Adriana Botnaru

Bachelor's degree in Cellular and Molecular Biology

Expression, purification and characterization of an enzyme isoform involved in cyclic nucleotide signaling in humans

Dissertation to obtain a master's degree in Biotechnology

Supervisor: Lígia Nobre, PhD, iBET

Co-supervisor: Peter Sandner, PhD, Bayer AG

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Dedicated to my parents

Resumo

A proteína solúvel guanilil cyclase (sGC), o principal receptor de óxido nítrico (NO) nas células de mamífero, é uma hemoproteína heterodimérica composta por uma subunidade α e outra β, que cataliza a conversão de GTP em cGMP. Quatro subunidades foram identificadas, mais especificamente, α_1, α_2, β_1 e a β_2; enquanto as isoformas α_1/β_1 e α_2/β_1 são as mais descritas e caracterizadas na literatura, a função da subunidade β_2 ainda é desconhecida. O objetivo deste trabalho é expressar e purificar a proteína humana β_2, e caracterizar a sua atividade. Para isso, baculovírus recombinantes que codificam as subunidades humanas produzidos e usados para infectar células de insecto Sf9. Vários testes em pequena escala foram realizados para otimizar a expressão da proteína, e a melhor condição foi utilizada para a produção da proteína em larga escala, o que permitiu a sua purificação da fração solúvel das células através de cromatografias de afinidade e de exclusão molecular.

A proteína purificada apresenta atividade catalítica na presença de 3 m*M* Mg^{2+} e 3 m*M* de Mn^{2+}, e foi identificada num formato monomérico, pórem, os resultaos obtidos também sugerem a existencia desta proteína num estado homodimerico. A proteína humana β_2 não apresenta características espectroscópicas de uma hemoproteína, e não responde ao NO, o que sugere que esta proteína não é capaz de ligar o grupo hemo, ao contrário do que foi observado com a subunidade β_2 murina. Para além disso, a proteína não é ativada por BAY 60-2770, que é um conhecido ativador de sGC. Adicionalmente, quando a subunidade β_2 foi co-expressa com α_2, não se conseguiu detetar atividade catalítica, ao contrário da isoforma α_2/β_1, que é uma enzima heterodimérica cataliticamente ativa e sensível ao NO, conforme descrito na literatura.

Assim, os resultados desta disertação indicam que a subnidade β_2 é uma enzima cataliticamente ativa sem a estrutura heterodimérica que é geralmente associada às proteínas sGC.

Palavras chave: guanilil cyclase solúvel; β_2; óxido nítrico; GMP cíclico;

Abstract

The soluble guanylyl cyclase (sGC), the main nitric oxide (NO) receptor in mammalian cells, is a heterodimeric hemoprotein composed of an α and β subunits, that catalyzes the conversion of guanosine-5'-triphosphate (GTP) into the second messenger 3',5'-cyclic guanosine monophosphate (cGMP). Four different subunits have been identified, namely, α_1, α_2, β_1 and β_2; while the α_1/β_1 and α_2/β_1 heterodimeric isoforms have been extensively characterized and their function well described, the role of β_2 subunit has remained elusive. The aim of this study is the expression and purification of the human β_2 subunit, and its functional characterization. To this end, recombinant baculovirus encoding the human β_2 subunit were generated and used to infect Sf9 insect cells. To optimize the expression of β_2 several small scale tests were performed, and the best expression condition was identified and used to produce and purify the enzyme via an affinity and a size exclusion chromatography.

The purified human β_2 protein is catalytically active in the presence of 3 m*M* Mg^{2+} and 3 m*M* Mn^{2+} and was identified in a monomeric form. However, results also suggest the presence of a homodimeric state. The human β_2 subunit lacks the spectroscopic features of a hemic protein and does not respond to NO, indicating that the protein does not bind the heme group, in opposition to the reported to rat β_2. Moreover, the protein is not activated by BAY 60-2770, which is a well know heme-free-sGC activator. In addition, co-expression tests of β_2 subunit with α_2 showed that α_2/β_2 complex does not seem to be active, unlike the α_2/β_1 heterodimeric isoform, which is a catalytically active and NO-sensitive enzyme, as previously reported.

The work presented in this master thesis suggests that human β_2 subunit is a catalytically active enzyme, lacking the heterodimeric structure usually reported to sGC proteins.

Keywords: soluble guanylyl cyclase; β_2; nitric oxide; cyclic GMP;

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Chapter I

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Abbreviations

AcMNPV- *Autographa californica multinucleopolyhedrovirus*

ALA- 5-aminolevulinic acid

BAC- Bacterial artificial chromosome

BSA- Bovine serum albumin

BV- Budded virus

Ca^{2+} / CaM- Calcium calmodulin complex

CC- Coiled coil domain

CFTR- Cystic fibrosis transmembrane conductance regulator

CGA-Cell growth arrest method

cGMP- 3',5'-Cyclic guanosine monophosphate

CO- Carbon monoxide

CV- Column volume

EDRF - Endothelial derived relaxing factor

eNOS/NOS3- Endothelial nitric oxide synthases

FBS- Fetal bovine serum

GC - Guanylyl cyclase

GFP- Green fluorescent protein

GTP- Guanosine-5'-triphosphate

H-NOX- Heme-nitric oxide/oxygen binding

hpi- Hours post infection

HPLC- High-performance liquid chromatography

iNOS/NOS2 – Inducible nitric oxide synthases

M- mol/L

M- Molecular weight marker

MB- Methylene blue

MOIs- Multiplicities of infection

mRNA- Messenger ribonucleic acid

MTT- Microplate tetrazolium assay

nNOS / NOS 1- Neuronal nitric oxide synthases

NO - Nitric oxide

NOS - nitric oxide synthases enzyme

NO-sGC - cGMP pathway- nitric oxide-soluble guanylyl cyclase-cyclic guanosine monophosphate

NPs- Natriuretic peptides

PAS - Per-Arnt-Sim domain

PDE- Phosphodiesterase

PEN-STREP- Penicillin-streptomycin antibiotic

pGC - Particulate guanylyl cyclase

PKG- cGMP-dependent protein kinase

PPi- Pyrophosphate

SEC- Size exclusion chromatography

Sf9 / Sf21- *Spodopterta frugiperda* cell lines

sGC – Soluble guanylyl cyclase

TCLD50- Tissue culture lethal dose 50

UV- Ultraviolet

WT- Wild type

Chapter I- Introduction

All cells from multicellular organisms have the capability to receive extracellular and intracellular signals and to respond accordingly, which will ultimately determine the cell survival, division, differentiation and functional activity. The binding of first messengers, such as hormones, cytokines, or neurotransmitters, to specific receptors will trigger a complex signal transduction mechanism that involves the formation of second messengers and the propagation of the signal in cells. One of the most common, widely distributed and physiological relevant second messenger systems are the cyclic nucleotide cAMP and cGMP.

I.1 The NO-sGC-cGMP signaling pathway

The 3',5'-cyclic guanosine monophosphate (cGMP) was first discovered in samples of rat urine but shortly after it was reported to be present in all organs and cell systems (Ashman *et al.*, 1963). By regulating the activity of cGMP-dependent protein kinase (PKG), cGMP-dependent phosphodiesterase (PDE) and cGMP-gated ion channels, cGMP was found to be a critical mediator of numerous physiological processes including smooth muscle relaxation, peripheral and central neurotransmission, platelet aggregation, cell proliferation and even phototransduction (Figure I.1). Impairment of this pathway can lead to cardiovascular, immunologic, neurodegenerative or metabolic diseases (Warner *et al.*, 1994; Wei *et al.*, 2002; Villalobo, 2006; Dangel *et al.*, 2010; Krishnan *et al.*, 2018).

The enzyme catalyzing the conversion of guanosine triphosphate (GTP) to cGMP is guanylyl cyclase (GC). Two different types of GC have been identified: the particulate guanylyl cyclase (pGC), which is a membrane-bound complex and is activated by natriuretic peptides (NPs), and the soluble guanylyl cyclase (sGC), a cytoplasmic protein, whose activity is stimulated by nitric oxide (NO) (Denninger and Marletta, 1999; Garbers *et al.*, 2006; Kots *et al.*, 2009; Steegborn, 2014).

The focus of this thesis will be on the sGC. Pharmacological modulation of the sGC enzyme began in the 19th century with the administration of organic nitrites and organic nitrates as clinical treatment of angina pectoris. This was more than 100 years before the discovery of the molecular structure of NO in 1980. Organic Nitrates and NO-donors relieve the pain associated with the disease by relaxation of the smooth muscle cells, more specifically, the concentration used affected the veins but not necessarily the arteries, and this lead to a reduction in the diastolic pressure, and a better perfusion of the myocardium (Ignarro *et al.*, 1999; Vaughn E. Nossaman, Bobby D. Nossaman, 2010). At that time, the biological molecule released by endothelial cells, that was inducing vasodilatation, was called endothelial derived relaxing factor (EDRF). Later, experimental evidence showed that NO and EDRF had a similar effect on pharmaceutical and biochemical levels: both lead to the increase of cGMP level. Only in 1998, the Nobel Prize in

Physiology and Medicine was awarded to Robert Furchgott, Louis Ignarro and Ferid Murad for their discovery on the identification of NO as a signaling molecule in the cardiovascular system and the NO-sGC-cGMP pathway (Furchgott, 1999; Murad, 1999; Szabo, 2010; Vaughn E. Nossaman, Bobby D. Nossaman, 2010).

The intracellular generation of NO occurs through the oxidation of L-arginine into L-citrulline by the nitric oxide synthases (NOS) enzymes. In humans, three different NOS were historically identified according to their tissue distribution: the neuronal (nNOS / NOS 1), inducible (iNOS / NOS 2) and endothelial (eNOS / NOS 3) (Palmer, Ashton and Moncada, 1988; Busse and Fleming, 1995). NO is a highly reactive molecule that has a very short lifetime. Diffusion of NO into the target cells and subsequent binding to the heme group of sGC leads to the activation of the protein, and consequent formation of cGMP and pyrophosphate (PPi), in a reaction dependent on the divalent cations magnesium (Mg^{2+}) or manganese (Mn^{2+}) (Waldman and Murad, 1987; Busse and Fleming, 1995; Denninger and Marletta, 1999).

As a central component of the NO-sGC-cGMP signaling pathway, sGC is a potential target for drug development. Stimulation of sGC and cGMP production might be a beneficial treatment approach in a large range of diseases (Murad, 2006). However, a better and complete understanding of the human sGC subunits/isoforms, structure, expression levels, and tissue distribution is vital for unveiling this molecule's potential as a therapeutic target and subsequent drug design of more selective and specific therapies (Denninger and Marletta, 1999). In September 2019 for the first time the full-length structure of the human sGC has been identified (Kang *et al.*, 2019).

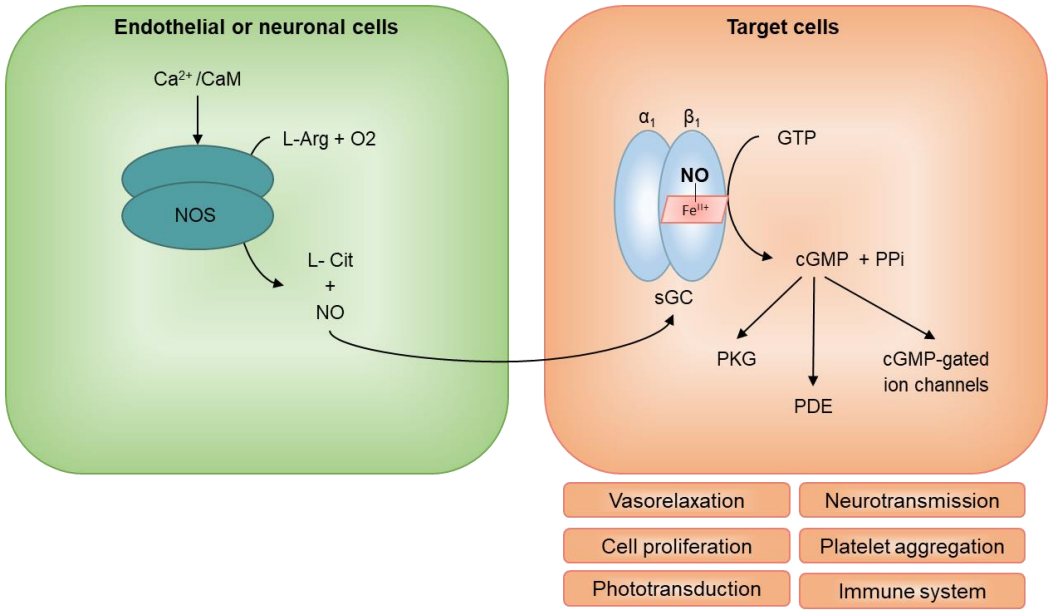

Figure I.1- The NO-sGC-cGMP pathway. The Calcium/Calmodulin complex (Ca^{2+}/CaM) binds to the NOS enzyme leading to the activation and oxidation of L-Arginine to L-Citrulline and NO. The NO molecule diffuses to the target cells and binds to the heme group of the sGC protein. This leads to activation of the sGC protein and conversion of GTP to cGMP, which targets and activates downstream proteins such as cGMP-dependent protein kinase (PKG), cGMP-dependent phosphodiesterase (PDE) and cGMP-gated ion channels. This pathway ultimately leads to modulation of several physiological processes such as: vasorelaxation, platelet aggregation, neurotransmission, phototransduction, cell proliferation and modulation of the immune system. Adapted from Derbyshire and Marletta 2012.

I.2 Soluble guanylyl cyclase

I.2.1 sGC subunits and isoforms

sGC is a heterodimeric protein of ~150 kDa composed by two subunits, the α subunit (~80 kDa) and the β subunit (~70 kDa). The later contains the heme binding domain to which the heme prosthetic group binds (Wedel *et al.*, 1994).

In humans, four different subunits were identified in sGC: two α subunits (α_1 and α_2) and two β subunits (β_1 and β_2), with 690, 732, 619 and 617 amino acids in length, respectively (from https://www.uniprot.org/ with the codes Q02108, P33402, Q02153, O75343 respectively). The human α_1/α_2 subunits share ~49% sequence identity while β_1/β_2 subunits share ~39% (Table I.1). Moreover, a high degree of identity is found between different subunits: for example, the identity of β_1 with α_1 and α_2 is 37% and 38% respectively (Table I.1). The highest sequence variability is found at the N-terminal of the α subunits and the greatest sequence identity is found at the C-terminal of both α and β subunits, revealing a high degree of conservation at the level of the catalytic domain of the protein (Figure VI.1 in Appendix) (Almeida, 2015).

Table I.1- Sequence identity between sGC human subunits. Results obtained by NCBI BLAST+ tool from EMBL-EBI (**https://www.ebi.ac.uk**).

Sequence Identity				
h- α_1	100%			
h- β_1	37%	100%		
h- α_2	49%	38%	100%	
h- β_2	39%	39%	32%	100%
sGC Subunits	**h- α_1**	**h- β_1**	**h- α_2**	**h- β_2**

The most physiologically relevant heterodimer α_1/β_1, also called GC1, was first isolated from the bovine lung and then from the lung and liver of rat (Koesling *et al.*, 1988, 1990; Nakane *et al.*, 1988, 1990). This α_1/β_1 heterodimer has been studied extensively and shown to be an essential player in the mechanism by which NO mediates vasorelaxation, lowers blood pressure and inhibits platelet aggregation via cGMP formation (Ignarro *et al.*, 1999; Lucas *et al.*, 2000). Moreover, the isoform has been overexpressed and purified *in vitro* by several groups (Lee, Martin and Murad, 2002; Russwurm and Koesling, 2005; Emmons *et al.*, 2009).

Co-expression tests showed that α_2 forms a catalytically active heterodimer with β_1 (GC2) and exhibits identical ligand-binding characteristics to α_1/β_1 heterodimer, suggesting a similar

and/or compensatory role in cells (Koglin and Behrends, 2000). The unique role of α_1/β_1 and α_2/β_1 sGC isoforms might be associated with the different subcellular and tissue distribution: although both are usually described as cytoplasmatic proteins, studies have suggested a membrane-bound localization of GC2 in specific tissues (Feussner *et al.*, 2001; Walter *et al.*, 2002; Mergia *et al.*, 2003).

The importance of each subunit was individually tested in knock-out mice. Mice deficient in either α_1 or α_2 subunits were viable and showed no significant changes in the life expectancies. In contrast, the knock-out of the β_1 subunit resulted in mice with severe intestinal dysfunction, which lead to death during the first months after birth. These results indicate that the absence of an α subunit could be compensated by the remaining one, yet the loss of the β_1 subunit lead to a complete loss of the sGC function, which enhances the importance of the β_1 for structural stability and protein assembly into a heterodimeric complex (Mergia *et al.*, 2006; Friebe *et al.*, 2007). Moreover, the importance of the heterodimerization between an α and a β subunit, in order to obtain a catalytically active protein, has been tested, and although homodimers of α/α and β/β can be formed, these complexes have no catalytic activity (Zabel, Hausler, *et al.*, 1999).

The β_2 subunit is the main focus of this thesis and will be further described in section I.3.

I.2.2 sGC structure architecture

The architecture of each subunit can be divided into four distinct domains: the Heme-nitric oxide/oxygen binding (H-NOX) domain at the N-terminal, a Per-Arnt-Sim (PAS) domain, a coiled-coil (CC) domain (also called helical domain) and a catalytic domain at the C-terminal (Figure I.2) (Derbyshire and Marletta, 2012; Marletta *et al.*, 2014; Montfort, Wales and Weichsel, 2016).

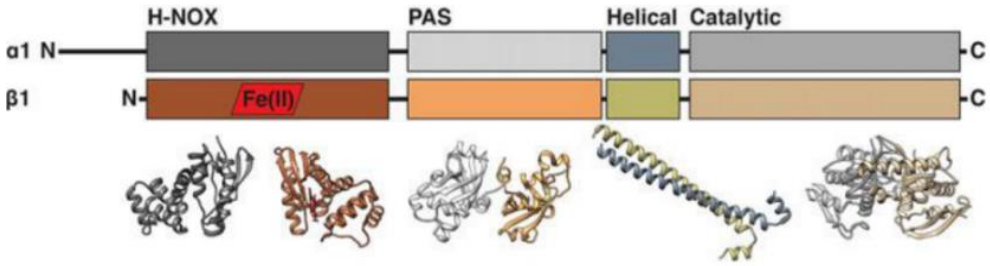

Figure I.2- Schematic and X-ray crystallographic model of the sGC domains. α_1 domain is shown in shades of grey, and β_1 domain in color. The Fe^{II+} heme group is shown in red in the H-NOX β_1 subunit. Figure adapted from Marletta *et al.* 2014.

While both α and β_1 subunits adopt a similar structure at the N-terminal, analogous to the bacterial H-NOX proteins, only the β_1 subunit contains a ferrous *b-type* heme-binding site. The heme coordinates β_1 subunit through a histidine residue, His105, forming a penta-coordinated complex that has an absorption maximum at 432 nm in the UV-visible spectrum (Iyer, Anantharaman and Aravind, 2003). The binding of the gaseous ligands to the ferrous heme results into a transient hexa-coordinated complex (absorption peak at 420 nm) that is followed by the cleavage of the ferrous-histidine bond, and fallouts into a penta-coordinated nitrosyl complex that has a UV-visible peak at 399 nm. Hence, the heme provides the ability to sense the gaseous ligands, such as NO and CO, and initiate the critic signaling process of activation of the catalytic domain at C-terminal of the protein (Iyer, Anantharaman and Aravind, 2003). The main difference between the CO and NO gaseous ligand is that the binding of CO to the heme group results in a 6-fold increase in the sGC activity, while the binding of NO results in the activation of the enzyme by several hundred-folds (Wedel *et al.*, 1994; Denninger and Marletta, 1999; Sömmer and Behrends, 2018).

The PAS domain is the central region of the sGC, closest to N-terminal, and is usually described as a mediator of protein-protein interactions (Ma *et al.*, 2008). This domain is involved in heterodimerization between the two subunits along with the helix coiled-coil domain (Underbakke, Iavarone and Marletta, 2013). Further studies revealed that the role of this domain in sGC function might also diminish the NO affinity to the heme group and/or participate in the heme insertion into the β_1 subunit through the heat shock protein 90 (hsp90) (Fritz *et al.*, 2013; Hazen *et al.*, 2015).

The other central region of the sGC, closest to the C-terminal, is the CC domain composed by two helices, one of each subunit. The orientation of the helixes was assessed as parallel, and several cross-links were found between the coiled-coiled and other sGC domains, which suggested that the major inter-domain contacts are between domains on opposite chains and that this structure is serving as an organizing center (Fritz *et al.*, 2013).

The C-terminal catalytic domain of the sGC contains the substrate-binding pocket located at the interface of both α and β_1 subunits, which are imperative for the formation of the sGC active site since both subunits contribute with key residues for the GTP binding. As the active site is at the heterodimer interface, a catalytic and a pseudo-catalytic pocket are formed. Although the pseudo-catalytic pocket has some ligand-binding affinity, it has no catalytic activity (Winger *et al.*, 2008; Fritz *et al.*, 2013). The catalytic domain of sGC requires divalent cations (Mg^{2+} or Mn^{2+}) as substrate co-factors and allosteric modulators in order to achieve maximum catalytic activity (Szabo, 2010; Vaughn E. Nossaman, Bobby D. Nossaman, 2010; Dupont *et al.*, 2014). The most likely physiological cation of sGC activity is Mg^{2+} due to its abundance in cells (Mg^{2+} concentration in cells is ~3.5 m*M* while Mn^{2+} is ~10 μ*M*) (Padh and Brenner, 1984).

I.2.3 Tissue distribution of sGC

To further unveil the specific function of each sGC isoform, cellular and tissue distribution studies were performed for each sGC subunit. Initially, most of the characterization studies were performed either in bovine or rat tissues, however current studies on the human localization have provided crucial information which is imperative for further advances in the field of using sGC as a therapeutic target.

The first study focusing on the localization of α and β subunits in human tissues revealed that, α_1 and β_1 are expressed in most tissues, although higher levels have been reported for β_1 subunit when compared to α_1. In contrast to the α_1 and β_1 subunits, α_2 localization seems to be less vast; it was specifically localized in the brain, lung, heart, spleen, uterus and placenta (Budworth *et al.*, 1999). Therefore, a higher expression of the β_1 when compared to α_1, in brain and in the placenta for example, might be explained by a high expression of α_2 in those tissues, suggesting a possible compensatory role for the α_2 subunit (Idriss *et al.*, 1992; Russwurm *et al.*, 1998; Zabel *et al.*, 1998; Mergia *et al.*, 2003).

Furthermore, studies in CHO cells, performed by Bellingham and Evans, showed that the different activities of the human α_1/β_1 and α_2/β_1 complexes are dependent on the sub-cellular localization, even though both isoforms have similar sensitivity to NO (Bellingham and Evans, 2007). Immunocytochemistry assay performed in that study showed that α_2/β_1 was localized into the plasma membrane, and by using NO-dependent activation of the ion channel cystic fibrosis transmembrane conductance regulator (CFTR) assay, it was shown that the generation of cGMP through the α_2/β_1 isoform was far more effective than through α_1/β_1. Thereby, this study suggested that the two isoforms mediate the cGMP generation through different sub-cellular locations: α_1/β_1 at the cytoplasmatic level and α_2/β_1 at the membrane level; therefore, each subunit might serve as a potential drug target, as it allows the development of more selective drugs (Russwurm *et al.*, 1998; Koesling *et al.*, 2004; Bellingham and Evans, 2007).

I.2.4 sGC modulation

The modulation of sGC activity began with the administration of organic nitrates for the treatment of angina pectoris as previously mentioned. Additionally, other compounds were identified as sGC modulators, such as CO, nitrosoalkanes or alkylisocyanides. Those compounds, also called nitrovasodilators or NO-donors, are still the drug of choice at clinical level to relive the symptoms of congestive heart failure, coronary artery disease and arterial hypertension. However, the rapid development of nitrate tolerance reduces the effectiveness of these compounds, possibly because of the oxidation of the heme group of the sGC protein (Figure I.3) (Jabs *et al.*, 2015; Buys *et al.*, 2018).

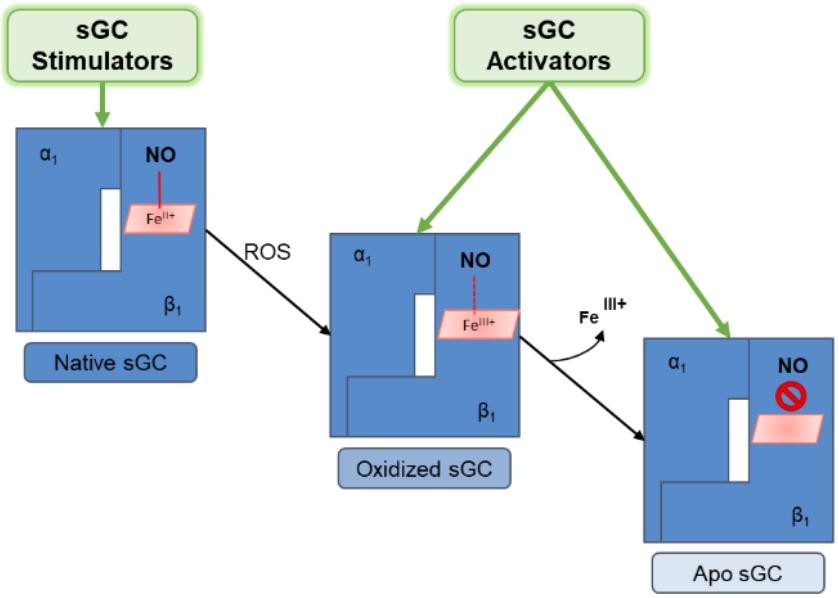

Figure I.3- Modulation of native sGC, oxidized sGC and apo sGC. Activity of native sGC is modulated by stimulators. Upon oxidative stress, the heme group is oxidized, and the binding to NO is less strong. Ultimately, the sGC losses the heme group and consequently its sensitivity to NO. Modulation of the oxidized sGC and heme free sGC (apo form) is obtained through use of activators. Adapted from Krishnan *et al.* 2018.

I.2.4.1 sGC Stimulators

In 1978, a benzylindazole derivative YC-1 (benzylindazole) was described as an inhibitor of platelet aggregation. Later it turned out, that YC-1 was stimulating sGC and activated the protein without coordinating the heme group (depending only on the reduced state of the heme prosthetic group). Chemical and pharmacological optimization programs at Bayer resulted in the identification of several pyrazolopyridines, such as BAY 41-2272 or BAY 41-8543 (Stasch *et al.*, 2001). These compounds were historically classified as sGC stimulators and this class stimulate sGC NO-independent molecules, but this stimulation is heme-dependent. Although they can increase the sGC activity in the absence of NO, these molecules can also act synergistically with NO, resulting in a powerful amplifier of the NO signaling. These molecules were initially promising drug candidates, albeit, it was found that their metabolism was disadvantageous since the metabolites formed upon the compound's degradation retained the ability to stimulate sGC. To overcome the problems associated with these compounds, structure/activity based research was pursued, and BAY 63-2521, also called Riociguat, was discovered (Figure I.4) (Nossaman and Kadowitz, 2013). This compound, now commercialized by Bayer as *Adempas*®, was approved by the Food and Drug Administration (FDA) for treatment of pulmonary hypertension in 2013, and it was the first marketed drug of this class (Schermuly *et al.*, 2008; Ghofrani *et al.*, 2013; Lai and Crews, 2017).

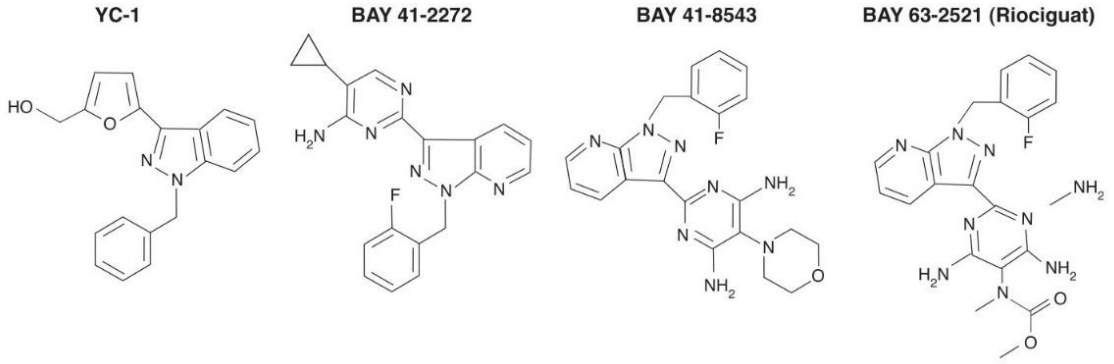

Figure I.4- Chemical structure of sGC stimulators. The structure of YC-1, BAY41-2272, BAY 41-8543 and BAY 63-2521 represented in the Figure from right to left (Nossaman and Kadowitz, 2013).

I.2.4.2 sGC Activators

Another class of sGC modulators are the so called sGC activators, which, in contrast to the stimulators, target the heme-oxidized or heme-deficient sGC resulting from oxidative stress (e.g. inflammation). Therefore, the activators are considered NO and heme independent modulators, and various compounds have already been identified: BAY 58-2667 (Cinaciguat), BAY 60-2770, S3448, TY55002 or HMR 1766 (Ataciguat). The mechanism of action of these compounds relies on their binding in the heme pocket, and therefore, acting as mimetic of the heme-NO complex and consequently promoting the active sGC conformation. Additionally, these compounds not only activate sGC and increase the catalytic activity, but also stabilize and protect the protein from degradation (Figure I.5) (Stasch *et al.*, 2002; Hoenicka and Schmid, 2008; Roy *et al.*, 2008; Meurer *et al.*, 2009; Thoonen *et al.*, 2015; Sawabe *et al.*, 2019).

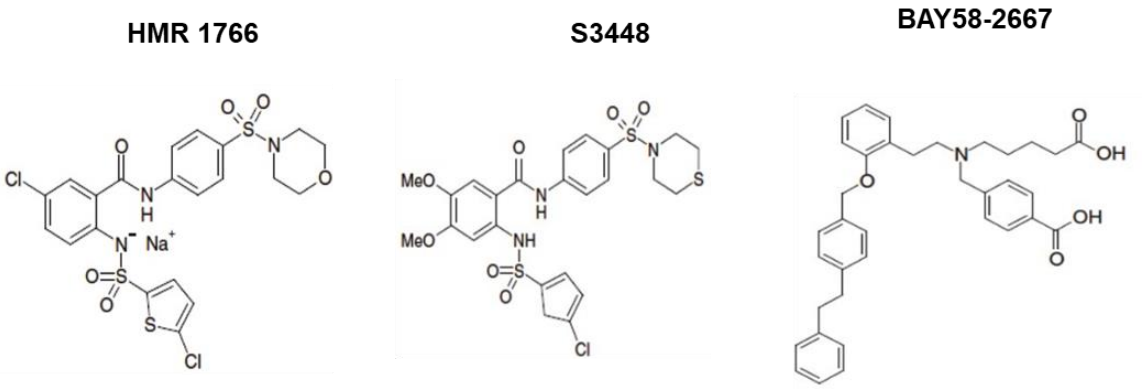

Figure I.5- Chemical structure of sGC activators. Structure of HMR 1766, S3448 and BAY58-2667 and are represented in the Figure from right to left. Adapted from (Thoonen, 2010).

I.2.4.3 sGC Inhibitors

Although there has been a significantly higher effort for the identification of specific sGC activators, compounds that inhibit sGC catalytic activity have also been reported. In general, the inhibitors can be divided in two classes: compounds that oxidize the heme group of sGC and compounds that bind to the catalytic domain.

The methylene blue (MB), YL83583, ODQ and NS-2028 are compounds known from the first group of sGC inhibitors. The MB and YL83583 mode of action relies on the generation of superoxide anions, which leads to the oxidation of NO and the heme group (Mayer, Brunner and Schmidt, 1993; Leinders-Zufall and Zufall, 2017). ODQ and NS-2028 compounds have a distinct mode of action, acting through oxidation of the ferrous heme group, which prevents the NO binding (Garthwaite *et al.*, 1995; Olesen *et al.*, 1998).

The second class of inhibitors, compounds that bind directly to the catalytic domain, include the quinoxaline derivative (D12) and specific organic phosphates and GTP analogs, such as 2,3-bisphosphoglyceric acid (2,3-BPG), inositol hexakisphosphate (IHP), guanosine tetraphosphate (G-tetra-P), 2'3'-dialdehyde derivative of GTP (Dial GTP) and inorganic triphosphate (P-P-P) (Brandwein *et al.*, 1982; Suzuki, Suematsu and Makino, 2001; Majumder *et al.*, 2009; Mota *et al.*, 2015). Although the mechanism of action is not fully known, it was suggested that D12 binds the catalytic domain and locks the protein in its basal conformation. The other inhibitory compounds that bind to the catalytic domain, are GTP-competitive, thus, acting as substrate analogues of GTP.

I.3 The β_2 subunit of sGC

The rat β_2 subunit of sGC was first cloned after homology screening of rat kidney (Yuen, Potter and Garbers, 1990). At that time, β_2 was predicted to have 682 amino acids, lacking the first 62 amino acids present in the N-terminal of β_1 subunit.

Later, sequence analysis revealed the existence of an earlier starting transcription site, which is highly conserved between all sGC β subunits (Koglin *et al.*, 2001). Alignment of the amino acid sequences of the rat sGC subunits showed that the rat β_2 subunit (742 amino acids) share 41%, 37% and 33% sequence identity with rat α_1, β_1 and α_2 (Table I.2). Moreover, it shows that rat β_2 presents the conserved His residue known to be the axial ligand of the heme group and that this molecule has an additional 86 amino acids at C-terminal that does not align with the β_1 subunit. In this C-terminal, an isoprenylation site (CVVL) was identified, which is absent in rat and human β_1 and also in human β_2 (Table VI.1 and VI.2 in Appendix). This isoprenylation site is commonly required for post-translational modification of *ras* family of proteins. Once this post-translational modification occurs, the *ras* proteins are translocated to the membrane plasma. Nevertheless, the existence of this sequence in unrelated proteins, such as the β_2 subunit, does

not guarantee the post-translational modification or translocation of the protein to the membrane. Furthermore, the high level of hydrophilic amino acids in the C-terminal of the β_2 subunit might not favor the membrane association (Yuen, Potter and Garbers, 1990; Koglin *et al.*, 2001).

Table I.2- Sequence similarity between sGC rat subunits isoforms. Results obtained by NCBI BLAST+ tool from EMBL-EBI (**https://embnet.vital-it.ch/**).

Sequence Identity				
r- α_1	100%			
r- β_1	36%	100%		
r- α_2	51%	38%	100%	
r- β_2	41%	37%	33%	100%
sGC Subunits	r- α_1	r- β_1	r- α_2	r- β_2

Rat β_2 subunit was showed to be mainly expressed in kidney (Yuen, Potter and Garbers, 1990; Behrends *et al.*, 2001), but expression of this isoform in liver, lung, brain, spleen and thymus was also reported (Yu *et al.* 2014; https://www.ncbi.nlm.nih.gov/gene/25206). In particular, an immunolocalization study performed by Mundel *et al.* 1995, showed the cytoplasmic localization of rat β_2 in the principal cells of the collecting duct (kidneys), but an immunohistochemistry assay also revealed the presence of β_2 in the sarcolemma region of the skeletal muscle fibers. Moreover, Kempfert *et al.* (2001) showed that the expression of β_2 in kidney tissues changes during development: expression of β_2 is detected in adult tissues and not in neonatal tissues (Behrends *et al.*, 2001). Similar results were observed for the α_1 and β_1 subunits in rat lung (Bloch *et al.*, 1997). Therefore, the exact and exclusive cellular localization of this subunit has yet to be confirmed (Mundel *et al.*, 1995; Feussner *et al.*, 2001).

Co-expression tests of β_2 with the other α_1 and α_2 sGC subunits does not seem to lead to the formation of active heterodimers (Denninger and Marletta, 1999; Behrends and Vehse, 2000; Russwurm and Koesling, 2005). Nevertheless, one study reported the formation of an active α_1/β_2 heterodimer with reduced NO sensitivity upon co-expression in COS cells (Gupta *et al.*, 1997). However, all the subsequent studies showed the opposite and suggested that the outcome obtained by Gupta *et al.* (1997) might have been due to the presence of a GFP protein attached to the N-terminal of the β_2 subunit. It was proposed that the GFP protein might have compensated for the domain that is missing in the N-terminal of rat β_2 sequence proposed, initially by *Yuenet et al.* (1990), and that was later shown to be important for heme binding (Yuen, Potter and Garbers, 1990; Behrends and Vehse, 2000; Koglin *et al.*, 2001).

More interestingly, Koglin *et al.* (2001) reported that the expression of the rat β_2 alone in the cytoplasmatic fractions of Sf9 cells resulted in a higher basal activity in the presence of Mn^{2+}

when compared to Mg^{2+}, the usual co-factor of α_1/β_1 and α_2/β_1 sGC isoforms. Also, the addition of NO lead to a 4-fold increase in the activity when compared to the activity of Sf9 cell extracts infected only with rat β_1 (Koglin *et al.*, 2001). Moreover, the addition of Tween 20 to the cytosolic fractions of the cells expressing the β_2 leads to the loss of the NO sensitivity while the addition of protoporphyrin IX restored the NO sensitivity. Altogether, these results strongly suggested that rat the β_2 subunit is able to bind heme and is NO responsive (Foerster *et al.*, 1996; Koglin *et al.*, 2001).

Fewer studies have targeted the human β_2 subunit, mainly because the expression of this protein is more restrict and significantly lower when compared with the human β_1. The gene (GUCY1B2) coding for this enzyme is localized on chromosome 13q14.2 and was described as a pseudogene. Additionally, studies on the mRNA expression in HeLa cells demonstrated that the expression of this subunit might be dependent on specific factors that trigger the mRNA transduction (Vazquez-Padron *et al.*, 2008). Expression of human β_2 was reported in kidney, lung, placenta, skin, spleen and stomach, and in lower amount in the urinary tract, brain, endometrium, lymph node, small intestine and appendix (Sönke Behrends and Vehse 2000b; Fagerberg *et al.* 2014; https://www.ncbi.nlm.nih.gov/gene/2974).

The human β_2 shares 82% of sequence similarity with the rat β_2 (Koglin *et al.* (2001) reported sequence) and 37% similarity with the rat β_1 (Figure V.3 in appendix). Sequence analysis revealed that the N-terminal of this isoform has the conserved His residue, known to coordinate the heme binding in β_1 and in rat β_2. However, a cysteine residue (Cys) known to be important for NO-sensitivity is lacking in the human sequence. Human and rat β_2 sequences alignment shows that the N and C-terminals of the human sequence is not as conserved when compared to the rat, since the human N-terminal lacks ~77 amino acids and the C-terminal lacks ~54 amino acids, including the isoprenylation site, present in the rat β_2 sequence. Despite of the sequence similarity, differences between them raises questions regarding the functionality of this subunit.

More interestingly, a 5'untranslated region (UTR) fragment of human β_2 isoform (74 amino acids long) was found in human gastric carcinoma and not in healthy tissues of the stomach, unlike β_1, which was identified in both types of tissues. This raises the possibility that expression of human β_2 might have a role in the tumorigenesis or in cell growth (Behrends and Vehse, 2000; Scholz, 2000). Although the β_2 subunit has been identified in human tissues, this subunit was never purified from the native tissues or from an artificially expression system, and its biological relevance, structural and biochemical function has remained elusive. Moreover, and to the best of our knowledge, no catalytic activity has been reported to the human β_2 subunit.

1.4 Aim of this thesis

The aim of this thesis is the production and purification of an sGC subunit, the human β_2, which so far has not been characterized and whose physiological function remains to be unveiled. In particular, the thesis focus will be on i) optimization of human β_2 expression in Sf9 cells using the baculovirus expression system; ii) purification process improvement to achieve a folded/functional protein and a high yield; and iii) functional characterization by NO and the sGC activator BAY 60-2770.

This master thesis aims to unveil the functional role of the human β_2 subunit and how its activity could be pharmacologically targeted.

Chapter II- Materials and Methods

With the aim of studying and characterizing biologically relevant proteins came the need to produce proteins at larger scales, for which, recombinant proteins expression systems became a tool. Choosing the appropriate method to express a recombinant protein is essential to obtain high expression yields as well as a correctly folded protein. Factors such as mass, type of posttranslational modifications (phosphorylation, glycosylation, lipidation), number of disulfide bonds and the destination of the expressed protein highly influence the decision on which expression system to implement. The main protein expression systems used nowadays are bacteria, yeast, insect and mammalian.

The baculovirus-mediated expression in insect cells is a vastly used tool to generate recombinant proteins. It allows the transient expression of the recombinant protein, both cytoplasmatic and secreted, with the majority of the post-translational modifications found in mammalian cells, and with the correct disulfide bonds (Berger, Fitzgerald and Richmond, 2004; Peleg, 2012).

II.1 Insect cell culture

Spodoptera frugiperda (Sf9) insect cells were routinely maintained in Sf900 III serum free medium from Gibco® (Thermo Fisher Scientific). Sf9 cells were sub-cultured twice a week with a starting inoculum of $0.3x10^6$ cells/mL and incubated in shake flasks containing 1/10 of the vessel volume (typically 50 mL in a 500 mL-shake flask), at 27ºC and 100 revolutions per minute (rpm) in a non-humidified environment (INNOVA incubator). Before reaching the maximum density of $3x10^6$ cells/mL, cells were sub-cultured. Basic cell culture technique was used as described in Growth and Maintenance of Insect Cell Lines (Invitrogen, 2002) under sterile conditions.

When indicated, culture was maintained in Sf900 III medium supplemented with 10% fetal bovine serum (FBS; Gibco™, Thermo Fisher Scientific) and 1% Penicillin-Streptomycin antibiotic (PEN-STREP; Gibco™, Thermo Fisher Scientific) to avoid contaminations. Three passages were usually performed before cells were employed in any experiment.

II.2 Baculovirus generation

II.2.1 Baculovirus expression system- The *flash*BAC technology

The baculovirus expression system was designed as a technique to express and produce functional recombinant proteins in Sf9 or Sf21 insect cells. The most commonly used virus to infect insect cells and transiently express the recombinant proteins is the baculovirus *Autographa californica multinucleopolyhedrovirus* (AcMNPV) which is a double-stranded, circular and non-

integrative DNA, with a size of 80 to 180 kbp, packed in a rod-shaped nucleocapsid (Fraser, 1987; Mansouri and Berger, 2018). The main protein present in the nucleocapsid matrix of the budded virus (BV) is polyhedrin, which is a non-essential protein for virus replication and whose expression is under the control of the powerful promoter *polh*.

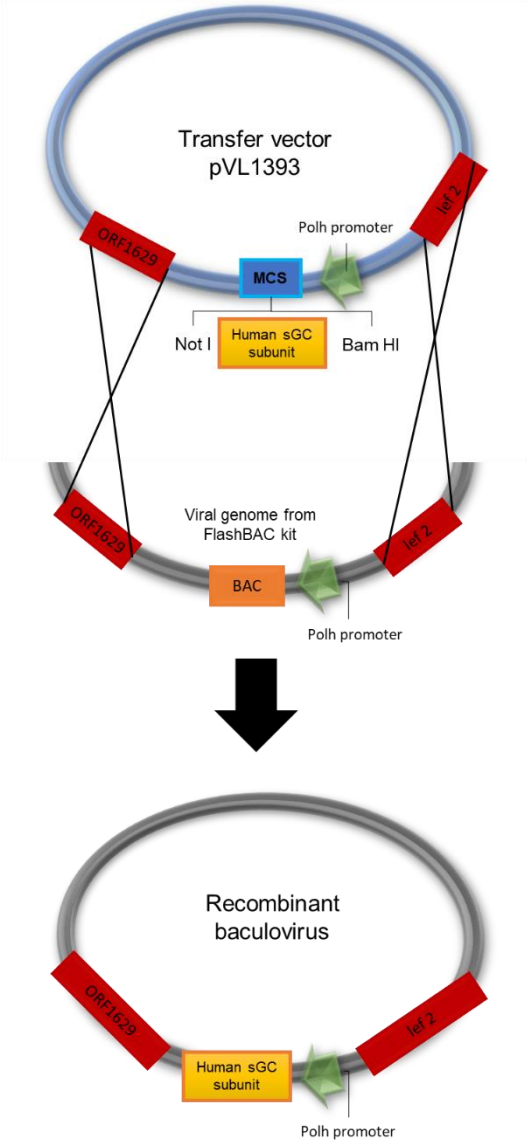

Figure II.1- Schematic representation of the *flash*BAC system. The co-transfection of the transfer vector pVL1393 (blue), containing the sGC gene of interest (yellow), and the *flash*BAC DNA (grey) that contains the bacterial artificial chromosome BAC (orange), in insect cells allows the homologous recombination and generation of the recombinant baculovirus. Adapted from Oxford Expression Technologies 2015.

The *flashBAC* technology was developed to allow the production of recombinant baculovirus particles in a one-step procedure. This system is based on the homologous recombination between the AcMNPV viral genome and a transfer vector, a plasmid carrying the gene encoding the recombinant protein which will be expressed under the control of the *polh*

promoter. The viral genome used in the *flash*BAC system lacks part of the ORF1629 gene, essential for the baculovirus replication in insect cells, and contains a bacterial artificial chromosome, BAC, replacing the polyhedrin coding sequence, which allows maintenance of the viral genome in bacteria. The BAC gene is flanked by the lef2 and ORF1629 sequences, which also exist in the transfer vector (in this work the pVL1393 was used). Upon infection of insect cells with the viral genome and the transfer vector, homologous recombination will occur between the lef2 sequences and the ORF1629 sequences, which will allow the insertion of the recombinant protein sequence in the viral genome, under control of *polh* promoter, and excision of the BAC chromosome (Figure II.1). By homologous recombination, the genome of the baculovirus is restored; ORF1629 restored gene allows recombinant baculovirus replication in insect cells. The non-recombinant virus cannot replicate, therefore all harvest baculovirus particles will express the gene of interest (Rohrman, 2013).

II.2.2 Recombinant proteins constructs

In this work, three different transfer vectors were designed by Dr. Gabriela Silva and their synthesis ordered at GeneArt. The cloning strategy consists of cloning the human β_2 or α_2 coding sequence flanked by the 5'-BamHI and 3'-NotI restriction sites of pVL1393 vector. Upstream of the sGC subunit a Kozak consensus sequence was introduced, and downstream, when indicated, a strep tag was added. For all nucleotide sequences, codon optimization was performed for expression in insect cells. The three transfer vectors generated were: pVL-hβ_2, pVL-hβ_2-strep and pVL-hα_2-strep. Each transfer vector was individually transformed in *Escherichia coli* (*E. coli*) Stellar competent cells, using routine laboratorial techniques. Bacterial stocks were prepared and stored at -80ºC.

Table II.1- Construct sequences. The coding sequence of sGC subunits are flanked by nucleotide sequences of BamHI restriction site and Kozak consensus sequence at 5' and at 3' by a Strep-Tag II, two STOP codons and a NotI restriction site.

Name	Designed construct in pVL1393 plasmid	Expected Molecular Weight
pVL-hβ_2-Strep	BamHI-Kozak- **Human-β_2**-Strep-Tag II- STOP-STOP-NotI	71 kDa
pVL-hβ_2	BamHI-Kozak**Human-β_2**-STOP-STOP-NotI	70 kDa
pVL-hα_2-Strep	BamHI-Kozak- **Human-α_2**-Strep-Tag II- STOP-STOP-NotI	83 kDa

II.2.3 Production of baculovirus stocks

Generation of the recombinant baculovirus was performed in Sf9 cells grown in Sf900 III medium at 27ºC. In 6-well plate (Ref 351146; Falcon®), 1.6 x10^6 Sf9 cells per well were seeded and allowed to attach for 1 h. The *flash*BAC DNA (100 ng) was mixed in Sf900 III medium with 500 ng transfer vector (both DNA's were filtered using a 2 µm filter (Pall Life Science) before use) and the mixture was incubated 20 min at room temperature (RT). Separately, 0.08 mg/mL of Cellfectin II (Invitrogen) was added to 100 µL of the same medium and incubated 20 min at RT. Both mixtures were combined and incubated 15 min at RT before being added drop-by-drop to the attached cells. The plate was incubated at 27ºC in a static incubator and in a plastic box with wet paper inside, to minimize evaporation of medium. Five days after co-transfection, cells were harvested and centrifuged at 500 xg for 5 min. The supernatant was transferred to a new falcon tube (under sterile conditions) and stored at 4ºC protected from light giving rise to the P1 baculovirus stock.

Amplification of the P1 recombinant baculovirus stocks was performed by inoculating 1x10^6 cells/mL of Sf9 in Sf900 III medium followed by infection with 200 µL of each P1 baculovirus stock. After 96 h incubation at 100 rpm (INNOVA incubator), the P2 baculovirus stocks were harvested by centrifugation at 500 xg for 10 min, and stored at 4ºC, protected from light. The generation and quantification of the baculovirus stocks is represented in Figure II.2.

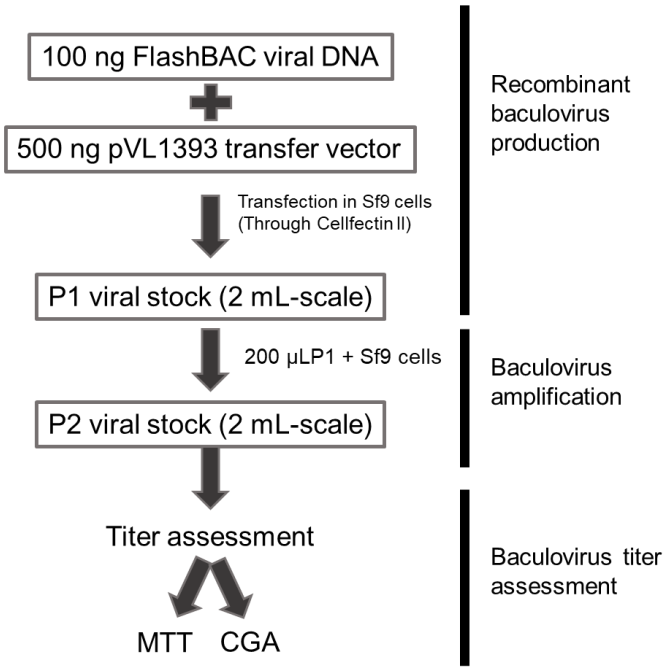

Figure II.2- Schematic representation of the generation and quantification of the recombinant baculovirus. Generation of the recombinant P1 baculovirus, amplification of the stock (P2) and titer assessment by two different methods: microculture tetrazolium assay (MTT) and cell growth arrest method (CGA).

II.2.4 Titration of the baculovirus stocks

Two different methods were used to titrate P2 baculovirus stocks: the Microculture Tetrazolium assay (MTT) which is based on the cell viability upon infection and the Cell Growth Arrest Method (CGA), based on the cell growth cessation induced by the viral particles (Mena *et al.*, 2003; Roldão *et al.*, 2009).

II.2.4.1 Microculture Tetrazolium assay

Serial dilutions (10^{-2} to 10^{-11}) of the P2 viral stocks were prepared in 96-well plates (Ref: 351172; Corning-Falcon®) containing 300 µL Sf900 III medium supplemented with 1% PEN-STEP. Sf9 cells, grown in the same medium, were seeded on 96-well plates at $5x10^4$ cells per well (triplicate plates were prepared) and incubated for 30 min at 27ºC in a plastic box with a wet paper inside. The supernatant was removed when cells were attached and 100 µL of the virus dilutions was added. One lane of 8 wells was left uninfected as a control. The plates were incubated for 6 days under the same conditions mentioned above. Thiazolyl blue tetrazolium bromide (Sigma–Aldrich) was added (0.5 µg/mL) and plates incubated for 4 h. The supernatant was removed, and formazan crystals were solubilized by adding 150 µL/well of dimethyl sulfoxide (Sigma–Aldrich). Plates were agitated 15 min and absorbance at 570 and 690 nm was measured using a TECAN microplate reader (TECAN Infinite 200Pro).

Collected data was analyzed using Prism 4 for Windows (GraphPad Software Inc., La Jolla, USA) to determine the virus dilution corresponding to 50% of growth inhibition, also known as tissue culture lethal dose 50 (TCLD50). The conversion of TCLD50 to viral titers (pfu/mL) was carried out using the mathematical regressions reported by Mena *et al.* 2003 and Roldão *et al.* 2009.

I.2.4.2 Cell Growth Arrest method

Sf9 insect cells, grown in Sf900 III medium, were inoculated at $1x10^6$ cells/mL and infected with 250 µL of virus serial dilutions ranging from $2x10^{-2}$ to $2x10^{-6}$ (previously prepared in the same medium). A negative control with uninfected cells was also set. The constructs were incubated for 24 h in the optimal growth conditions, after which the number of viable cells was calculated by cell counting in a hemocytometer using trypan blue exclusion dye method to assess when the cells growth stops (Gibco™, Thermo Fisher Scientific). Cell concentration was determined in triplicate, and the raw data was analyzed using the 4-parameter method as described by Possee and King (1992) and Roldão *et al.* (2009), in order to estimate the viral titers.

II.3 Protein expression in Sf9 insect cells

II.3.1 Small scale expression tests

Small scale expression tests were performed with $2x10^6$ Sf9 cells/mL transiently infected with the P2 baculovirus stocks expressing human β_2 with a strep tag at C-terminal (P2-hβ_2-Strep) at different multiplicities of infection (MOIs), using three different media and incubated at 27ºC and 100 rpm, in a final volume of 12,5-25 mL. When indicated, 0.1 m*M* of filtrated 5-aminolevulin acid (ALA; SIGMA), a precursor for the heme biosynthesis, was added 30 min before infection (Table II.2).

Another set of expression tests of human β_2-strep was performed in which the incubation temperature after infection was lowered to 22ºC, in order to decrease the metabolism and allow improvement of the solubility of the protein (Table II.3).

Table II.2- Small scale test of hβ_2-strep: simple expression conditions tested.

Condition	1	2	3	4	5	6	7	8
Single Expression	P2-hβ_2-Strep							
MOI	0.02	0.2	0.4	0.02	0.2	0.4	0.2	0.4
Media	Sf900 III			Sf900 III +10% FBS +1% PEN-STREP			Sf900 III +10% FBS +1% PEN-STREP + ALA	
Temperature	27ºC							
Harvest point	72 hpi							

Table II.3- Small scale test of hβ_2-strep: comparison using 22ºC and 27ºC.

Condition	1	2	3	4	5	6	7	8
Single Expression	P2-hβ_2-Strep							
MOI	0.4	40	0.4	40	0.4	40	0.4	40
Media	Sf900 III				Sf900 III +10% FBS +1% PEN-STREP			
Temperature	22ºC		27ºC		22ºC		27ºC	
Harvest point	48 hpi; 72 hpi; 96 hpi							

Using the same protocol described above, co-expression the following tests were performed: Sf9 insect cells were co-infected with P2-hα_2-strep and P2-hβ_2 or P2-rβ_1 (available at iBET/Bayer laboratory) in order to co-express the two sGC subunits together, α_2 and β_2 or β_1. The conditions tested are listed in Tables II.4 and II.5, respectively.

Table II.4- Small scale co-expression test of hα2-strep with hβ2.

Condition	1	2	3
Co-expression	P2-hα_2-Strep/P2-β_2		
MOI	0.2 : 0.2	0.02 : 0.02	0.02 : 0.02
Media	Sf900 III	Sf900 III +10% FBS +1% PEN-STREP	Sf900 III +10% FBS +1% PEN-STREP + ALA
Temperature	27ºC		
Harvest point	72 hpi		

Table II.5- Small scale test of hα_2-strep: co-expression conditions tested with rβ_1.

Condition	1	2
Co-expression	P2-hα_2-Strep/ P2-rβ_1	
MOI	0.02 : 1	0.02 : 1
Media	Sf900 III +10% FBS +1% Pen-Strep + ALA	Sf900 III +10% FBS +1% Pen-Strep
Temperature	27ºC	
Harvest point	72 hpi	

II.3.2 Sf9 cell extracts preparation: separation of the soluble fraction from the insoluble fraction

Two million cells were harvested by centrifugation 5 min at 200 xg (Eppendorf® MiniSpin®). Pellet was resuspended at $1.4x10^7$ cells/mL in Sonication Buffer containing 50 m*M* triethylamine hydrochloride (TEA-HCl) at pH 7.6, 0.5 m*M* ethylenediamine tetra-acetic acid (EDTA), 0.2 m*M* phenyl-methyl-sulfonyl fluoride (PMSF), 1 µ*M* Pepstatin A, 1 µ*M* Leupeptin and 7 m*M* glutathione (GSH), and sonicated at 60% amplitude, for 6 s, on ice (Branson 450 Digital Sonifier adapted with a sound enclosure from Marshall Scientific). The soluble fraction of the cell extracts was separated from insoluble fraction by centrifugation at 13000 xg, 15 min, 4ºC (Centrifuge 5415 R; eppendorf). The supernatant (soluble fraction) was collected and used to analyze the expression of sGC subunits by western blot and to measure the guanylyl cyclase activity. The pellet (insoluble fraction) was resuspended in 0.5 mL of Dulbecco's phosphate-buffered saline (DPBS; Gibco™) or 140 µL Sonication buffer and used directly for the western blot. When indicated the resuspended pellets were also sonicated, and a new soluble fraction was extracted by centrifugation at 13000 xg, 15 min, 4ºC and analyzed.

II.3.3 Detergents screening

In order to increase the solubility of the human β_2 protein, several detergents were tested. Detergents are polar molecules, with a polar head and a long hydrophobic carbon chain that can bind hydrophobic regions of membrane protein. Usually the use of these allows membrane proteins to remain embed in the formed micelles in an aqueous solution, thus increasing their solubilization. The protein-detergent complex can be isolated and purified. According to the type of the polar headgroup, these compounds can be classified as: nonionic, anionic, cationic or zwitterionic. Typically, anionic detergents are harsher than the nonionic or zwitterionic ones since they lead to total membrane disruption and denaturation of the proteins. The selection of the right detergent is crucial for effective solubilization and purification of membrane proteins (Linke, 2009; Kubicek *et al.*, 2014).

Human β_2-strep was expressed in 50 mL and 1.5 L batches using a MOI 0.4 and incubated at 22ºC. Seventy two hpi cells were harvested by centrifugation at 800 xg for 15 min and resuspended in a final concentration of ~$3x10^7$ cells/mL in lysis buffer (50 m*M* TEA-HCl, 1 m*M* EDTA, 10 m*M* dithiothreitol (DTT) supplemented with an anti-proteases cocktail from Roche (1 Tablet per 50 mL of buffer)). Cells were distributed in individual falcons, and detergents were added (Table II.6). Cells were lysed by sonication at 60% amplitude, on ice, for 6 s cycles, and this procedure was repeated until cell debris were detected microscopically (Branson 450 Digital Sonifier adapted with a sound enclosure from Marshall Scientific). Cell homogenates were incubated at RT for 60 min, after which the soluble fractions were obtained as described in section II.3.2.

Table II.6- List of detergents tested in the solubilization of human β2-strep protein.

Detergent	Classification	Concentration used (mM)
Triton	nonionic	1%; 0.5%
Nonyl phenoxypolyethoxylethanol (NP-40)	nonionic	0.58
Tween 20	nonionic	0.12
Tween 80	nonionic	0.02
Brij 35	nonionic	0.18
Chaps	zwitterionic	20
Digitonin	nonionic	0.02
n-Dodecyl-β-maltoside (DDM)	nonionic	34
Octyl glucoside (OG)	nonionic	40
n-Dodecyl-phosphocholine (FC-12)	ionic	18
Decyl maltoside (DM)	nonionic	3.2
N-octyl-B-D-thioglucoside (OTG)	nonionic	4
6-Cyclohexyl-1-Hexyl-β-D-maltoside (Cymal-6)	nonionic	1.12
N,N-Dimethyl-n-dodecylamine N-oxide (LDAO)	nonionic	3
Nonylglucoside (NG)	nonionic	40

II.3.4 Scale up expression of human β_2

Large scale productions were performed with the best expression condition identified in the small scale tests. Briefly, 2 x10^6 cell/mL of Sf9 cells, grown in Sf900 III medium, were infected with human P2-hβ_2-strep at MOI 0.4, and incubated for 72 h at 27ºC and 100 rpm agitation.

II.4 Large scale protein purification

The development of an optimal protocol for protein purification is an essential pre-requisite in order to achieve a high yield of the recombinant protein. The protein purification process can vary from a simple one-step procedure to several-step process, in order to achieve the desired purity and yield. Each step in the purification leads to some loss of the product, and consequently to a decrease in the final yield, therefore, experience has shown that a minimum of four well-chosen purification steps are enough even for the most changeling proteins. Moreover, even though recombinant proteins are produced in expression systems that facilitate their subsequent purification (like the use of tags), there are still some challenges in the purification process, such as: protein solubility, structural integrity and biological activity.

Most common purification processes include four different steps: sample preparation, clarification, capture/purification and polishing. The first step is performed in order to extract the protein from the cell culture. Next, in the clarification step the target product is isolated and concentrated. Usually, filtration or centrifugation techniques are applied and additionally, the product is stabilized in a buffer that allow further purification steps. The intermediate purification step consists in the removal of most of the impurities and the main technique used for this purpose is chromatography. Different chromatographic methods can be employed depending on the protein properties, and combination of different chromatographic techniques, with different selectivity, can be a powerful tool in the purification process. In particular, affinity chromatography is a method with high selectivity and high capacity that allows the separation of a specific molecule, from a complex solution containing many other components, by specific affinity interaction between the target protein and a ligand that is immobilized on a solid support (Urh, Simpson and Zhao, 2009; Hermanson, 2013; Duong-Ly and Gabelli, 2015). In the final polishing step, the main objective is to achieve a high purity of the protein mainly by removing aggregates and any remaining impurities. Size exclusion chromatography (SEC), also called gel filtration, is a method highly used to separate the particles according to their size. This technique is based on how efficiently the molecule penetrates the pores of the resin: smaller size molecules will penetrate the small pores in the resin, therefore will be retained in the resin for longer time, while larger molecules will be rapidly eluted without being retained in the resin (Kim, Chang and Koo, 2000; Nagy and Vékey, 2008; Issaq and Veenstra, 2013).

I.4.1 Human β_2 purification

Purification of the Human β_2 protein was performed as described below. Briefly, insect cells were collected by centrifugation at 800 xg for 15 min at 4 ºC, and pellet resuspended in ice-cold lysis buffer (~50 mL per L of culture) at a final concentration of ~3x10^7 cell/mL. Cells were homogenized using one of the two methods: homogenization at 600 bar, three times in AVESTIN C5 homogenizer or sonication at 60% amplitude, for 6s cycles, on ice (cycles repeated until cell debris was detected microscopically). After lysis, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) was added to inhibit protein proteolysis. Also, 250 n*M* Avidin were added to scavenger the biotin from the sample in order to prevent its binding to the strep-tag resin, that might impair the purification. The mixture was incubated 30 min at 4ºC under constant agitation after which a clarification step by ultracentrifugation at 30000 xg, 90 min, 4 ºC (Beckman Coulter™ Optima™ LE-80k Ultracentrifuge) was performed. The supernatant was collected and filtered through Vaccum CAP 0.2 µm (Pall Life Science).

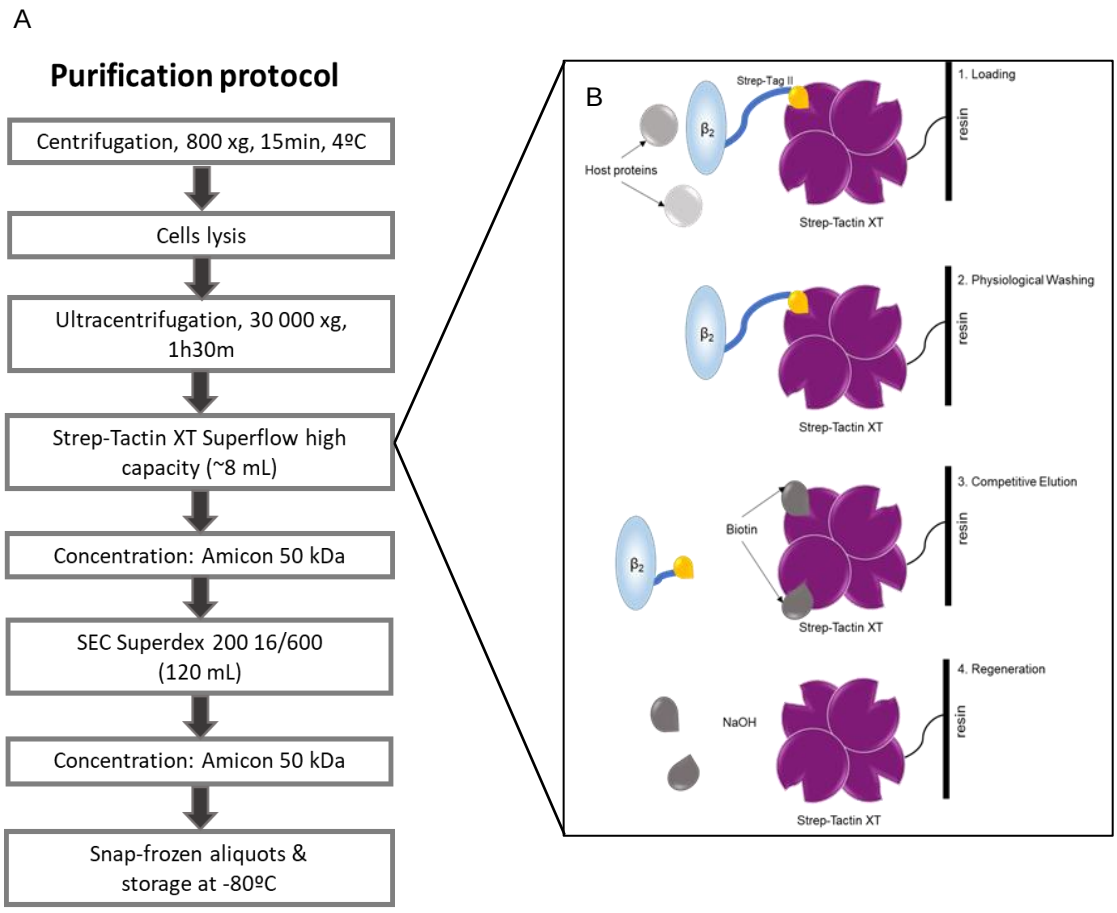

Figure II.3- Schematic representation of the purification process. A: Protocol for the purification of human β_2- Strep Tag II. **B:** Strep-Tactin XT super flow high capacity purification cycle. In the first step, cells supernatant is loaded on to the column. The human β_2 protein binds to the resin by the strep-tag II. In the washing step all unspecific host proteins are removed. In the elution step, the biotin specifically competes for the biotin binding pocket and the β_2 protein is eluted. In the fourth step, the column is regenerated by the application of NaOH that removes the biotin from the binding pocket. Adapted from Manual Strep-Tactin XT-Purification manual (https://www.iba-lifesciences.com/).

Protein was purified using an Äkta Purifier 100 System (GE Healthcare) with an 8 mL StrepTactin®XT Superflow high capacity column (IBA) at 4ºC. This technology relies on the non-covalent interaction between the Strep-tag peptide, attached to the C-terminal of recombinant protein, and the engineered streptavidin resin. This system allows a rapid one-step elution of the recombinant proteins that allows achieving a high degree of purity. Moreover, as the peptide Strep-tag is only 8 amino acids long and has a neutral isoelectric point it does not interfere with the folding or the function of the protein. Additionally, this system allows the purification of metalloproteins, multi complex proteins (with several subunits) and membrane proteins independently of the expression host. The StrepTactin®XT column was equilibrated with at least 2 column volumes (CV) of washing buffer (100 m*M* Tris-hydrochloride (Tris-HCl) pH at 8, 1 *M* sodium chloride (NaCl), 1 m*M* EDTA, 1 m*M* Benzamidin and 10 m*M* DTT) and the filtered supernatant was loaded overnight. After extensively washing using ~12 CV of washing buffer, the bound protein was eluted with 50 m*M* biotin at 1 mL/min. After elution the column was regenerated with 10 m*M* NaOH freshly prepared (Figure II.3-B). The eluted fractions were pooled and concentrated in the presence of 10% glycerol using an Amicon 50 kDa in successive centrifugations at 3500 rpm, 5 min up to ~2 mL (centrifuge 5810R; eppendorf). Before loading the pool onto a size exclusion column, the sample was filtered through a 0.2 µm filter (Millipore).

Size exclusion chromatography (SEC) was performed on an Äkta Pure System (GE Healthcare) using a 120 mL HiLoad 16/600 Superdex™ 200 pg column (GE Healthcare) at RT. The matrix of this column is composed of dextran and cross-linked agarose, which combined allow a high resolution separation of proteins by size ranging from 10 to 600 kDa. The HiLoad 16/600 Superdex™ column was equilibrated with 2 CV of SEC buffer (50 m*M* TEA-HCl pH at 7.6, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* Benzamidin, 10 m*M* DTT and 10% Glycerol) and the concentrated and filtered pool from the affinity chromatography was injected using a 2 mL loop. The eluted fractions were pooled and concentrated by successive centrifugations at 3500 rpm, 5 min, using 50 kDa Amicon filter units. Aliquots of the final product were prepared, snap frozen in liquid nitrogen and stored at -80ºC until further use.

II.5 Standard analytics for protein characterization

II.5.1 Protein Quantification

Protein's concentration was assessed by Bradford assay. A calibration curve with bovine serum albumin (BSA; Thermo Scientific™) was prepared by diluting the protein (0 to 1 mg/mL) in DPBS. On a 96-wells plate (Nunc™MicroWell™96-Well microplate; Ref: 243656; Thermo Scientific) 5 µL of each sample was loaded; the BSA dilutions were applied in triplicate and the protein samples in duplicate. After pipetting the samples, 150 µL of Bradford Reagent (Pierce™; Thermo Scientific™) was added, and the plate incubated for 30 min at RT, protected from the light. This assay relies on the binding of Coomassie to the basic amino acids from the proteins,

which results in a shift of the absorption maximum of the dye (from 465 to 595 nm) and results in a change in color from brown to blue (Bradford, 1976). The absorbance was measured at 595 nm (TECAN Infinite 200Pro). A linear regression between the absorbance and the BSA protein concentration was traced, which allowed to estimate the total protein concentration in the samples tested.

II.5.2 SDS-PAGE and western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using Nu-PAGE 10% Bis-Tris protein gels (Invitrogen™). Before the run, all the samples were boiled in a dry bath, 5 min at 99ºC with Laeemli buffer 1x (Invitrogen™). Precision Plus Protein™ All Blue Standards and Unstained were used to visualize the molecular weights range. The gel was run at 150 volts (V) for 2 h in 1x MOPS buffer (Invitrogen™). The gels were stained using Instant Blue (Expedeon) following the manufacturers' protocol. To remove the staining background an additional washing step was introduced leaving the gel in bidistilled water for at least 2 h.

The western blot technique was carried out using the same gels and protocol as described before. After running the gel, the proteins were transferred using the iBlot 2 transfer stacks PVDF membrane system (Invitrogen™) and the iBlot™ 2 Gel Transfer device (Invitrogen™), at 20 V for 7 min following the manufacturers' protocol.

Once transferred, the membrane was blocked for 1 h in 5% (w/v) non-fat milk in 1x tris-buffered saline with 0.1% Tween (TBS-T 0.1%). At the same time, the gel was stained with Instant Blue (Expedeon) to check for transfer efficiency. Afterwards, the membrane was incubated with the primary antibody (1:1000 in 5% (w/v) non-fat milk in TBS-T 0.1%) on a roller at 4ºC. To stain human β_2 subunit, the anti-GC β_2 antibody from Invitrogen™ (AB_2553570) was used, while to stain human α_2 subunit, the anti-GC α_2 antibody from Abcam (ab42108) was utilized. To stain the strep-tag attached at the C-terminal of the α_2 subunit, anti-streptavidin (S6390; Sigma-Aldrich) and anti-strep-tag HPR conjugated (2-1509-001; IBA) antibodies were used accordingly to the recommended antibodies protocols. To stain the β_1 subunit the anti-β_1 antibody from SIGMA (G4405) was used accordingly to the *in house* developed protocol. After overnight incubation, the membrane was washed three times for 10 min, with agitation in 1x TBS-T 0.1% and incubated with the secondary antibody anti-rabbit IgG (a-1969; SIGMA Aldrich), for one hour with gentle agitation at RT. This antibody was prepared in 5% (w/v) non-fat milk in TBS-T 0.1% using a 1:500 dilution. Next, the blot was washed twice in 1x TBS-T 0.1% and the membrane incubated for 15 min with gentle agitation in 1x TBST 0.1% with Precision Strep-Tactin-HPR conjugate (BIO-RAD) at 1:5000. To remove the Strep-Tactin-HPR conjugate, the membrane was washed three times for 15 min with agitation in 1x TBST 0.1% and 2 h with 1x TBS-T 0.1% before developing to remove strep-tactin background.

The membrane was developed using Western Lighting ® Western Blot Chemiluminescence (PerkinElmer) reagent prepared at 1:1 ratio of Enhanced Luminol Reagent and the Oxidizing reagent. The images were obtained with ChemiDoc MP (Bio-Rad). The exposure time was adapted as required in signal accumulation mode. Additionally, quantitative analysis of the western blot bands was performed by Image J software.

II.5.3 Guanylyl cyclase activity assay

Guanylyl cyclase activity was determined via high-performance liquid chromatography (HPLC) method by measuring the amount of cGMP formed from GTP in reactions containing 50 m*M* TEA-HCl at pH 7.6, 10 m*M* DTT, 3 m*M* $MgCl_2$ or 3 m*M* $MnCl_2$ and 1 m*M* GTP. Briefly, enzymatic reactions were prepared in a final volume of 100 µl and initiated by the addition of 10% (v/v) of the soluble fraction of Sf9-infected cells or purified protein. Reactions were incubated for 10 min at 37ºC in a dry bath (Thermomixer of Eppendorf) and stopped by the addition of 0.5 *M* of cold sodium acetate ($C_2H_3NaO_2$) (1:1). After centrifugation at 13000 xg, for 2 min at RT, the supernatant was collected and transferred to HPLC tubes (Waters 12x32mm).

Fifty microliters of each reaction were loaded (at 0.8 mL/min) in a Nova-Pak C18 4µm, 3.9 x 300mn chromatographic column (Waters) connected to a HPLC Waters 2695 Separation Module-Alliance, equipped with Photodiode Array Detector, Waters 2996. The substrate (GTP) was separated from the product cGMP in 100 m*M* phosphate buffer, pH 4.3 containing methanol (90:10) and cGMP formation monitored at 254 nm. A calibration curve was prepared by using known concentrations of cGMP (0 ,0.2 ,0.4 ,1 ,2 ,3 ,6 ,9 ,12 ,16 and 20 nmol) in assays containing the same reaction mixture. After peak integration, peak areas were converted in nmol cGMP formed per min *per* mg of protein (nmol cGMP min-1 mg-1).

When indicated, guanylyl cyclase activity was assayed in the presence of 100 µ*M* NO (stimulator) or 1-5 µ*M* BAY 60-2770 (activators). In these cases, reactions in the presence of the vehicles NaOH and DMSO were also performed.

Table II.7- List of the sGC modulators tested, the molecular weight and type of modulation.

sGC Modulators	Molecular Weight (g/mol)	Type of modulator
NO	30.01	Stimulator
BAY 60- 2770	623.645	Activator

II.5.4 UV-Visible spectrometry

The spectra were traced using the NanoDrop™2000C Spectrophotometer (Thermo Scientific™).

II.5.5 Mass Spectrometry

The mass spectrometry analysis was performed by the analytic services of iBET (ASU) using TripleTOF 6600 mass spectrometer.

For protein identification, the generated mass spectra results were processed using Protein Pilot Software v. 5.0 (Sciex) and the search for the protein identity was performed against the SwissProt (reviewed) protein sequence database for human, supplemented with the specific human β_2 sequence (construct sequence). Peptide identification was considered with >95% confidence.

Chapter III- Results and Discussion

The first part of this work focused on the generation of the baculovirus stocks and assessment of the best expression condition for production of human β_2 subunit in Sf9 insect cells via the baculovirus expression system. The second part aimed at large scale production and purification of human β_2 subunit. Simultaneously, functional characterization of human β_2 subunit was conducted on Sf9 cell extracts expressing the enzyme and on the purified protein.

III.1 Expression of sGC subunits from baculovirus stocks

The expression of the desired recombinant subunits was analyzed by western blot as described in section II.5.2 in Sf9 cell extracts after infection with the P1-hβ_2-strep, P1-hβ_2 and P1-hα_2-strep. The results presented in Figure III.1 showed that human β_2 and β_2-strep are expressed in Sf9 cells; two bands of different molecular weights (MW) were detected using the specific anti-β_2 antibody: one at ~71 kDa and another at ~140 kDa. The predicted MW of recombinant human β_2 subunit is ~71 kDa and hence, the identified bands suggest the presence of both the monomeric and dimeric forms of the protein. Sf9 non-infected cells (empty Sf9) were used as the negative control and analysis of this sample revealed no bands as expected. A sample of purified rat α_1/β_1 sGC heterodimer (available at iBET/Bayer lab) was also tested to assess it as a possible positive control. The heterodimeric protein was stained with anti-β_2 antibody; hence, it was used hereafter as positive control in all the following experiments. This result indicates that the anti-β_2 antibody used was able to detect the rat α_1 and/or β_1 alone or in complex which reinforces the high degree of identity reported between the subunits and species (Behrends and Vehse, 2000). Reports have shown that in particular, human β_2 shares 37% and 41% identity with rat β_1 and α_1, respectively.

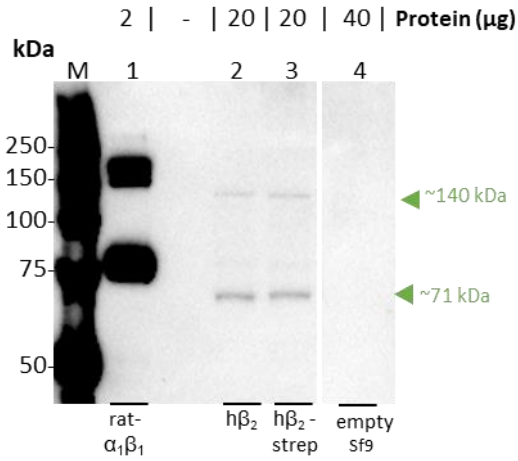

Figure III.1- Expression of human β2 subunit in Sf9 cells infected with P1 baculovirus stock. Soluble fractions of infected Sf9 cells were loaded in SDS-page and western blot membrane was stained with anti-β_2 antibody (exposure time: 120 s). M- molecular weight marker; Lane 1 - purified rat α_1/β_1 (2 µg); Lane 2 and 3 - soluble fraction of Sf9 cells infected with P1-hβ_2 and P1-hβ_2-strep respectively (20 µg); Lane 4 - soluble fraction of uninfected Sf9 cells (40 µg)**.**

The expression of the human α_2 from Sf9 cells infected with P1-hα_2-strep was also analyzed by western blot using the anti-α_2 antibody. However, detection of human α_2 was inconclusive. The anti-α_2 antibody seems to be very unspecific as several bands were also detected in samples containing non-infected cells (data not shown). Taking advantage of the strep tag present at C-terminal of α_2 subunit, other antibodies targeting the strep tag were tested, namely anti-streptavidin and anti-strep-tag HPR conjugated. The results presented in Figure III.2-A show that the anti-streptavidin antibody does not stain the strep-tag present in the C-terminal of α_1 subunit of the rat α_1/β_1 protein (used herein as positive control). In addition, this antibody detected bands at ~70-100 kDa in cell extracts expressing human α_2-strep subunit and in Sf9 empty cell extracts, showing a high degree of unspecificity under the conditions tested. Unlike the anti-streptavidin antibody, the anti-strep-tag antibody stained the strep tag present in the rat α_1 subunit (the positive control) however, it also showed some unspecificity, as β_1, which lacks the strep tag, was also marked (~70kDa band) (Figure III.2-B). Although the positive control was detected, the strep tag present in the C terminal of hα_2-strep was not stained. Based on these results, the expression of human α_2 was not possible to detect by western blot using the tested antibodies. This data lead to propose two hypotheses: either the human α_2 subunit has a very low expression level in Sf9 cells or it is very unstable. Previous data had reported that the rat α_1 subunit was shown to be very unstable and prone to degradation when expressed in Sf9 insect cells in the absence of its counterpart the β_1 subunit (Zabel, Häusler, *et al.*, 1999). As such, and due to the homology between these two proteins we could account/estimate that the inability to detect hα_2-strep in Sf9 cell extracts might not be due to the lack of expression of α_2 but instead due to a high instability of this subunit.

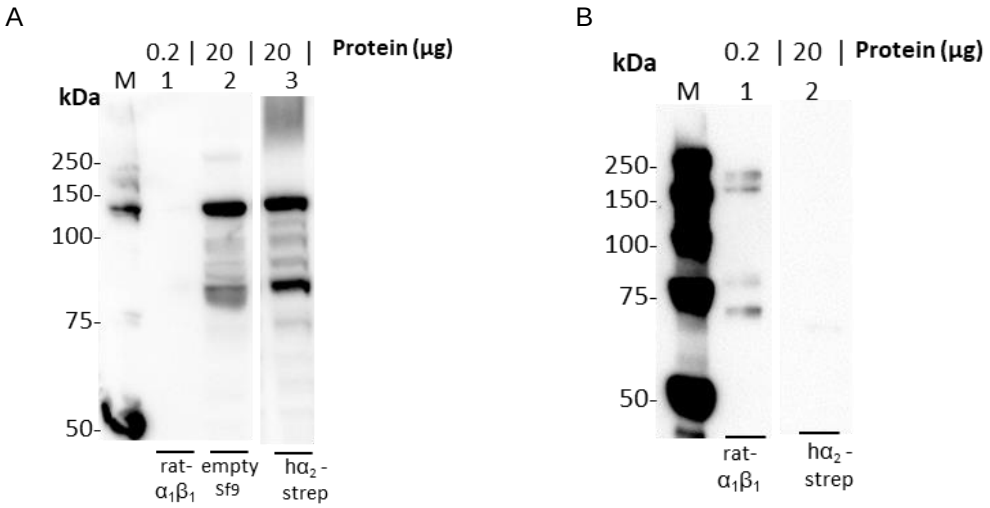

Figure III.2- Expression of human α_2 subunit in Sf9 cells infected with P1 baculovirus stock. Soluble fractions of infected-Sf9 cells were loaded in SDS-page and western blot membrane was stained with: anti-streptavidin antibody (exposition time: 12 s) in **A:** M- molecular weight marker; Lane 1 - purified rat α_1-strep/β_1 (0.2 µg); Lane 2 - soluble fraction of uninfected Sf9 cell (20 µg)**;** Lane 3 - soluble fraction of Sf9 cells infected with P1-hα_2-strep (20 µg) and in **B** with anti-Strep-tag antibody (exposition time 160 s): M- molecular weight marker; Lane 1 - purified rat α_1-strep/β_1 (0.2 µg); Lane 2 - soluble fraction of Sf9 cells infected with P1-hα_2-strep (20 µg).

III.2 Quantification of Baculovirus stocks

The P2 baculovirus stocks expressing the human β_2 subunit (P2-hβ_2-strep and P2-hβ_2) and the human α_2 (P2-hα_2-strep) were titrated by the CGA and MTT methods (described in section II.2.4), but the titer obtained by the two methods revealed not to be as robust as expected giving the variation of ~1 log (Table III.1). Nevertheless, the values obtained in two independent experiments for each method did not vary significatively, showing accuracy in the technical point of view. The MTT has been described as a method that presents low error, less dependency on the operator, and relies on the use of 24 replicate wells for each virus dilution, thus being a highly recommended method to quantify baculovirus (Mena *et al.*, 2003; Roldão *et al.*, 2009). Based on this, the titers used in this thesis were determined by MTT.

Table III.1- P2 baculovirus stocks quantification. The titer of each baculovirus stock was assessed by the CGA and MTT method individually. (*) The roundup value used is the average value between the MTT results.

	CGA method	MTT method	Value used *
	pfu x 10^9/mL	pfu x 10^9/mL	pfu x 10^9/mL
h-β_2-strep	8.7	0.11	0.2
	8.5	0.17	
h-β_2	27	0.95	1
	34	-	
h-α_2-strep	32	0.76	0.8
	45	-	

III.3 Small scale expression tests

III.3.1 Optimization of recombinant human β_2 subunit expression

Small scale expression tests were conducted on Sf9 cells in order to optimize the production of the human β_2-strep protein. Sf9 cells were grown in non-supplemented and FBS-supplemented Sf900 III medium and infected with P2-β_2-strep at different MOIs. The medium was supplemented with 10% FBS since this allows higher culture densities to be reached. Moreover, this supplement is also used in the medium currently used to express other sGC isoforms (*in house protocols*) (Lee, Martin and Murad, 2002). Low MOIs are commonly preferred in expression experiments since they lead to the infection of a fraction of the cell culture, hence, allowing the remaining part to continue to duplicate. Consequently, cell density increases, and the cells that were not initially infected will be so in a secondary infection by the baculovirus generated from the first infection. This approach was shown to be beneficial in the production of complex proteins (Chen *et al.*, 2013).

Details of each expression condition are shown in Table II.2 from methods section. Sf9 non-infected cells were used as negative control. The expression of hβ_2-strep was analyzed after 72 hpi (ideal time of harvest assessed by *in house* protocol for other sGC subunits) by western blot using the anti-β_2 antibody (Figure III.3-A). The results showed that expression of human β_2-strep is ~2 times lower in FBS-supplemented medium. Moreover, increase in the MOI from 0.02 to 0.2 enhanced the expression of the subunit ~2 times in Sf900 III, while no significant differences were observed in the supplemented medium. The guanylyl cyclase activity was measured in the same cell extracts as described in II.5.3 section, in the presence of 3 m*M* of Mn^{2+} since it was reported that manganese is the preferential co-factor of rat β_2. When indicated, 100 µ*M* NO (stimulator of sGC α_1/β_1) and 1 µ*M* BAY 60-2770 (activator of the heme free-sGC α_1/β_1) was also added to the enzymatic reaction. Results showed that the guanylyl cyclase activity of human β_2 is higher than the reported for rat β_2 by Koglin *et al.* (2001) and, in contrast to rat β_2, the human protein does not seem to be activated neither by NO nor by the activator BAY 60-2770 (Figure III.3-B). Moreover, regardless of the expression condition of human β_2 -strep (different media and MOI), the basal activity is very similar between all samples, and does not correlate with the amount of protein identified by western blot.

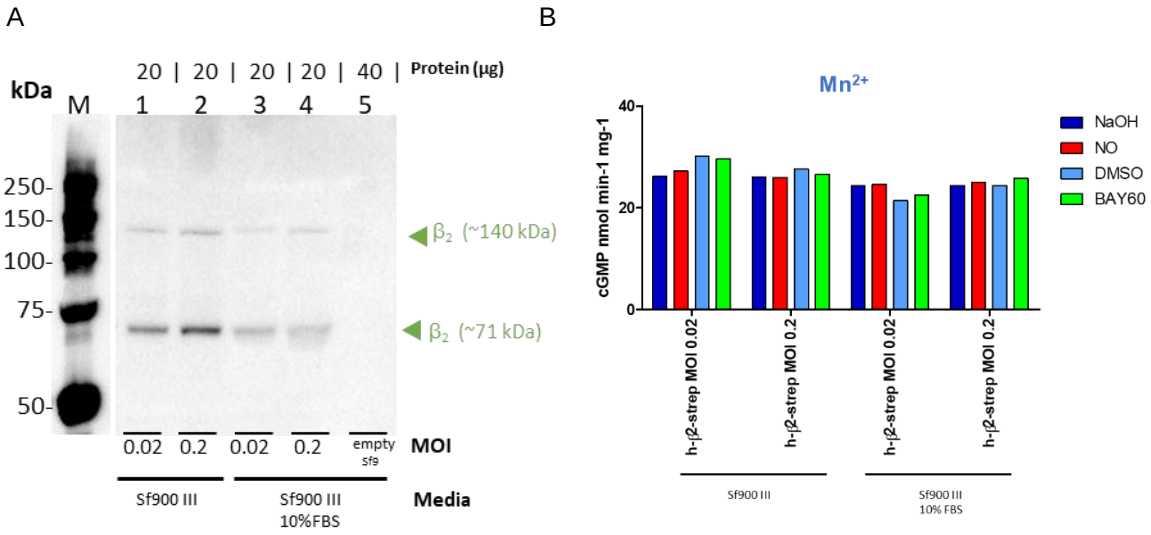

Figure III.3- Expression of human β_2 at MOI 0.02 and 0.2 in Sf900 III medium, and Sf900 III-FBS supplemented medium. A: Western blot stained with anti-β_2 antibody (exposure time: 120 s). M- molecular weight marker; Lane 1-4 - soluble fraction of Sf9 cells expressing human β_2 at MOI 0.02 and 0.2 in two different media Sf900 III and Sf900III supplemented with 10% FBS; Lane 5 - soluble fraction of Sf9 uninfected cells. **B**: Guanylyl cyclase activity of soluble cell extracts of Sf9 infected cells was measured in reactions containing 3 m*M* of Mn^{2+} and in the presence of 100 µ*M* NaOH, 100 µ*M* NO, 0.1% (v/v) DMSO or 1 µ*M* BAY 60-2770. The enzymatic reaction was started with the addition of the soluble fraction of Sf9 cell extracts (10% (v/v) of the total volume) and incubated for 10 min at 37ºC.

To further increase the expression of β_2 in Sf9 cells, a higher MOI was tested. Moreover, a medium supplemented with ALA, the precursor of the heme biosynthesis was also tested even though it is unknown if human β_2 has the ability to bind heme (Behrends and Vehse, 2000). Western blot results presented in Figure III.4-A showed that the expression of the human β_2-strep subunit in Sf9 cells decreased upon the supplementation of the Sf900 III medium, either with FBS alone (as observed previously in Figure III.3) or in combination with ALA. Under this later condition the reduction of β_2 expression was even more pronounced (~3 times lower). On the other hand, the increase of the MOI from 0.2 to 0.4 lead to a ~2 fold increment of human β_2-strep in Sf900 III medium.

Assessment of the guanylyl cyclase activity showed that Sf9 non-infected cells presented the highest basal activity when grown in Sf900 III medium. After supplementing the medium, the activity of non-infected cells decreased. Moreover, the cells expressing human β_2 were not activated neither by NO nor BAY 60-2770. The expression of the enzyme seems to decrease the basal activity of Sf9 cells grown in non-supplemented medium, but this correlation is not observed in cells grown in FBS and FBS/ALA-supplemented media, as no differences were observed in non-infected and infected -Sf9 cell extracts. (Figure III.4-B). Even though cell extracts expressing the human β_2-strep do not seem to respond to any of the tested compounds, independently of the media or the MOI used, a higher protein expression was observed when using Sf900 III non-supplemented medium after infecting Sf9 cells at MOI 0.2 and 0.4. The lack of induction by NO

in cell extracts grown in FBS/ALA-supplemented medium and expressing human β_2, suggests that this subunit might not bind heme, unlike the rat β_2 reported by (Koglin *et al.*, 2001).

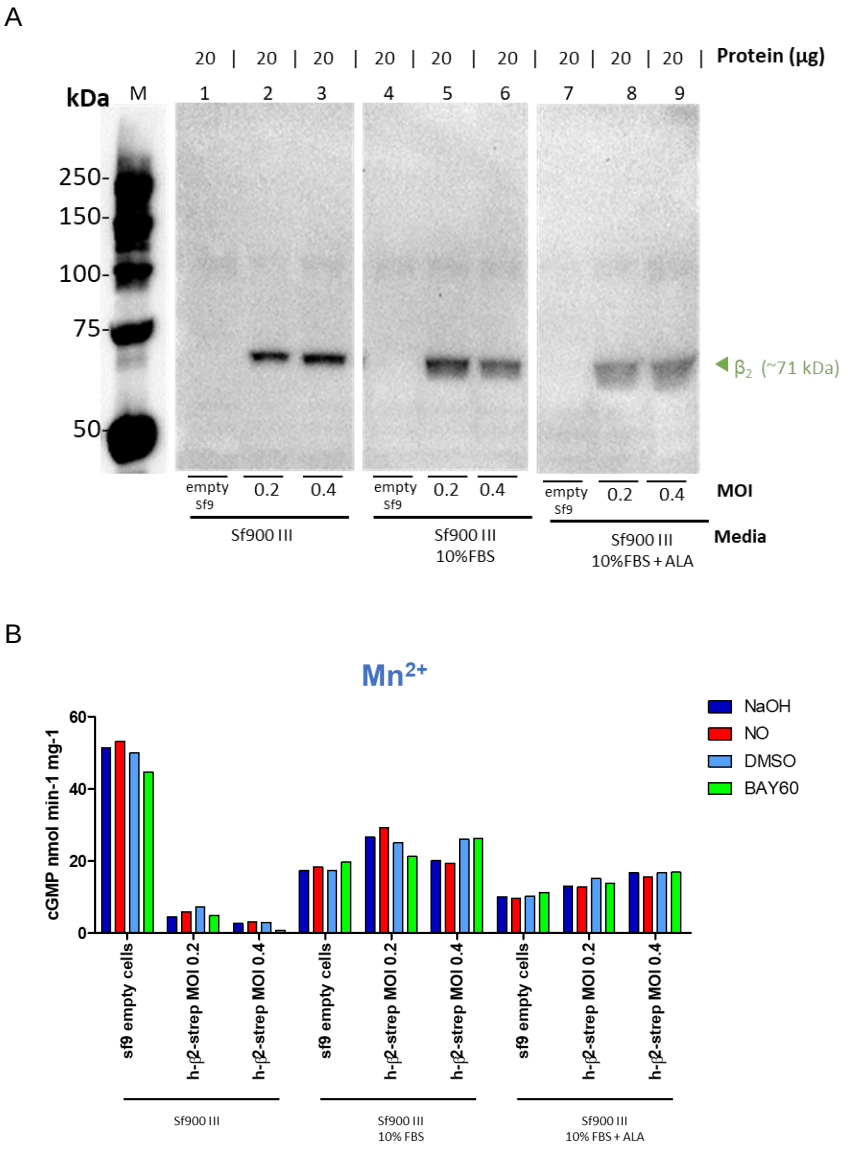

Figure III.4- Expression of human β_2 in Sf9 cells infected at MOI 0.2 and 0.4 and grown in non-supplemented or FBS and FBS/ALA supplemented Sf900 III medium. A: Western blot membrane was stained with anti-β_2 antibody (exposure time: 120 s). M- molecular weight marker; Lanes 2-3, 5-6 and 8-9 - soluble fraction of Sf9 cells infected with baculovirus expressing human β_2 at MOI 0.2 and 0.4 in Sf900 III medium (lane 2-3) and FBS- or FBS/ALA-supplemented medium (Lanes 5-6 and 8-9, respectively); Lanes 1,4 and 7 - soluble fraction of Sf9 uninfected cells in each of the tested media. **B:** Guanylyl cyclase activity performed on the cell extracts infected with P2-hβ_2-strep. Activity was measured in the presence of 3 m*M* of Mn^{2+} under basal (NaOH or DMSO) and stimulated conditions (100 µ*M* NO or 1 µ*M* BAY 60-2770). The enzymatic reactions were started with the addition of the soluble fraction of Sf9 cell extracts (10% (v/v) of the total volume) and incubated for 10 min at 37ºC.

III.3.2 Expression of human α_2 subunit

As stated before, expression of human α_2-strep subunit in Sf9 cell infected with P1-hα_2-strep was not detected by western blot. Previous studies have reported the formation of an α_2/β_1 active heterodimer both in rat and in human tissues but no evidence of an active α_2/β_2 heterodimer has been reported (Harteneck *et al.*, 1991; Koglin *et al.*, 2001). Therefore, small scale co-expression tests of the human α_2 subunit with the β subunits were tested, in order to analyze the possible formation of an α_2/β_1 heterodimer and/or α_2/β_2 heterodimer.

Sf9 cells, grown in supplemented media (FBS or FBS/ALA) were co-infected with P2-hα_2-strep (MOI 0.02) and P2-hβ_1 (MOI 1) (available at iBET/Bayer lab) or P2-hβ_2 (MOI 0.02). The soluble fractions of the Sf9 cell extracts were collected and analyzed by western blot (Figure III.5), and for activity (Figure III.6). Sf9 non-infected cells, grown in both media, were used as negative control for proteins expression. As positive control of the co-expression of human α_2 with β_1 or β_2, Sf9 cells, grown in Sf900 III FBS/ALA supplemented medium, were infected with baculovirus stocks expressing the rat α_1/β_1 subunits (MOI 2:2), and Sf9 cells, grown in Sf900 III FBS supplemented medium were infected with baculovirus stock expressing the rat α_1/β_1* subunits (MOI 0.5:1). β_1* has a mutation which allows the expression of the heme-free form of sGC. Baculovirus stocks of P2-rat α_1, P2-rat β_1 and P2-rat β_1* were available at iBET/Bayer lab. Moreover, the expression of hβ_2-strep (MOI 0.4) was used as positive control for human β_2 expression.

The expression of hα_2-strep subunit was evaluated by western blot using the anti-α_2 antibody, however the protein was still not detected whether co-expressed with hβ_1 or hβ_2. Even though, an unspecific ~50 kDa band was detected in all tested cell extracts, including in the non-infected cells (Figure III.5-A). To confirm the expression of the β subunits in co-transfected cells, western blot using anti-β_1 antibody or anti-β_2 antibody (Figure III.5-B and C) was also performed. The expression of β_1 was confirmed by the stained band at ~75 kDa in all tested conditions (Figure III.5-B). The expression of human β_2 was only possible in Sf9 cells grown in FBS supplemented medium (Figure III.5-C). This might be due to the very low MOI utilized and also due to the fact that expression of the subunit is strongly reduced in Sf9 cells grown in FBS/ALA Sf900 III-supplemented medium, as shown previously (Figure III.4).

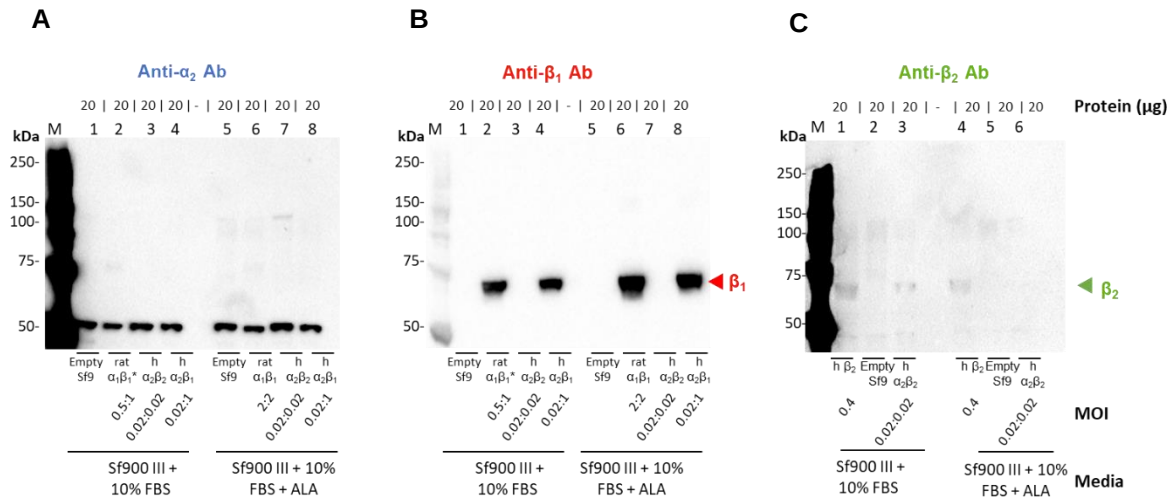

Figure III.5- Western blot results of the co-expression tests of human α_2 with rat β_1 and human β_2 in two different media. A/B: Western blot membranes stained with anti-α_2 and anti-β_1 antibodies, respectively (exposure time: 250 s in **A** and 1 s in **B**). M- molecular weight marker; Lanes 1,5 - soluble fraction of Sf9 uninfected cells in both media; Lane 2 - soluble fraction of Sf9 cells co-expressing rat heme-free-sGC (α_1/β_1*) and grown in Sf900 III supplemented with 10% FBS medium; Lanes 3,7 - soluble fraction of Sf9 cells co-expressing human $\alpha_2\beta_2$ (MOI 0.02:0.02) in both media; Lanes 4,8 - soluble fraction of Sf9 cells co-expressing human α_2/β_1 subunits (MOI 0.02:1) in both media; Lane 6 - soluble fraction of Sf9 cells co-expressing rat α_1/β_1 subunits in Sf900 III supplemented with 10% FBS and ALA. **C:** Western blot membrane stained with anti-β_2 antibody (exposition time: 250 s). M- molecular weight marker; Lanes 1,4 - soluble fraction of Sf9 cells expressing human β_2 subunit (MOI 0.4) in both tested media; Lanes 2,5 - soluble fraction of Sf9 uninfected cells in both media respectively; Lanes 3,6 - soluble fraction of Sf9 cells co-expressing human α_2/β_2 subunits (MOI 0.02:0.02) in both media.

Activity of soluble extracts resultant from the co-transfection experiments were analyzed in the presence of Mn^{2+} and Mg^{2+} (Figure III.6) as described in section II.5.3. Sf9 cells showed higher guanylyl cyclase activity in the presence of Mn^{2+} when compared to Mg^{2+}, whether cells were grown in the presence of FBS or FBS/ ALA supplemented medium. Co-expression of α_2 and β_1 in Sf9 cells, grown in both media, lead to an increase of the guanylyl cyclase activity in the presence of NO and BAY 60-2770. In particular, when activity was assayed in the presence of Mn^{2+}, no significant differences were observed in the activation fold, whether using NO or BAY 60-2770, in cells grown in FBS supplemented medium. However, an increase in the NO response was observed when cells were grown in medium supplemented with FBS/ALA. Similar results were obtained when the activity assays was performed with the Mg^{2+} co-factor; cell extracts co-infected with α_2/β_1 were more stimulated by NO and less by BAY 60-2770 when cells were grown in FBS/ALA supplemented medium. These results strongly suggest that the α_2/β_1 complex binds heme and is able to function as the α_1/β_1 heterodimer. However, the activation fold of the α_2/β_1 protein seems to be lower than that for the rat α_1/β_1 heterodimer specially in the presence of Mg^{2+}. This effect was also previously reported by Harteneck *et al.*, (1991) and in (1995). Moreover, the α_2/β_1 heterodimer also showed to respond to BAY 60-2770, independently of the growth media tested, which suggests the presence of a heme-free form in Sf9 cells expressing α_2/β_1. Therefore, the co-expression of α_2-strep/β_1 indeed seemed to result in an active heterodimer that responds to NO, even though the expression of the α_2 was not possible to detect by western blot (Figure

III.5-A). Nevertheless, the activity results obtained clearly show that the α_2 subunit is being expressed in Sf9 cells together with β_1 (Russwurm *et al.*, 1998; Koglin and Behrends, 2000; Haase *et al.*, 2010).

The human α_2/β_2 heterodimer, expressed in Sf9 cell (infected with P2-hα_2-strep and P2-hβ_2) and grown in FBS- and FBS/ALA-supplemented medium, has similar basal activity when compared to Sf9 empty cells, and does not seem to respond to NO nor BAY 60-2770 compound. The expression of α_2/β_2 in Sf900 III FBS and ALA supplemented medium seems to lead to a slight increase in the basal activity in the presence of Mn^{2+} when compared to Sf9 empty cells. No further differences were observed in the activity measured in the presence of Mg^{2+}.

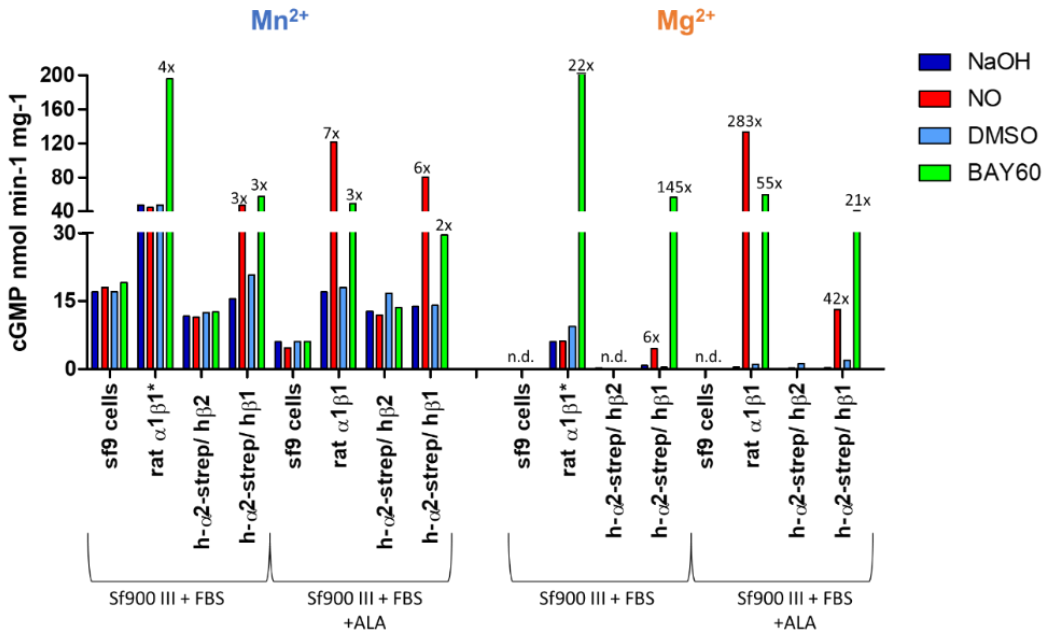

Figure III.6- Guanylyl cyclase activity on Sf9 cells co-expressing α_2/β_1 or α_2/β_2. Activity was measured in the presence of 3 m*M* of Mn^{2+} or Mg^{2+} under basal (NaOH and DMSO) and stimulated conditions (100 µ*M* NO and 1 µ*M* BAY 60-2770). The enzymatic reactions were started with the addition of the soluble fraction of Sf9 cell extracts (10% (v/v) of the total volume) and incubated for 10 min at 37ºC. n.d: non detected.

As mentioned above, hβ_2 expression was achieved in the absence of a second subunit when Sf9 cells were grown in Sf900 III non-supplemented medium and at MOI 0.4. Therefore, co-expression of the α_2-strep and β_2 might also be higher in those conditions (higher MOI and non-supplemented medium). In order to confirm this hypothesis, small scale co-expression tests were performed in Sf9 cells grown in Sf900 III medium and infected with P2-hα_2-strep and P2-hβ_2 at MOI 0.2:0.2. Under this condition, in addition to the 50 kDa unspecific band, two bands at ~71 and ~80 kDa were also stained by the anti-α_2 antibody, suggesting that the later band corresponds to the α_2 subunit (Figure III.7-A). However, the guanylyl cyclase activity measured in those cell extracts was lower than in Sf9 empty cells (showed in Figure III.6) when assessed in the presence of Mg^{2+} or Mn^{2+} and did not respond to treatment with NO or BAY 60-2770 (Figure III.7-B). Therefore, although α_2 seems to be expressed together with β_2, the activity results might indicate

that α_2/β_2 does not form a functional heterodimer which is in accordance to previously reported results (Koglin *et al.*, 2001). Another possible explanation for this outcome could be that GTP is not the preferential substrate for this complex (Mittal *et al.*, 1979), and therefore the registered catalytic activity is very low, and for this probably the HPLC is not the most suitable method to measure these values.

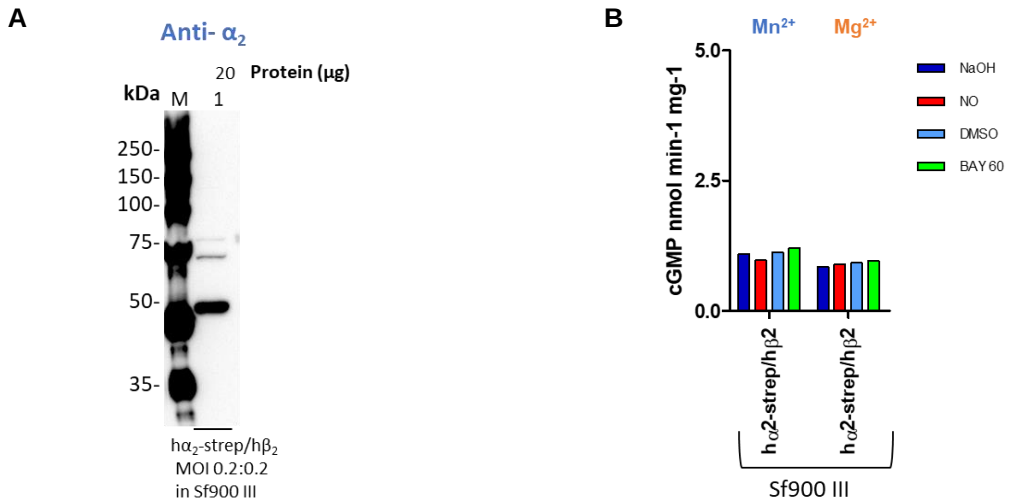

Figure III.7- Results of small scale co-expression tests of human α_2-strep with β_2 at MOI 0.2:0.2 in Sf900 III medium. A: Western blot results stained against anti-α_2 antibody (exposure time:160 s). M- molecular weight marker; Lane 1 - soluble fraction of Sf9 cell extracts co-expressing hα_2-strep/β_2 (MOI 0.2:0.2) (20µg) **B:** Guanylyl cyclase activity was measured in the presence of 3 m*M* of Mn^{2+} or Mg^{+2} under basal (NaOH and DMSO) and stimulated conditions (100 µ*M* NO and 1 µ*M* BAY 60-2770). The enzymatic reaction was started with the addition of the soluble fraction of Sf9 cell extracts (10% (v/v) of the total volume) and incubated for 10 min at 37ºC.

III.4- Large scale production and purification of human β_2-strep

III.4.1 First purification process: lysis by sonication

The best expression condition of human β_2 protein, determined in section III.2.1, was used to produce the protein in a large scale batch of 3.4 L. Sf9 cell culture, grown in Sf900 III medium, was transiently infected with P2-hβ_2-strep baculovirus stock at MOI 0.4. Purification of human β_2 protein was performed as described in methods section II.4. All purification steps were simultaneously analyzed by SDS-page, western blot using the anti-β_2 antibody, and basal activity was measured in the presence of 3 m*M* Mg^{2+} or Mn^{2+}.

After harvesting, cells were lysed through sonication and the soluble fraction loaded into the affinity Strep-tactin column. The human β_2-strep was eluted in a single peak (Figure III.8-A) and SDS-page showed the presence of several contaminants with different MW, not allowing an accurate identification of human β_2-strep (Figure III.8-B). Nevertheless, fractions A8 to E3 were

pooled into Pool D (Figure III.8-A) and human β_2-strep subunit was identified by western blot in that pool (Figure III.8-C). Western blot results also showed a high amount of hβ_2-strep in the insoluble fraction (pellet; lane 2) and no protein in the soluble fraction (loading; lane 1). This indicates that most of protein was retained in the membrane fractions of the Sf9 cells and that only a very small part of the expressed protein was loaded in the affinity column. This outcome impaired the following purification steps, and the overall yield of the purification process. Analysis of Pool D (Figure III.8-C; lane 5 and 6) indicated the presence of hβ_2-strep with three different molecular weights. The expected monomeric form at ~71 kDa, a second band at ~75 kDa, which might indicate the occurrence of post-translational modifications of hβ_2 protein, leading to the formation of a heavy form, and a third band at ~140 kDa, also previously identified in cell extracts (Figure III.3), suggesting a possible dimeric form of hβ_2-strep.

Guanylyl cyclase activity was measured in the same samples analyzed by western blot (Figure III.8-D). Higher basal activity was measured in the presence of Mn^{2+} when compared to Mg^{2+}, which is in agreement with the results reported by Koglin *et al.* (2001) in Sf9 cell extracts expressing the rat β_2 subunit. However, the increase of purity, after the affinity chromatography step, seems to lead to an increment in the catalytic activity in the presence of Mg^{2+}, as Pool D conc presents similar activity in the presence of both co-factors tested. Moreover, the sample presenting the lower activity is the one resultant from the insoluble fraction, which is also the fraction containing most of the expressed protein, revealing an inverse correlation between the expression of the protein and the increase of the basal activity in the presence of Mn^{2+}.

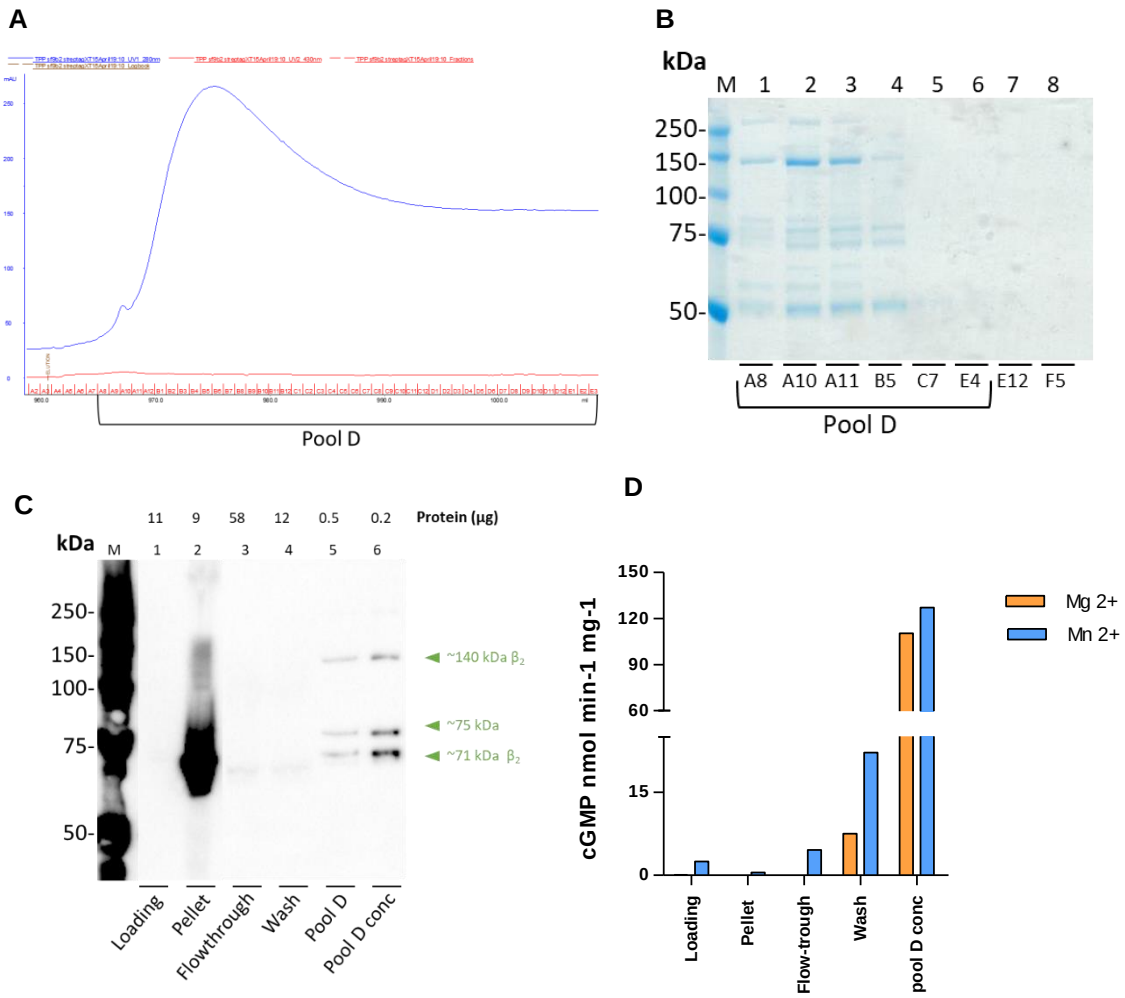

Figure III.8- Results of the first purification process of hβ$_2$-strep: first purification steps and affinity chromatography. A: Chromatogram of the eluted fractions. **B:** SDS-Page stained gel loaded with 15 µL of each fraction; M- molecular weight marker. **C**: Western blot stained with the anti-β2 antibody (exposition time: 27 s): M- molecular weight marker; Lane 1 - soluble fraction after the ultracentrifugation step which was loaded into the affinity chromatography (loading; 11 µg); Lane 2 - insoluble fraction obtained after the ultracentrifugation: small pellet resuspended in 1 mL DPBS (Pellet; 9 µg); Lane 3 - flowthrough (58 µg); Lane 4 - wash of the affinity column (12 µg); Lane 5 - Pool D (0.5 µg); lane 6 - Pool D concentrated (0.2 µg); **D:** Guanylyl cyclase activity in the presence of 3 m*M* Mg^{2+} and 3 m*M* Mn^{2+}. The enzymatic reaction was started with the addition 10% (v/v) of the total volume of each sample and incubated for 10 min at 37ºC.

In the following purification step, size exclusion chromatography was performed in order to separate the contaminant proteins accordingly to their molecular weights. One peak and a broad band were identified in the chromatogram (Figure III.9-A), and two pools were made accordingly, Pool E1 and Pool E2. The elution volume of the Pool E1 corresponds to the void volume of the column and suggests the existence of oligomeric forms of the protein; the broad band of Pool E2 was eluted at ~200 kDa, suggesting a dimeric form for the human hβ$_2$-strep protein. These two pools were further analyzed by SDS-page and western blot. Total protein was quantified by Bradford method and guanylyl cyclase activity was measured in the presence of both co-factors (Figure III.9-B, C and D).

SDS-page of both concentrated pools (Pool E1 conc and E2 conc) indicates that the purity of the samples was very low, since several bands were detected (Figure III.9-B), but western blot results confirmed the presence of hβ$_2$-strep subunit at the different MWs. The two bands at ~71 and ~75 kDa correspond to the monomeric forms of the protein, while the highest MW band at ~140 kDa might indicate the dimeric form of hβ$_2$-strep (Figure III.9-C). Moreover, Pool E2 conc seems to have ~7 fold more hβ$_2$-strep than Pool E1, however, both pools present similar basal activity in the presence of Mn^{2+} and Mg^{2+} (Figure III.9-D). Overall, the yield of the purification process was very low (~3%). The results indicate that the lysis method (sonication) was inefficient, since a high amount of the protein was retained in the insoluble fraction of the Sf9 cells during the first purification step. In order to increase the solubilization of hβ$_2$-strep protein in the soluble fraction and increase the overall purification yield, a new lysis method was tested.

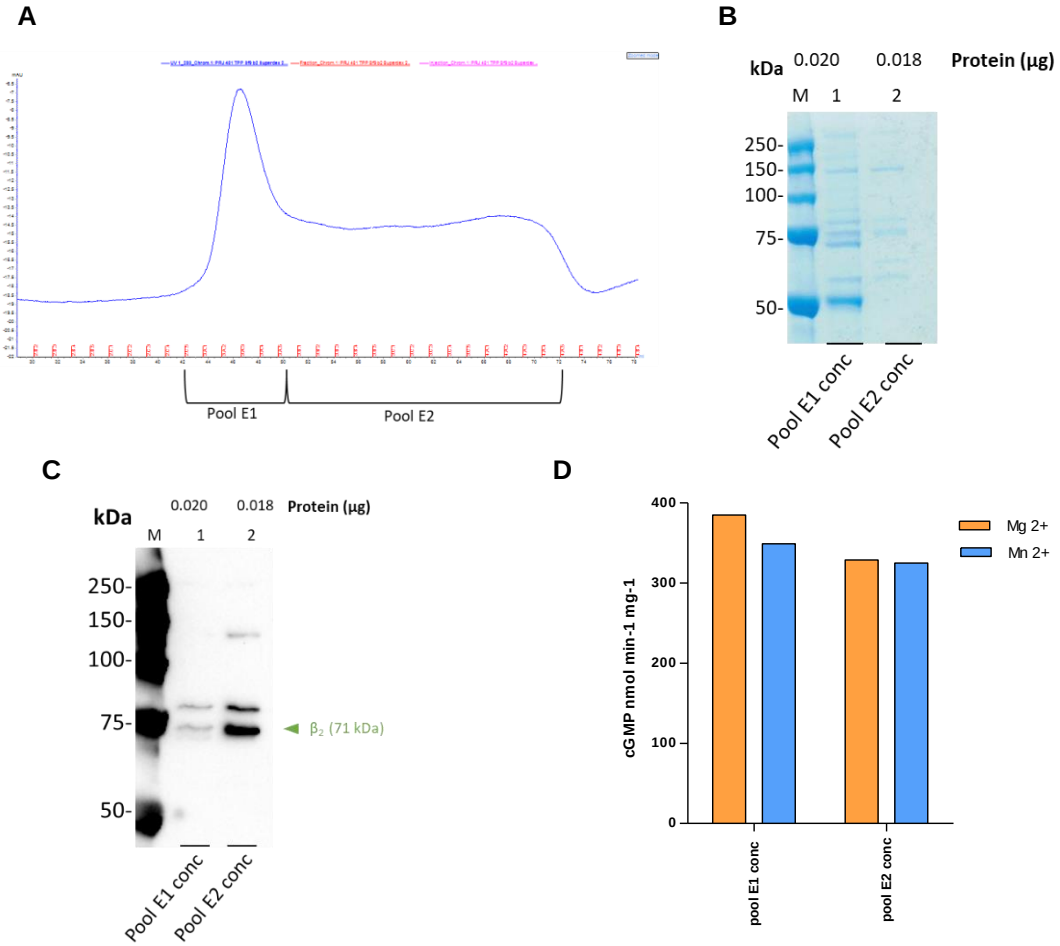

Figure III.9- Results of the first purification process of hβ$_2$-strep: SEC and final characterization A: SEC chromatogram; **B:** SDS-Page stained gel of the concentrated E pools. M- molecular weight marker. **C:** Western blot of the concentrated E pools staining the h-β$_2$-strep protein with the anti-β$_2$ antibody (exposure time: 27 s); M- molecular weight marker. **D:** Guanylyl cyclase activity in the presence of 3 m*M* Mg^{2+} and 3 m*M* Mn^{2+}. The enzymatic reaction was started with the addition of Pool E1 or Pool E2 samples (10% (v/v) of the total volume) and incubated for 10 min at 37ºC.

III.4.2 Second purification process: lysis by homogenization and solubilization by 1% Triton X-100

To overcome the lack of solubility of the recombinant human β_2-strep a new purification process was performed, in which the cells were lysed through a new method: homogenization at 600 bar (AVESTIN C5 homogenizer) and in the presence of a detergent, Triton X-100. Triton X-100 is one of the most used detergents in laboratories, and sGC studies have reported its use to solubilize the protein from native tissues (Theilig *et al.*, 2001; Thoonen *et al.*, 2015). Based on this Sf9 cells, infected with P2-hβ_2-strep baculovirus stock at MOI 0.4 and incubated at 27ºC, were incubated with 1% Triton X-100, at 4ºC for 1 hour with gentle agitation before being mechanically disrupted in a homogenizer. The soluble fraction, obtained by ultracentrifugation, was loaded into the affinity chromatography (as described in the section II.1.4.1 in methods chapter) and all the purification steps were analyzed by SDS-page, western blot and guanylyl cyclase activity. Analysis by western blot (Figure III.10- A) reveled a high amount of human β_2-strep protein in the lysate (lane 1), which confirms the efficient expression of the protein in Sf9 cells. However, after ultracentrifugation, human β_2-strep was detected again in both soluble and insoluble fractions of the cell lysate. Moreover, the amount of protein stained in the soluble fraction is ~11 times less than the one in the lysate. This result indicates that ~90% of the expressed hβ_2-strep subunit was retained in the membrane fraction of Sf9 cells, even after treatment with 1% Triton X-100 and lysis by homogenization. Although a strong band was detected in the insoluble fraction when using the anti-β_2 antibody, a direct comparison cannot be performed as a small fraction of the pellet, was resuspended in PBS, sonicated and then extracted after centrifugation. Guanylyl cyclase activity was evaluated in the same samples (Figure 10-B) and no differences were observed in the activity measurements in the presence of Mg^{2+} or Mn^{2+}. Moreover, the tested samples did not seem to respond to NO nor to the BAY 60-2770 compound. The membrane fraction presented again the lowest basal activity, which does not correlate with the high amount of human β_2-strep stained (Figure III.10-A, lane 3).

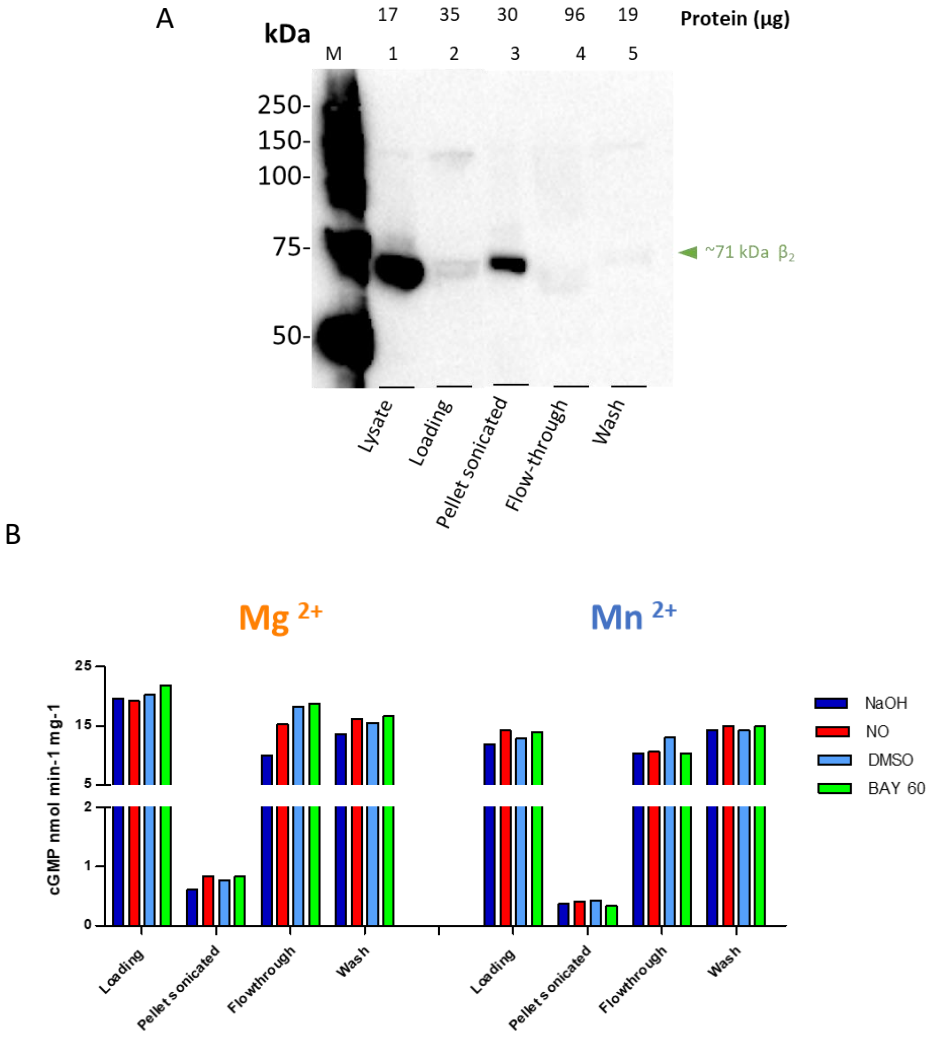

Figure III.10- Results of the second purification process of hβ2-strep: first purification steps. A: Western Blot stained with anti-β_2 antibody (exposure time: 117 s): M- molecular weight marker; Lane 1: lysate obtained after detergent treatment and cell lysis (17 µg); Lane 2: loading- the soluble fraction obtained after ultracentrifugation (35 µg); Lane 3: supernatant of a small sample of the pellet sonicated (30 µg); Lane 4- flow-through (96 µg); Lane 5: wash (19 µg); **B:** Guanylyl cyclase activity measured with the same samples in the presence of 3 m*M* Mg^{2+} and 3 m*M* Mn^{2+} and in the presence of NO (100 µ*M*) and BAY 60-2770 compound (1 µ*M*). The enzymatic reaction was started with the addition of the samples (10% (v/v) of the total volume) and incubated for 10 min at 37ºC.

Although the amount of protein in the soluble fraction was still very low, the sample was loaded into the affinity column and the eluted fractions were analyzed by SDS-Page and western blot (Figure III.10-A and B). The SDS-page results showed an increase of the amount of protein (when compared with the first purification; Figure III. 8-B). The human β_2-strep was eluted in a single peak (Figure III.11-A) and western blot results confirmed the presence of the protein with three different MW (Figure III.11-C): ~71 kDa, ~75 kDa and at ~140 kDa. All the fractions containing the protein were pooled into Pool D and the guanylyl cyclase activity measured showed that hβ_2-strep presents a similar basal activity in the presence of both Mg^{2+} and Mn^{2+} (Figure 11-D). Moreover, Pool D conc did not seem to respond to NO nor to BAY 60-2770 compound.

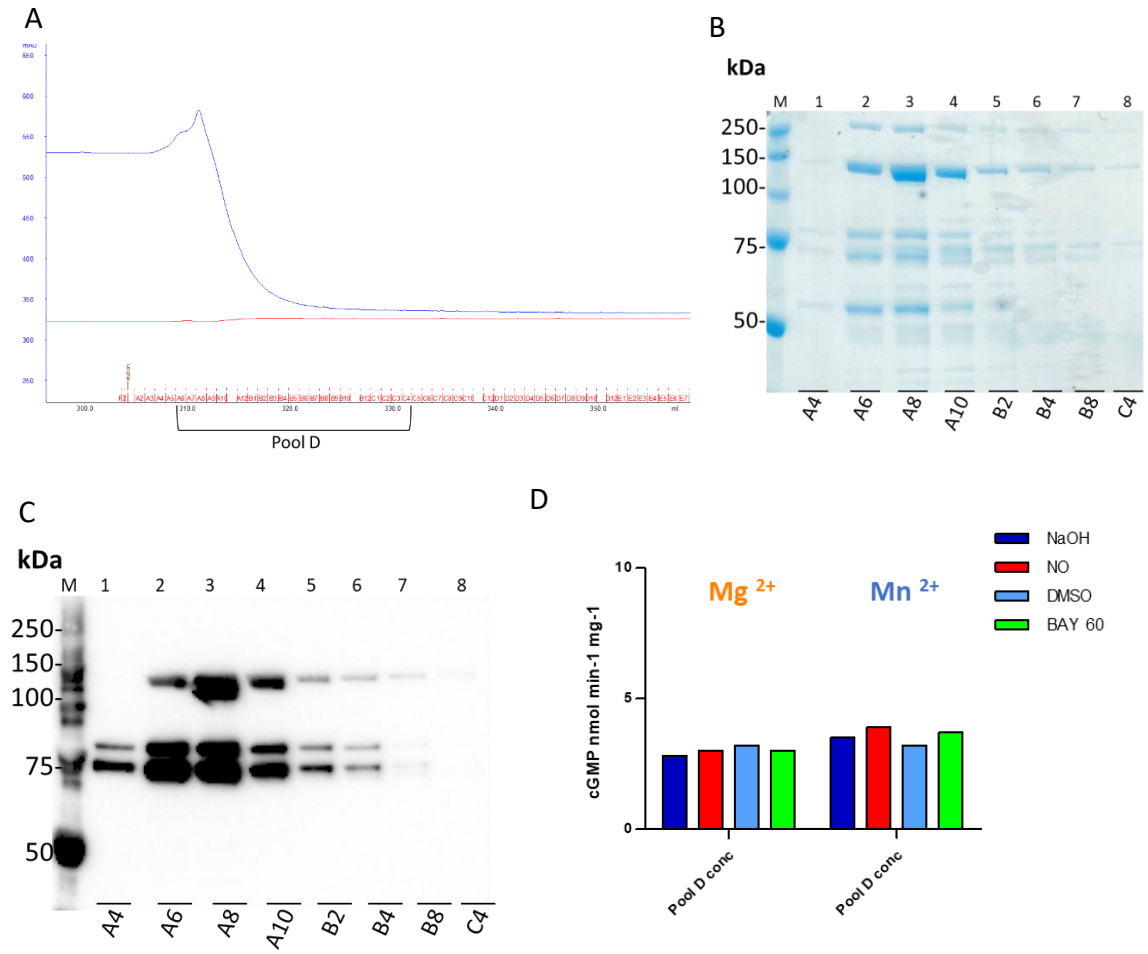

Figure III.11- Results of the second purification process of hβ$_2$-strep: affinity chromatography. A: Chromatogram of the eluted fractions. **B**: SDS-Page gel, stained with all blue, loaded with 15 µL of fractions from the affinity chromatograph. **C:** Western blot stained with anti-β$_2$ antibody (exposure time: 10 s); 15 µL of fractions from the affinity chromatography were loaded in each well. **D:** Guanylyl cyclase activity was measured in pool D concentrated in the in the conditions described above. The enzymatic reaction was started with the addition of the soluble fraction of Sf9 cell extracts (10% (v/v) of the total volume) and incubated for 10 min at 37ºC.

The concentrated pool D was loaded into the SEC column, and the chromatogram revealed two different peaks and a very small broad band (Figure III.12-A). The two first peaks, were eluted at low elution volumes suggesting the presence of high MW oligomeric forms. These results might also suggest an incorrect folding of the protein, however, this is also commonly observed when detergents are used in the extraction of proteins, since they form micelles with the proteins (Lin and Guidotti, 2009; Kubicek *et al.*, 2014). Fractions of all pools were analyzed by SDS-page and western blot (Figure III.12- B and C). SDS-page results stained different MW bands however, only the bands at ~71 and ~75 kDa were stained with the anti-β$_2$ antibody.

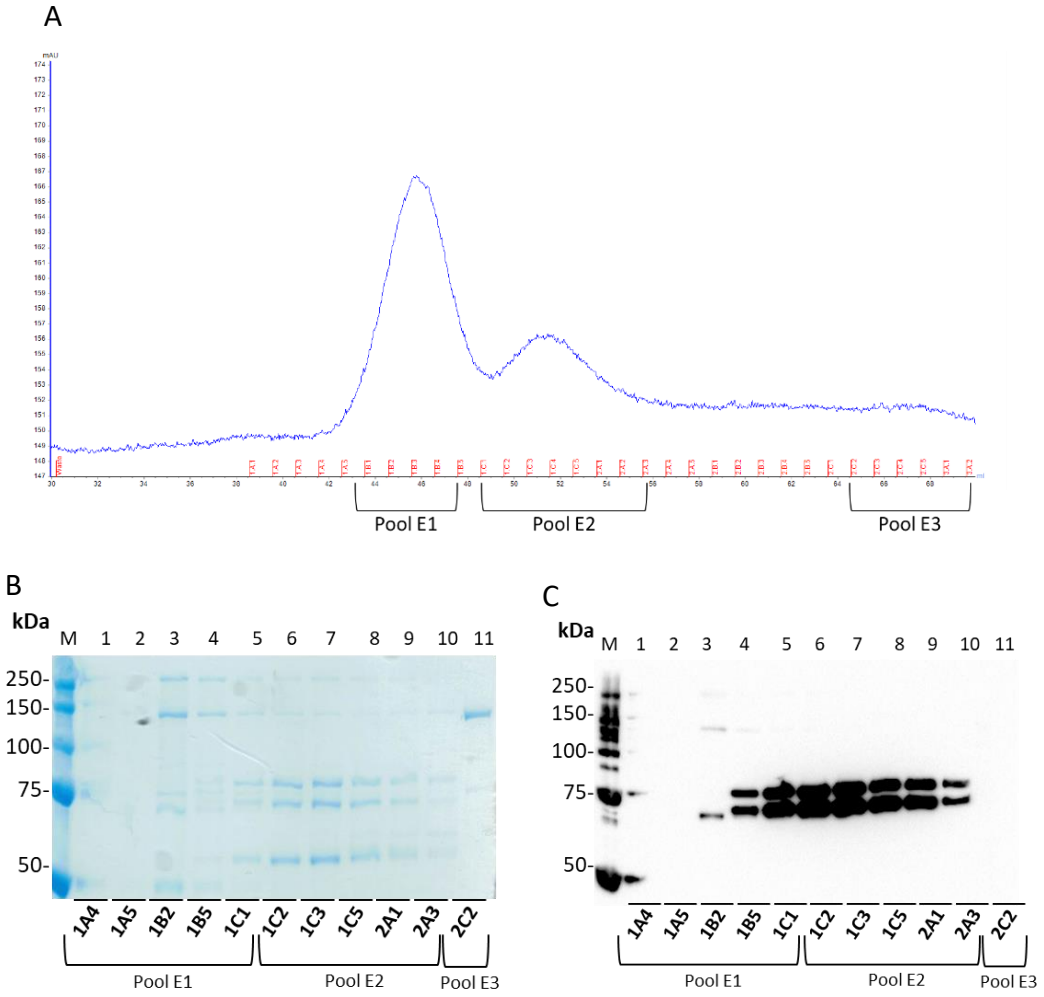

Figure III.12- Results of the second purification process of hβ$_2$-strep: SEC chromatography. A: Chromatogram of the eluted fractions from SEC. **B and C:** SDS-Page gel and western blot stained with anti-β$_2$ antibody (exposure time: 118 s);15 µL of fractions from the SEC chromatography were loaded in each well. M- molecular weight marker.

The selected fractions were pooled, concentrated and further analyzed by SDS-page, western blot, UV-visible and mass spectrometry (Figure III.13). SDS-page analysis of the concentrated pools (Figure III.13-A) showed bands at different MWs (~71-75 kDa and ~140 kDa) in Pool E2 and E3 and no band was detected in Pool E1. Parallel analysis of the same samples by western blot suggest the presence of human β$_2$-strep in all pools at ~71kDa, ~75kDa and ~140kDa (Figure III.13-B). While Pool E2 seems to contain the highest amount of β$_2$ in the monomeric form (~71-75 kDa), Pool E3 contains the highest amount of dimeric β$_2$ form (~140 kDa).

The presence of human β$_2$-strep protein was confirmed by mass spectrometry analysis. The data showed that human β$_2$-strep was only identified in one of the pools; in band 1 and 2 of the pool E2 conc (lane 2). Therefore, the ~71-75 kDa bands stained by the anti-β$_2$ antibody in the western blot (in all pools) are indeed the human β$_2$ -strep protein, however, the stained band at

~140 kDa and ~250 kDa bands might be unspecific. Although the mass spectrometry results suggest that the stained band at ~140 kDa in Pool E3 does not correspond to human β_2-strep, this is highly unlikely, since the protein was frequently stained by western blot in cell extracts infected with P2-hβ_2-strep, and not in Sf9 uninfected cells. A possible explication for these results is that the concentration of human β_2-strep at dimeric state is less abundant than the contaminant protein or it could be bound to it not allowing its correct identification by mass spectrometry.

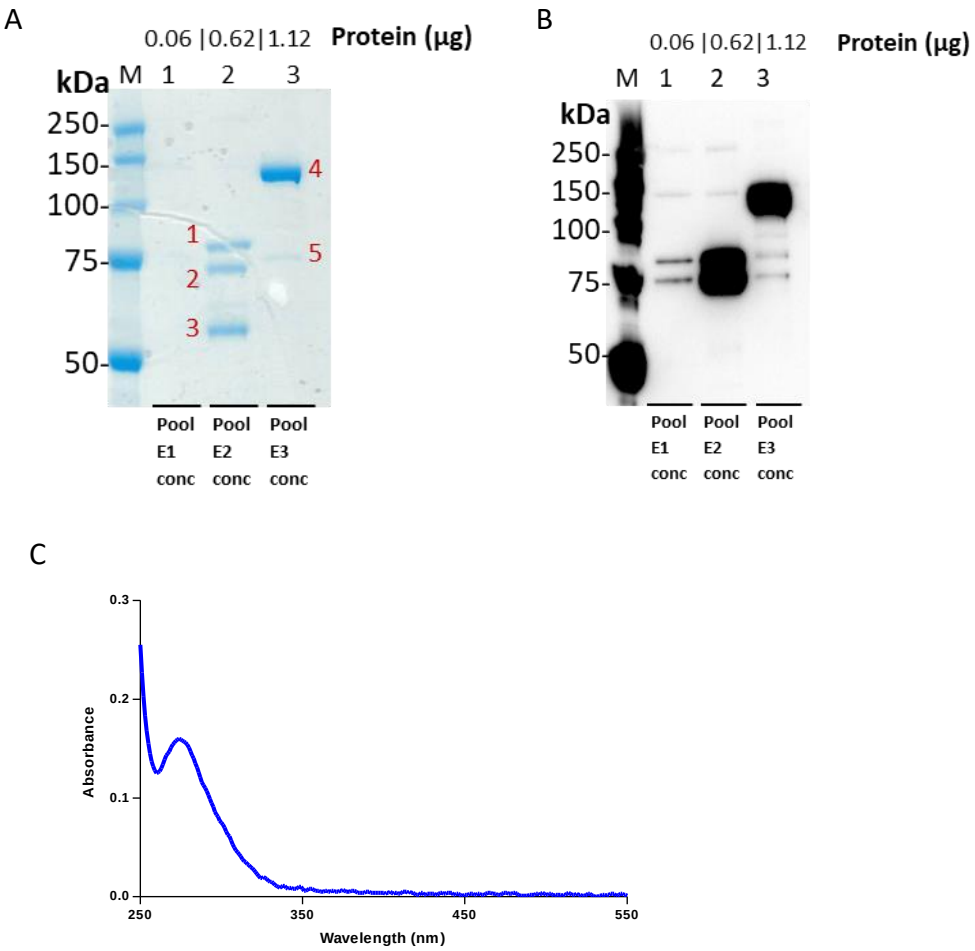

Figure III.13- Results of the second purification process of hβ_2-strep: final characterization. A: SDS-page, stained with all blue, loaded with the concentrated pool E1, E2 and E3; numbers in red mark the bands analyzed by mass spectrometry. M- molecular weight marker. **B:** The same samples were analyzed by western blot stained with anti-β_2 antibody (exposure time: 46 s). **C:** UV-visible spectra of the concentrated samples from the SEC chromatography.

Therefore, in this second purification approach, human β_2-strep enzyme was successfully purified through lysis of the cell by homogenization and 1% Triton-X100, which seemed to lead to an increase of the human β_2-strep protein in the soluble fraction. The overall yield of the purification process was ~10%, which is an improvement compared to the previous process (~3%). The purity of the purified human β_2-strep was higher than in previous purification; in particular, purity of Pool E2 is ~48% as assessed by SDS-Page, and only one contaminant was detected. Moreover, UV-visible spectra of concentrated Pool E2 (Figure 13-C) indicates that the

purified human β_2-strep protein does not contain heme, since no 430 nm peak was detected. Moreover, although the human β_2-strep seems to be able to convert GTP into cGMP, the guanylyl cyclase activity measured in Pool E2 conc showed that the protein presents similar basal activity in the presence of both co-factors and does not seem to respond to NO nor to BAY 60-2770 compound.

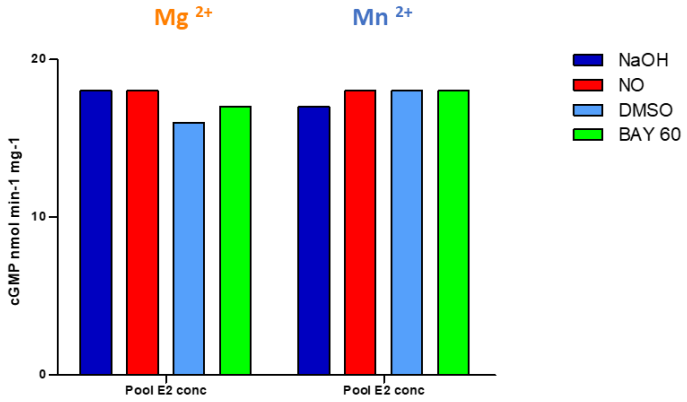

Figure III.14- Screening for potential modulators of purified hβ$_2$-strep protein. Guanylyl cyclase activity was measured in pool E2 conc in the presence of 3 m*M* Mg^{2+} and 3 m*M* Mn^{2+} and in the presence of NO (100 µ*M*) and BAY 60-2770 compound (1 µ*M*). The enzymatic reaction was started with the addition of the purified protein from Pool E2 (10% (v/v) of the total volume) and incubated for 10 min at 37ºC. n.d: non detected.

III.5 Optimization of human β_2 subunit expression

III.5.1 Expression human β_2 subunit at 22ºC

Since human β_2-strep was retained in the membrane fractions of Sf9 cells and did not seem to have induced catalytic activity, additional expression tests were performed, in order to study two other different variables: the increase of the MOI and the decrease of the incubation temperature after infection. We hypothesized that expression at 22ºC would slow down the expression of the protein, leading to the correct folding of the enzyme and increasing the protein solubility (Cain *et al.*, 1999). A higher MOI of 40 was also tested to exponentially increase the protein expression, so that the overall yield of the expression would not decrease too much when cells were grown under low temperature.

To perform these expression tests, a new P2-hβ$_2$-strep baculovirus stock was generated; the viral titer was $0.4x10^9$ pfu/mL, as assessed by MTT assay, which is similar to the previously P2 baculovirus stock generated ($0.2x10^9$ pfu/mL; Table III.1). The expression of human β_2 was analyzed by western blot in Sf9 cell extracts infected with P2-hβ$_2$-strep at MOI 0.4 and 40 and left to grow at 22 or 27ºC (Figure III.15-A). Sf9 non-infected cells were used as negative control.

The results show that, independently of the MOI utilized, incubation at 22ºC lead to ~2 times decrease of $h\beta_2$ expression when compared to the protein expression at 27ºC. On the other hand, the infection of Sf9 cells at the high MOI of 40 lead to a ~2/3 fold increase in $h\beta_2$ expression. Moreover, unlike the other tested conditions (in which only one band was detected at ~71 kDa) at MOI 0.4 and 27 ºC - condition selected to the large scale production, the human β_2 seemed to be expressed also with a size of ~75 kDa.

The assessment of the guanylyl cyclase activity in these cell extracts (Figure 15-B) showed that Sf9 cells present a higher basal activity in the presence of Mn^{2+} than in the presence of Mg^{2+} but no significant differences were observed after Sf9 infection with P2-$h\beta_2$-strep. The major difference was observed when Sf9 cells were infected at MOI 0.4 and cells grown at 27ºC. Under this condition the activity measured in the presence of Mg^{2+} was higher than in the Sf9 cells, while the activity determined in the presence of Mn^{2+} was lower. Interestingly it was also in this condition that two bands at ~71 and ~75 kDa were detected by western blot. As the infection of Sf9 cells at MOI 40 would not be substantiable for a large scale production, infection of Sf9 cells at MOI 0.4 with P2-h β_2-strep baculovirus stock remains the condition of choice for further expression tests.

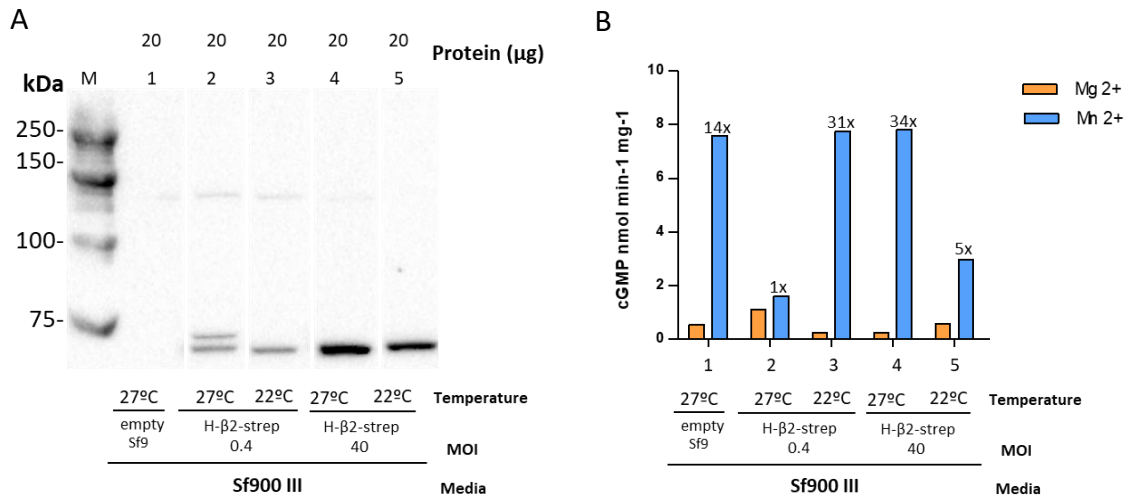

Figure III.15- Expression tests of $h\beta_2$-strep at MOI 0.4 and 40 in Sf9 cells and incubation at 22ºC and 27ºC. A: Western blot stained with the anti-β_2 antibody (exposure time: 150 s): M- molecular weight marker; Lane 1- Soluble fraction of Sf9 uninfected cells; Lane 2 and 3- soluble fraction of Sf9 cells expressing human β_2 subunit at MOI 0.4 at 27ºC and 22ºC respectively; Lane 4 and 5 - soluble fraction of Sf9 cells expressing human β_2 subunit at MOI 40 at 27ºC and 22ºC respectively. **B:** Basal catalytic activity measured in the soluble fraction of Sf9 cells infected with P2-$h\beta_2$-strep. Assay was performed in the presence of 3 m*M* Mg^{2+} and 3 m*M* Mn^{2+}. The enzymatic reaction was started with the addition of the soluble fraction of Sf9 cell extracts (10% (v/v) of the total volume) and incubated for 10 min at 37ºC.

III.5.2 Detergents screening for solubilization of human β_2 subunit

Results from the large scale purification processes showed a high amount of human β_2 protein retained in the membrane fractions of Sf9 cells infected with P2-hβ_2-strep, therefore, further optimization of the lysis method was necessary to the increase the protein solubility in the soluble fractions of Sf9 cells. Although 1% Triton X-100 lead to increase of the protein in the soluble fractions, a high amount of protein was still retained in the insoluble fractions of Sf9 cells. Different detergents are commonly used to increase the protein solubility depending on the specific type of interaction of the protein with the membrane of the cells (intracellular localization, transmembrane domain or covalent binding). Therefore, a thorough screening assay was performed to test several detergents (Table II.6 in methods section) in Sf9 cells infected with P2-hβ_2-strep baculovirus at MOI 0.4 and incubated at 22ºC.

The soluble fractions of Sf9 cells after infection were analyzed by western blot using anti-β_2 antibody (Figure III.16- A and B) and untreated Sf9 cells expressing human β_2 were used as control. Results showed that human β_2-strep was detected at ~71 kDa and at ~140 kDa in all soluble fractions. The results showed that for a group of detergents (Triton, DDM, OG, FC-12, NG, DM, OTG, Cymal-6 and LDAO) an increase of the band stained at 140 kDa (4-14 times) was observed when compared with untreated cell extracts (Figure III.16-C in green). Only a few detergents, namely FC-12, and Cymal-6 seem to lead to a modest increase of the monomeric form of human β_2 (~3 times) (Figure III.16-C in blue). However, total amount of protein did not vary significantly; only a ~3 fold increase was observed after treatment with FC-12 and Cymal-6 (Figure III.16-C in yellow).

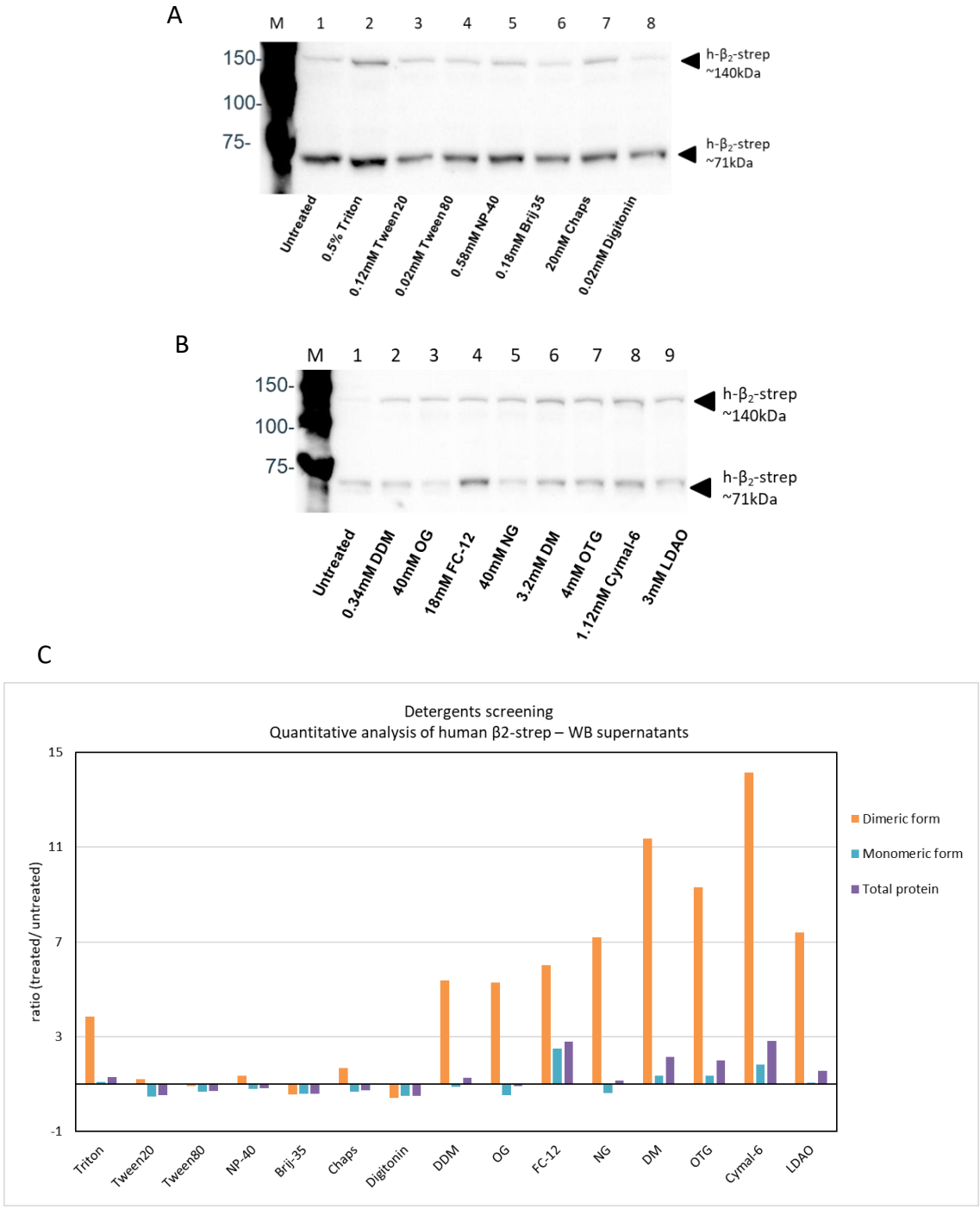

Figure III.16- Western blot results from the detergent screening assay. Western blot was stained with anti- β_2 antibody. M- molecular weight marker. **A:** Sf9 cells expressing human β_2 -strep were treated with Triton, Tween 20, Tween 80, NP-40, Brij-35, Chaps and Digitonin; (exposure time: 42 s). **B:** Sf9 cells expressing human β_2-strep were treated with DDM, OG, FC-12, NG, DM, OTG, Cymal-6 and LDAO; (exposure time: 15 s). **C:** Quantitative analysis of the western blot bands.

Basal guanylyl cyclase activity was measured in the presence of Mg^{2+} and Mn^{2+} (Figure III.17) which revealed that the treatment with the different detergents did not affect the protein activity when compared to untreated cells. Moreover, it was observed that cells expressing human β_2-strep presented a higher basal activity in the presence of Mn^{2+}. The exception was observed

when cell extracts were treated with Triton X-100 and Chaps, which seems to lead to an increase of the basal activity in the presence of Mg^{2+}. Therefore, it seems that there is no correlation between human β_2 -strep expression and activity measured. Although some detergents seem to lead to an increase in the amount of human β_2-strep solubilized protein, it does not translate into a higher basal activity. Based on these results, the use of new detergents tested did not present any advantage over the first one tested, Triton X-100.

As the translations of the mRNA coding the human β_2 were reported to be through an IRES-mediated translational mechanism (Vazquez-Padron *et al.*, 2008), further expression tests will be performed in different expression conditions (e.g. media) in order to determine the optimal condition that could trigger protein translation.

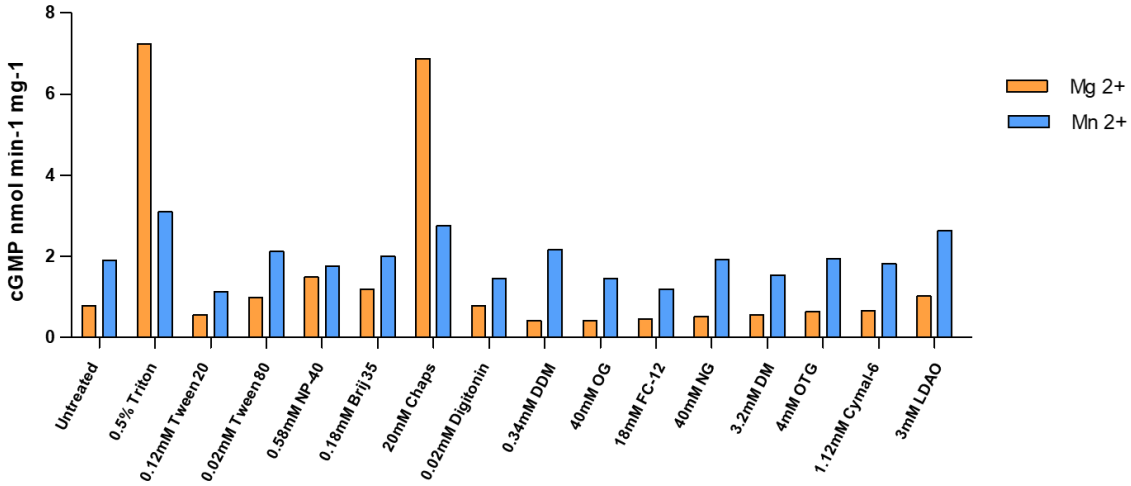

Figure III.17- Detergents screening to increase the human β_2-strep solubilization. Basal guanylyl cyclase was measured in the presence of 3 m*M* Mg^{2+} and 3 m*M* Mn^{2+}. The enzymatic reaction was started with the addition of the soluble fraction of Sf9 cell extracts (10% (v/v) of the total volume) and incubated for 10 min at 37ºC.

Chapter IV- Conclusions and Future Perspectives

While several studies on the rat sGC β_2 subunit have been reported, human β_2 has never been described as a catalytically active enzyme. Even though the sequence alignment indicates some degree of identity between the rat β_2 and β_1 subunits, the gene encoding for the β_2 subunit was firstly described as a pseudogene, and its expression questioned. Aiming at the study and characterization of this neglected subunit, we successfully expressed and purified the human β_2 enzyme for the first time via the baculovirus expression system.

To achieve this, recombinant baculovirus stocks were successfully produced and titrated and several small scale expression tests allowed the identification of the best expression condition for the human β_2 subunit: infection of Sf9 cells grown in Sf900 III medium at MOI 0.4 and incubation at 27ºC. The expressed human β_2 protein was identified in Sf9 cells extracts at ~71 and ~140 kDa by western blot, which suggested the presence of a monomeric form and a dimeric form of the protein.

In the second part of the work, large scale productions were performed using the identified expression conditions and purification was conducted via an affinity and a size exclusion chromatography. In the first purification process, results indicated that a large amount of the protein was retained in the insoluble fraction of the Sf9 cells, and that the achieved purity and the overall yield were very low leading to the need for optimization. A second large scale purification process was performed, in which a new lysis method including the addition of the detergent Triton X-100 was used. Even though a high amount of protein was still lost in the insoluble fraction of the Sf9 cells, the overall yield of the process was ~10%, and a higher purity was achieved in the final pool (~48%), when compared to the previous process. The UV-visible spectrum indicated that the purified human β_2 protein does not contain heme, and, although mass spectrometry confirmed the presence of the human β_2 protein in the monomeric forms (~71 and ~75 kDa), western blot detected the protein not only in the monomeric form, but also in a possible dimeric form (~140 kDa). The existence of the ~75 kDa band might suggest the occurrence of post-translational modifications even if this band was only stained after the purification of the protein and only once in cell extracts. Although the cell extracts expressing the human β_2 protein presented higher basal activity in the presence of Mn^{2+} and even higher than the reported to rat β_2 (Koglin *et al.*, 2001), upon the purification of the enzyme the basal activity became similar in the presence of both Mg^{2+} and Mn^{2+} (~20 nmol/min^{-1}/mg^{-1}). Nevertheless, this result suggests that the enzyme was able to convert GTP into cGMP, but further biochemical studies need to be performed to demonstrate that human β_2 is a catalytically active enzyme without the need to form a complex with a second subunit. Interestingly, upon the increase of the protein purity, we observed the existence of a heavy form of the protein (the ~75kDa band) and the increase of the basal activity in the response to Mg^{2+}. Whether these two events are related, and whether the same is observed with the homologous rat protein has yet to be further investigated. Additionally, screening for potential modulators of the purified human β_2 was performed in the presence of both

divalent cations, however, the protein does not seem to be activated by NO (sGC stimulator) nor by BAY60-2770 (sGC activator).

Even though the purification was successful, a high amount of human β_2 was highly retained in the membrane fractions of the cells, even after treatment with Triton X-100, therefore several steps could be further optimized to increase the yield of the purification process. We started by screening for other solubilizing agents, and the obtained results indicated that some detergents lead to an increase of the human β_2-strep in the monomeric form, while others in the dimeric form. However, none of the detergents tested showed an increase in the total amount of protein that would also translate into a higher basal activity of human β_2-strep. Therefore, new methodologies are currently being evaluated, namely the purification of the protein from the insoluble fraction using re-folding technologies. We also hypothesized that the expression of the protein at lower temperatures could lead to the production of human β_2 in a more soluble conformation and with higher catalytic activity. Results obtained with the soluble fractions of infected Sf9 cells indicated that the expression at 22ºC did not lead to a higher basal activity in the presence of Mn^{2+}, but rather led to a decrease in the amount of the expressed protein. Therefore, further expression tests using different media, temperatures and MOIs might allow for the production of a more active protein.

Additionally, the α_2/β_1 heterodimer was also expressed in Sf9 cells, and catalytic activity was detected after treatment with NO and the sGC activator BAY 60-2770, both in the presence of Mn^{2+} and Mg^{2+}. Our data showed that similar to results reported in the literature, this complex seems to contain heme, however the catalytic activity is several fold lower than the reported to rat α_1/β_1 complex (Harteneck *et al.*, 1991; Koglin and Behrends, 2000). The expression of the β_2 with α_2 was also tested, and although the complex seems to exist (as observed by western blot results), no catalytic activity was detected in those cell extracts. This result might be justified by the incapability to measure such low activity values (due to the detection limit of the HPLC). However, as the GTP is not the only substrate reported for the sGC enzymes, the low values obtained might also be due to the failure to identify the ideal substrate for this complex (Mittal *et al.*, 1979).

To the best of our knowledge, this is the first study reporting the purification of catalytically active human β_2 via the baculovirus expression system in Sf9 cells. Further optimization of the expression and purification of the human enzyme will allow to study the effect of sGC modulating compounds on the β_2 activity. This further characterization of the protein could finally contribute to unveil a potential functional role of the human β_2 subunit and the use of this protein as a pharmacological target.

Chapter V- References

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Chapter VI- Appendix

Figure VII.1- Human sGC subunits sequence alignment. (https://www.ebi.ac.uk/Tools**)**

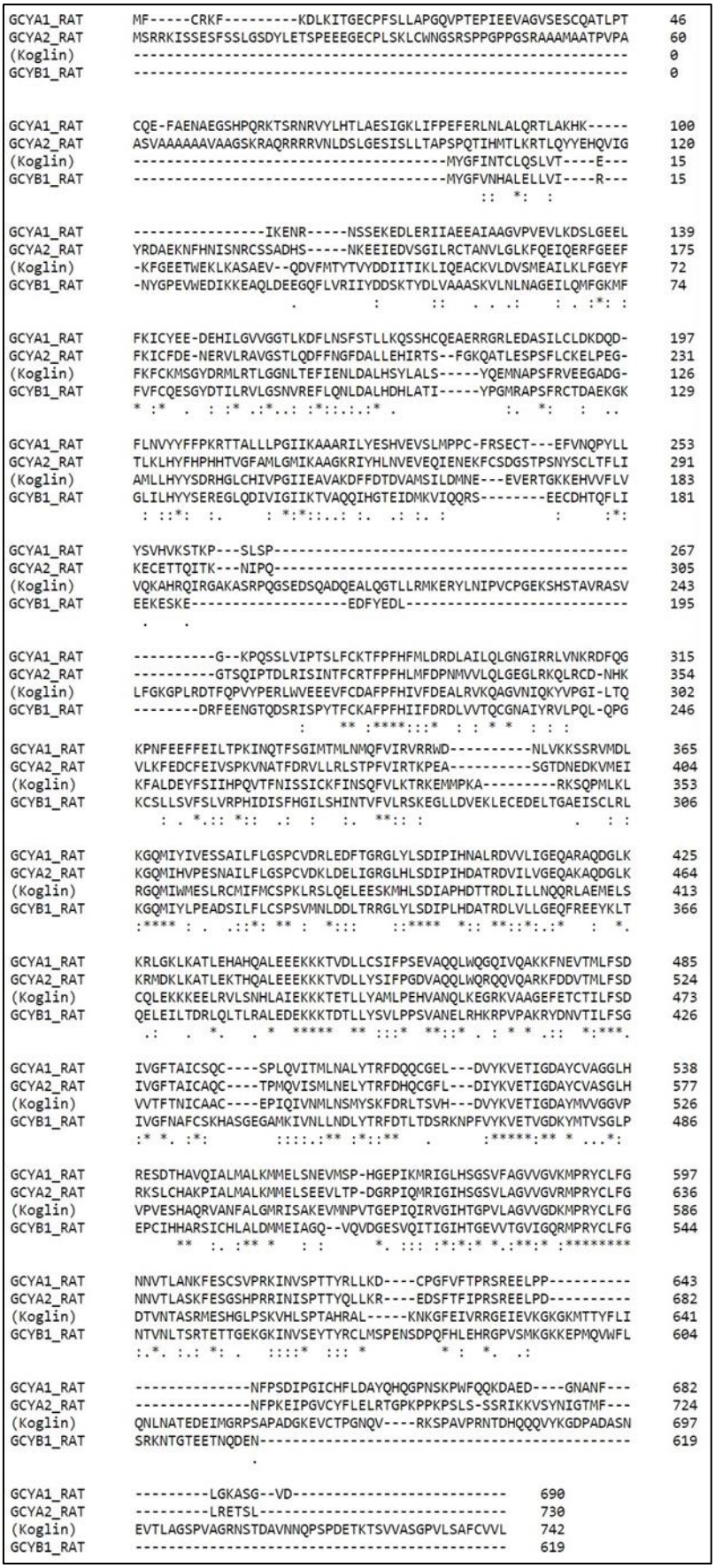

Figure VII.2- Rat sGC subunits sequence alignment. (https://www.ebi.ac.uk/Tools**)-** Koglin is the rat β_2 sequence described by Koglin et al. (2001).

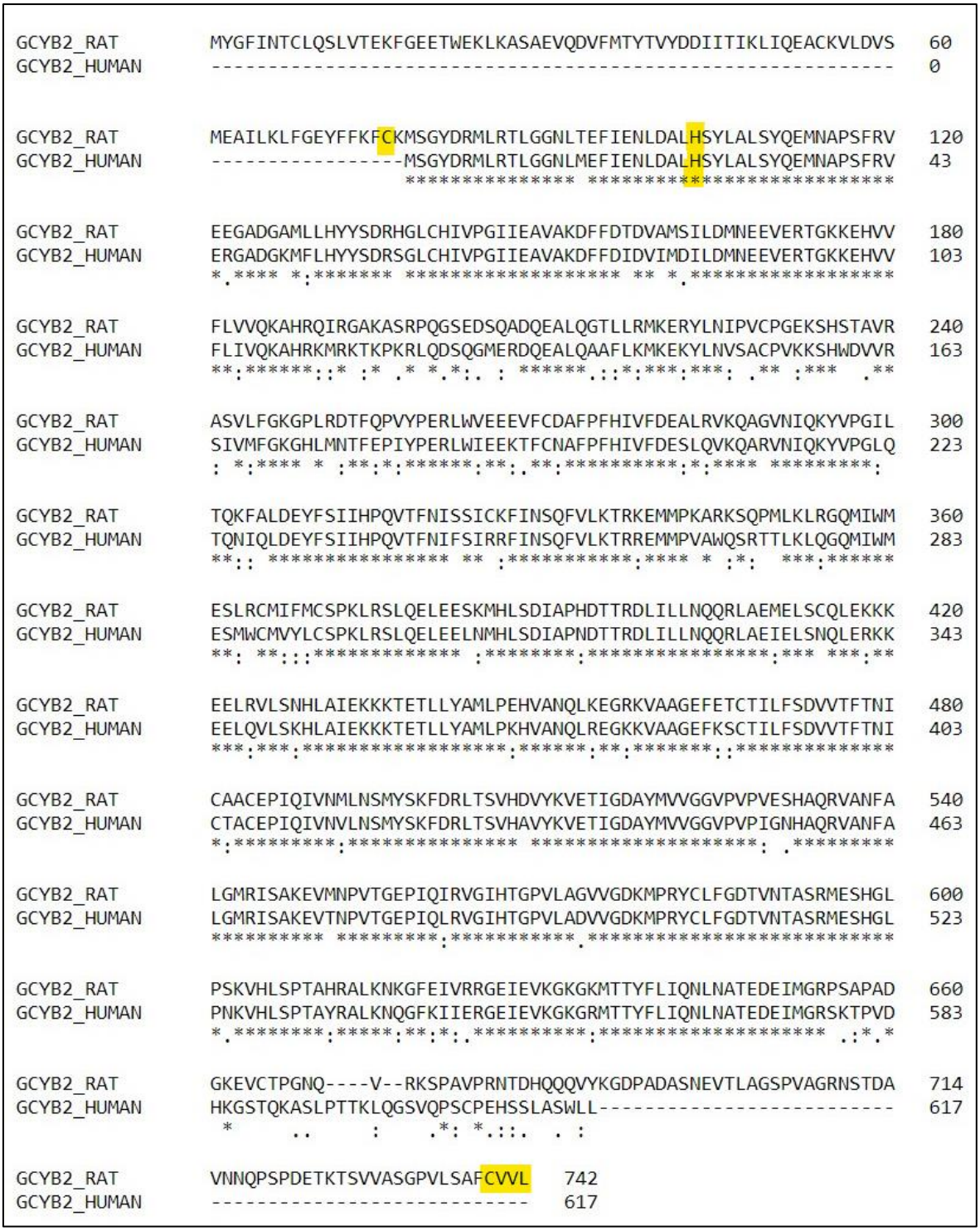

Figure VII.3- Rat GUCY1B2 and human GUCYB2 sequence alignment. The rat sequence is the reported by Koglin et al (2001), which binds heme and is NO-sensitive. In yellow are marked the Cys residue important for NO-sensitivity; His residue important for heme binding; the CVVL isoprenylation site (https://www.ebi.ac.uk/Tools/msa/clustalo/).