to be of particular interest, due to the connection to heme-proteins and their functions. The proteins of interest in this thesis all have a distinctive absorbance spectrum and are visibly colored, either due to heme coordination (Antonini & Brunori, 1971) or due to the chromophore of the A1M protein, which confers a yellow-brown color (Kwasek *et al.*, 2007; Åkerström *et al.*, 1995). In fact, the first evidence of success when expressing the heme proteins of Hb and Mb in plants would be found by visual observation of the color of the extracts.

Characteristic features of heme-protein absorbance spectra include the Soret- (γ-) peak in the ultraviolet and the α- and β-peaks in the visible range of the spectrum (Antonini & Brunori, 1971). These absorption bands originate from the absorbance of the heme but are sensitive to the interaction between heme and other molecules, such as bound gaseous ligands or coordinating proteins, and can be used to investigate these interactions (Comer & Zhang, 2018). For the production of recombinant heme-protein, an absorbance spectrum identical or very similar to the native protein would therefore be a strong indicator of correct heme coordination, in turn implying correct folding of the protein. In paper I, II, and IV, the absorbance spectra were used to investigate the heterologously expressed heme-proteins and to test the ligand binding capability and redox status, as their respective deoxy-, oxy- carboxy-

and met- spectra are distinctive (Antonini & Brunori, 1971) and can be easily differentiated. In paper I measurements of the gradual shift from oxy- to met-Mb spectrum were used to test the oxidative stability and determine the rate of autoxidation (Brantley *et al.*, 1993). Similarly, chemical and pH stability could be estimated by observing the loss of heme coordination at harsh conditions. The binding of heme to A1M also affects its absorbance, with a resulting redshift of the Soret peak, which can also be affected by an N-terminal his-tag (Karnaukhova *et al.*, 2014). This was used in Paper III to test heme binding. The absorbance spectra of plant produced A1M was also compared to that of A1M from other sources, in order to investigate how the bound chromophore would depend on the expression system.

4. Results and Discussion

4.1 Myoglobin (Paper I)

In Paper I, it was shown that the functional Mb protein could be successfully expressed in the leaves of *N. benthamiana,* following agroinfiltration or agrospray treatment with the pJL-TRBO vector carrying the human Mb gene. Both the cytosol accumulated construct and the construct targeted to chloroplasts were expressed at comparable levels, as indicated by SDS-PAGE and Western blot, and displayed the expected absorbance spectrum (Antonini & Brunori, 1971; Hardman *et al.*, 1966). The chloroplast targeted construct displayed some heterogeneity in the Western blot though, possibly due to incomplete removal of the signal peptide, and it was therefore not analyzed further.

A cost-effective protocol was developed for the purification of Mb expressed in leaves, where most of the host cell proteins were removed by heat precipitation, after which the Mb protein could be concentrated and unwanted small molecules removed, using a cross-flow diafiltration setup. Precipitation of contaminants by ammonium sulfate addition, and a shift in pH were also performed in conjunction with the diafiltration. These relatively low-cost and scalable techniques ensured a comparatively high degree of purity and concentration of the target protein, even without the use of chromatography, which could be of interest in particular for nutritional applications. Anion-exchange chromatography was used as a final step to provide the high purity required for protein analysis and possible future medical applications. SDS-PAGE and absorbance spectroscopy analyses of the extracts or Mb solutions were used to determine purity. Absorbance

spectroscopy was also used to determine the final yield of Mb following purification, ~210 mg/kg for agroinfiltration and about 60-80 mg/kg for agrospray (per fresh leaf weight). The yield of the purification was probably around 50-70% (for agrospray), based on estimations of the absorbance spectra in the extracts.

While the yield of Mb protein obtained here was not exceptionally high for heterologous expression using the pJL-TRBO (Lindbo, 2007), it is interesting to note that the plant was capable of supplying this amount of heme. Something that has been an issue in *E. coli* for instance, which requires expression of additional proteins or supplementation of heme or its precursor during recombinant Hb production (Varnado *et al.*, 2013). It was also capable of supplying it for both cytosol accumulated, and chloroplast targeted Mb, although the cellular location of heme binding event was not determined for the latter. In fact, no heme protein peak was discerned from the absorbance spectra of the extracts from the untreated *N. benthamiana*, which suggests that the produced Mb is the primary source of soluble heme-protein present in the treated leaves. Whether the overall heme level of the plant was increased as an effect of the expression of Mb or whether a pool of heme of sufficient size for incorporation into Mb without additional biosynthesis was available in the plant was not determined. The results seem promising however, and if the increase corresponds to an increase in nutritionally available heme, the amounts of Mb-bound heme present in tobacco could mean a significant increase to the nutritional value of the plant. Plant produced Mb isolated with the low cost methods described here could also be an interesting nutritional, or culinary, additive to other foods, such as meat substitutes; though a thorough cost analysis would need to be done to assess the economic feasibility of such an approach.

Following purification, the protein was shown to be functional i.e. capable of binding and releasing its gaseous ligands O_2 and CO. The observed absorbance spectra conformed closely to previously reported spectra of sperm whale Mb (Antonini & Brunori, 1971; Hardman *et al.*, 1966), with the only significant difference a small shoulder around 620 nm, probably resulting from a small population of degraded Mb. Data on circular dichroism, autoxidation rate, temperature and chemical stability were also collected for the purified Mb and compared to literature sources in Paper I.

The results from the analyses were all in line with the properties of a correctly folded Mb. Overall, the protein analysis indicated that the plant was capable of expressing a functional and correctly folded human Mb. Figure 3 shows Mb colored extracts and some of the steps of the purification process.

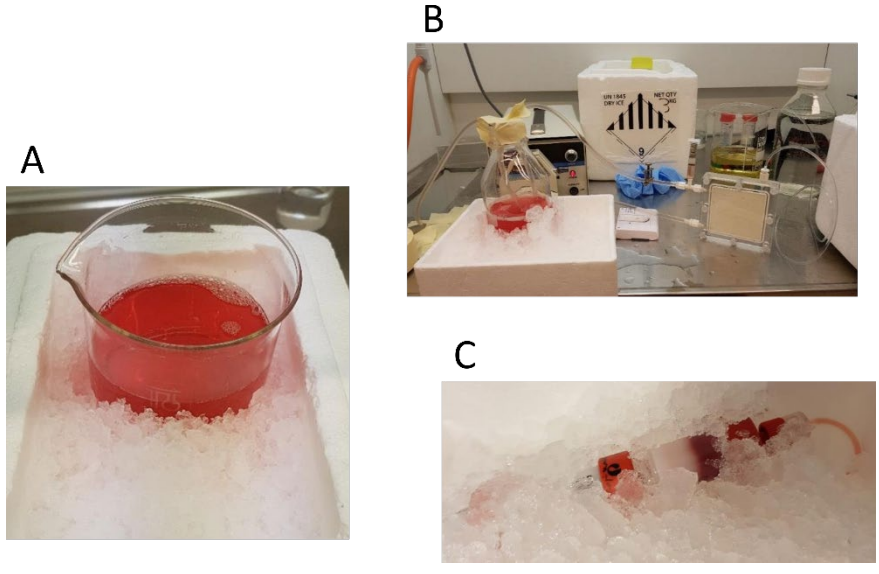

Figure 3. Mb extraction and purification. **A.** Tobacco extract colored red by expressed Mb following agroinfiltration. **B.** Diafiltration setup during operation. **C.** Anion exchange chromatography of Mb, in ice bath (Photos: Magnus Carlsson).

4.2 Hemoglobin (Paper II, IV)

In Paper II the expression and analysis of fHbF is described. The expression in this case relied on agrospray and the pJL-TRBO vector. The protein extract was heat treated at 60°C similarly to Mb, and the fHbF protein was then purified by two steps of ion-exchange chromatography, with a preceding dialysis step to facilitate column binding. SDS-PAGE and Western blot were used to confirm expression of the target protein and monitor the purity. Following purification, it was determined to be functionally active by absorbance spectroscopy, in the presence or absence of its ligands. Along with the circular dichroism spectrum, this suggests that

the protein was correctly folded, indicating that the fusion of the subunits worked well. As for the Mb in Paper I, substantial amounts of heme appeared to be available for incorporation in fHbF and a yield of up to ~80-100 mg/kg highly purified fHbF was obtained.

An important part of the analysis in Paper II is the animal test, which provides data on the circulation time of the fHbF, and serves as a proof of concept of a plant produced HBOC. Following injection, the fHbF appeared to be removed quickly via the kidneys however, in spite of the larger minimum molecular size of the fused protein. In addition to blood samples, where a decrease in the fHbF level was seen, urine samples from the mice were observed to contain Hb, detectable by spectroscopy, likely consisting of a mix of ferric and ferrous fHbF. To study the impact of the treatment on the mice, indicators (albumin to creatinine ratio (ACR) and blood urea nitrogen (BUN)) of kidney damage were analyzed and showed an increase following the injection, but seem to have reverted to normal levels after 24h. Mouse weight and body temperature did not appear to be significantly affected by the treatment.

A important advantage of the plant system compared to bacterial expression systems when it comes to HBOC uses, is the lack of endotoxins (Varnado *et al.*, 2013). Some endotoxin could perhaps be introduced by the *Agrobacterium* or other sources during production and purification though. The purified fHbF was found to contain less than 0.031 EU / mg fHbF, using a limulus amebocyte lysate (LAL) assay. For reference a maximum level of 5 EU per kg of body weight per hour is indicated for intravenous pharmaceutical use (Petsch & Anspach, 2000). This suggests that the plant production platform and used purification methodology produced a very low level of endotoxins, possibly low enough for intravenous use in the high doses expected for HBOC use, though some minor additional purification step might be needed.

XTENylated fHbF was produced with agroinfiltration as reported in Paper IV, primarily relying on the pEAQ-*HT* vector. As discussed in the materials and methods section this protein design is intended to prolong the circulation time of the fHbF when used as an HBOC. As for fHbF, heating was successfully used as an initial purification step, which was in this case

followed by immobilized metal affinity chromatography (IMAC), using the attached his-tag, and its functional status was confirmed by ligand binding and absorbance spectroscopy. The XTEN-tag did thus not appear to prevent the functional activity of the protein. Expression of the XTEN-tag has not previously been demonstrated in plants. In this study, the expression of the tag seemed to work well and a yield of about 60 mg/kg purified H_6-XTEN-fHbF was achieved.

Dynamic light scattering was used to assess the change in hydrodynamic radius of the molecule. The observed values of 5.3 ± 0.2 for fHbF and 12.2 ± 0.8 for H_6-XTEN-fHbF indicated a substantial increase in size. The XTEN tag is also expected to substantially increase the negative charges on the protein under physiological conditions, as the theoretical pKa changes from 7.96 for fHbF to 4.70 for H_6-XTEN-fHbF (calculated with protparam online tool (Gasteiger *et al.*, 2005)). The glomerular filtration barrier tends to be selectively permeable based on both size and charge, with large negatively charged macromolecules better retained in circulation (Scott & Quaggin, 2015). The attachment of the XTEN tag could therefore be expected to have a substantial effect on circulation time of the fHbF. The heat stability of fHbF appeared to be moderately increased by the attachment of the XTEN tag. Thermostability has been suggested to be another property that can be improved by XTENylation (Podust *et al.*, 2016; Schellenberger *et al.*, 2009). For an HBOC it would be an additional beneficial factor, particularly when heat steps are used for purification. Overall Paper IV showed that production of an XTENylated protein was possible using the plant system and that XTENylation could be a promising approach to improving HBOC retention time. However, this comes at a cost of a larger and more complex protein to produce.

4.3 α_1-Microglobulin (Paper III)

As described in Paper III A1M was successfully expressed using the pJL-TRBO vector and agroinfiltration of *N. benthamiana*. The untargeted, cytosol accumulated, construct, A1M-NB1 had a significantly higher yield than the ER- or apoplast-targeted A1M-NB2 and A1M-NB3. The latter two constructs appeared to have an increased size, perhaps indicating successful glycosylation. Due to its higher yield A1M-NB1 became the focus of further protein purification and analysis work conducted in Paper III. A1M-NB1 was

purified by three chromatography steps, IMAC, anion-exchange and size exclusion chromatography before analysis and yielded ~50 mg highly purified A1M-NB1 per kg fresh leaf material.

The size exclusion chromatography also served as an analytical step, and showed that A1M-NB1 was present as an equilibrium of monomer, dimer and aggregates, which is characteristic for native A1M (Ekström & Berggård, 1977). Native A1M has three cysteine residues (Kaumeyer *et al.*, 1986), two of which form an internal cysteine bond (Mendez *et al.*, 1982), while the third, Cys34, is located on the surface and believed to be involved in the function of A1M (Gunnarsson *et al.*, 2017). A1M-NB1, displayed properties in line with this; Ellman's test results indicated the presence of a single free surface cysteine while a shift in mobility in SDS-PAGE between reduced and non-reduced conditions, suggested that an internal cysteine bond is present. A1M-NB1 further displayed thermal stability properties and a hydrodynamic radius similar to *E. coli* produced A1M-035 (Åkerström *et al.*, 2019), on which its sequence was based, also indicating that the plant produced A1M was correctly folded.

An interesting feature of A1M is its chromophore, an unknown compound that binds to the protein, in a heterogeneous fashion, and confers a yellow-brown color, and a characteristic absorbance spectrum (Kwasek *et al.*, 2007; Åkerström *et al.*, 1995). Purified A1M-NB1 similarly displayed a faint yellow-brown color. Absorbance and fluorescence spectroscopy revealed both similarities and differences pertaining to the chromophore, compared to A1M produced in other organisms (Kwasek *et al.*, 2007; Åkerström *et al.*, 1995). The absorbance spectrum of A1M-NB1 displayed a similar overall shape as the *E. coli* produced A1M-WT (Kwasek *et al.*, 2007) which was also tested, but the chromophore absorbance appeared to be elevated in comparison, most likely due to additional bound chromophore, and featured a bulge or shoulder around 390 nm unlike the bacterial A1M. With the higher chromophore absorbance the spectrum may therefore be more reminiscent of A1M from mammalian or insect cell culture sources (Åkerström *et al.*, 1995), although these too lack the pronounced shoulder present in the tobacco produced A1M. Moreover, the fluorescence spectra, following excitation at 350 nm, showed an increase in fluorescence around 400-550 nm, compared to the *E. coli* produced sample, indicating a higher level of bound chromophore, similarly to the absorbance data. However the profile of the spectrum differed substantially compared to *E. coli* produced A1M

(Kwasek *et al.*, 2007) and human urine A1M (Berggård *et al.*, 1999), perhaps an indication that the bound compounds differ between A1M originating in the different organisms, and reflecting the different compounds available in the cells.

In Paper III, the tobacco produced A1M-NB1 was shown to be functionally active. The heme binding capability of A1M (Karnaukhova *et al.*, 2014) is of particular interest for HBOC applications and was tested with several methods. Binding of heme to proteins is detectable by observing the effect on the Soret peak, tryptophan fluorescence, which can be quenched, due to proximity of bound heme, and by gel mobility shifts (Comer & Zhang, 2018). When combined with varying amounts of free heme and analyzed with absorbance spectroscopy, a shift in the heme peak was observed, consistent with heme binding by A1M (Åkerström *et al.*, 2019; Karnaukhova *et al.*, 2014), with the resulting heme-A1M spectra closely matching those of the similar, *E. coli* produced A1M-035 (Åkerström *et al.*, 2019). The fluorescence quenching and migration shift associated with heme binding of A1M (Åkerström *et al.*, 2019; Rutardottir *et al.*, 2016; Karnaukhova *et al.*, 2014) were also observed, and matched the *E. coli* sample tested in parallel, though a slightly lesser effect on migration was seen, perhaps caused by the additional chromophore. In a further test of function, the protective effect of A1M-NB1 on RBCs against spontaneous, heme-induced and osmotic stress was evaluated. The tobacco produced A1M showed efficacy, resulting in a lower stress level, as has been previously demonstrated for recombinant A1M (Kristiansson *et al.*, 2020). The effect against different types of stress implies that the mechanism is probably not attributable to heme binding alone, and indicates that the tobacco-produced protein is capable of producing the protective effects via other mechanisms, based on other functions attributed to A1M, i.e. radical binding and a reductase activity (Åkerström & Gram, 2014).

5. Conclusions and future perspectives

The proteins of tested (Mb, fHbF, H_6-XTEN-fHbF, A1M) were all successfully expressed through transient expression in *N. benthamiana* using viral vectors delivered by *Agrobacterium* and displayed functional activity. The heme-proteins could be produced at a relatively high yield, without requiring supplementation of heme or heme precursor. The endotoxin level in the purified fHbF sample appeared to be at low or acceptable levels, even considering that very large doses would be expected for HBOC use, and an initial animal test served as a proof of concept of use of the plant produced Hb as an HBOC, though additional modification is required to improve the circulation time. Solutions based on protein engineering, such as the XTEN tag, could be one way forward and the demonstration in this thesis work that it could be expressed in the plant system without affecting the fHbF protein's function is promising. The A1M protein meanwhile could have many possible applications due to its detoxifying heme binding and other functions. Based on the data in paper III, production of A1M for medical applications in plants appears to be feasible. The plant is capable of producing the A1M protein in a soluble form, and does not require refolding from inclusion bodies, as has been the case for production in *E. coli* (Kwasek *et al.*, 2007) and that impact the yield negatively. The higher level of bound chromophore may however be a drawback. The glycosylation strategy, though only explored superficially in Paper III, could also be an advantage, and open future options for an improved product.

Overall, the results from Paper I-IV indicate that the plant production system may have an important role in the future development of HBOC's, and at least in the case of the heme scavenger A1M, in other pharmaceutical contexts. Nutritional applications could also be a possibility for plant produced heme-proteins, based on the data, either involving biofortification, or a partially or extensively purified heme-protein. Whether this is

economically feasible will need to be evaluated in further studies, although the demonstration of an inexpensive isolation method for Mb might help in this regard. As the production scale and the purity requirements would inevitably be different from the type of production typically considered in molecular farming and the production cost might be lowered due to economies of scale this would need careful consideration. It is likely that further optimization, and perhaps use of stable transformation techniques would be required for nutritional applications to be feasible.

Further increasing the yield in the transient expression system could involve methodological improvements to the cultivation or the agroinfection procedure, or improvements based on molecular techniques, such as engineering of the heme biosynthetic pathway. For the latter approach, a more thorough investigation into how the heme synthesis rate was affected by the massive expression of heme binding proteins following the *Agrobacterium* treatments would be a reasonable first step. Whether the heme biosynthesis was upregulated following the treatments and whether it was a limiting factor for the heme-protein production following the infiltration are important questions that remain to be explored. It might also be interesting to test expression using other methods, relying on stable expression instead of the burst seen during transient expression, as this might shed some light on whether heme biosynthesis, or other factors, such as protein stability are the major determinants of yield. Due to the high amounts that would be required in a blood substitute or nutrition context, stable transformation techniques might also be worth testing as a way to further decrease the cost of production in the plant expression systems. One approach that could be of interest is the stable integration of an activatable viral vector into the plant genome, which has been done previously with promising results (Dugdale *et al.*, 2013). Chloroplast transformation could also be an interesting option to explore, due to its potential of achieving high protein yields. It might also have other advantages for the expression of heme-proteins, due to being the main site of heme biosynthesis and the presence of enzymatic systems for controlling ROS generated by the photosynthesis machinery (Asada, 2006).

Another question that is raised by considering the plant as an expression system for HBOCs is whether the system could offer new possibilities for protein engineering. One possibility could be the use of the plant

glycosylation machinery as another tool to modify the properties of Hbs. The use of viral vectors for the production also begs the question of whether viral-like particles (VLP's), which are often discussed in a drug delivery context, and often based on plant virus capsids (Ibrahim *et al.*, 2019; Rohovie *et al.*, 2017) or other similar protein structures could be interesting as artificial RBCs to better control toxicity and function. Using targeting peptides to package the HBOC into organelles, such as vaults (Muñoz-Juan *et al.*, 2019), or even isolated chloroplasts might also open up possibilities for drug delivery. While all of these are very speculative ideas, to produce functional HBOCs people have tried using a wide selection of chemical or genetic modifications, from simple surface decoration to nanoparticles and Hb from humans, animals and even worms (Alayash, 2019). Plant production of HBOCs and other heme binding proteins could be an additional tool. Although safe and effective HBOC's are elusive, if we keep trying, in the end, we will get there.

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Popular science summary

Blood donations are often critical for saving lives, bringing the blood from a donor to a patient in a safe and effective way is often challenging. Even if blood is very good at its task when it circulates in the body, it has several issues as a pharmaceutical, for example, it cannot be stored very long and a major effort is required to ensure, for example, that no diseases are transferred along with the blood. For this reason, people have worked on developing pharmaceuticals that can fulfill blood's most acute and critical function, delivering oxygen. These have been termed oxygen therapeutics. Unfortunately, these oxygen therapeutics have mostly remained a dream, as it is challenging to develop a safe oxygen therapeutic. Often Hemoglobin is used, but without the red blood cells wherein this oxygen binding protein is normally found. Even if protein for pharmaceutical purposes can be produced in many different organisms, such as bacteria, or cultured human cells, plants have several abilities that make them particularly interesting, for example the possibility of large scale and safe production. This is particularly important for this type of pharmaceutical as there is so much hemoglobin in the blood that one needs to replace.

In this project, we have worked on producing a number of human heme-binding proteins for use either as such pharmaceuticals, or to assist in their function. To do this we have used plants, more specifically tobacco. To get the plant to produce the human proteins, we first inserted the DNA sequence that codes for the protein into a plasmid vector, a small circular DNA molecule, containing elements from viruses. This vector was then inserted into a bacterium, *Agrobacterium tumefaciens, which* has a natural ability to insert DNA into plants. The plants are then treated with the *Agrobacterium*, which makes the plant start to produce the protein that the original DNA sequence coded for, which can then be extracted from the plant.

In the thesis project, we produced myoglobin, a red heme-protein that is found in muscles, A1M, a heme binding protein that circulates in the blood and protects against toxic compounds, and different variants of hemoglobin. The project showed that these proteins could be produced and could be folded correctly in the plant, and retained its natural functions, which means that plants could be a good future alternative to produce this type of oxygen carrying pharmaceutical. As the plant was capable of supplying a sizable amount of heme for the heme-protein production, it could also be interesting to produce these proteins for iron-nutrition purposes. This is because heme is absorbed better by the body than other types of iron, and is otherwise mostly found in large amounts in meat and blood.

Populärvetenskaplig sammanfattning

Bloddonationer är ofta kritiska för att rädda liv, men för att få blodet från en donator till en patient på ett säkert och effektivt sätt är ofta utmanande. Även om blod är väldigt bra på sin uppgift då det cirkulerar i blodomloppet så har det flera tillkortakommanden som ett läkemedel, till exempel så kan man inte lagra det särskilt länge, och det behövs en stor industri för att säkerställa exempelvis att smittbara sjukdomar inte kommer med. Det är därför som man länge har arbetat med att ta fram läkemedel som kan uppfylla blodets mest akuta och kritiska funktion, att leverera syre. Tyvärr så har detta i stort förblivit en dröm, då det är utmanande att producera en säker "syreleverantör". Ofta använder man hemoglobin, fast utan de röda blodkroppar som normalt innesluter detta syrebindande protein, på så vis får man ett proteinläkemedel, liknande de som läkemedelsindustrin har stor erfarenhet av. Även om man kan producera protein för läkemedelsändamål i många olika organismer, som till exempel bakterier eller i odlade människoceller, så har växter flera egenskaper som gör dem särskilt intressenta, som till exempel möjligheten till storskalig och säker produktion. Detta är särskilt viktigt för denna typ av läkemedel då det finns så mycket hemoglobin det blod man vill ersätta.

I det här projektet så har vi arbetat med att producera ett antal mänskliga heme-bindande protein för användning antingen som ett sådant läkemedel, eller i samband med det. För att göra detta så har vi använt växter, närmare bestämt tobak. För att få växten att producera de mänskliga proteinerna så har vi först fört in de DNA sekvenser som kodar för dem i en plasmidvektor, en cirkulär sträng av DNA, vilken har designats med komponenter från ett växtvirus. Denna plasmid, har vi sedan låtit tas upp av en bakterie, *Agrobakterium tumefaciens*, som har en naturlig förmåga att föra in DNA i växter. Därefter behandlar man växterna med dessa bakterier, vilka får

växten att börja producera proteinet man är ute efter vilket därefter kan utvinnas från växten.

I projektet så producerade vi myoglobin, ett rött heme protein som finns i muskler, A1M, ett heme-bindande protein som cirkulerar i blodet och skyddar mot gifter, och olika varianter av hemoglobin, det protein som ger blodet, och de röda blodkropparna sin färg. Projektet visade att alla dessa proteiner kunde produceras och vikas ihop rätt i växten, och behöll sina naturliga funktioner, vilket betyder att växten skulle kunna vara ett bra framtida alternativ för att producera denna typ av syre transporterande läkemedel. Det är också intressent att se att växten kunde tillhandahålla så mycket av den järnhaltiga molekylen heme, som ju också är intressent för järn-nutrition, då den tas upp bättre i kroppen än andra typer av järn, och annars mest brukar sägas finnas i stora mängder i kött och blod.

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Plants have long attracted interest as a platform for production of protein pharmaceuticals. Production in plants could be a particularly interesting option for hemoglobin based oxygen carriers (HBOC), a class of pharmaceuticals sometimes termed “blood substitute”. In this thesis, we have aimed to produce and characterize human heme-binding proteins of interest in an HBOC context, with possible additional nutritional uses. The proteins were successfully produced in *Nicotiana benthamiana* by *Agrobacterium*-mediated transient expression. The isolated proteins were functional and highly pure.

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