Universidade de Aveiro Departamento de Química

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Rafael Neves Francisco

Solubilização de proteínas de membrana utilizando líquidos iónicos

Solubilization of membrane proteins using ionic liquids

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo biotecnologia industrial e ambiental, realizada sob a orientação científica da Doutora Mara Guadalupe Freire Martins, Investigadora Auxiliar do Departamento de Química, CICECO, da Universidade de Aveiro e coorientação do Doutor Ricardo Simão Vieira-Pires Investigador principal do grupo Biotecnologia Estrutural, Centro de Neurociências e Biologia Celular (CNBC/UC), da Universidade de Coimbra

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palavras-chave Proteínas de membrana, Líquidos iónicos, Solubilização, Bacteriorodopsinas

resumo

O objetivo principal deste trabalho consistiu no estudo da capacidade de líquidos iónicos para extrair proteínas de membrana de membranas biológicas, assim como em manter a sua integridade em solução aquosa.

Para a avaliação da capacidade de extração, foram selecionadas quatro proteínas para sobreexpressar em *Escherichia coli*, nomeadamente *Outer Membrane Protein F* (OmpF) e *Outer Membrane Protein C* (OmpC), que são proteínas em barril-β, e duas bacteriorodopsinas, *Haloarcula marismortui* (*Hm*BRI) e *Haloarcula walsbyi* (*Hw*BR), que são compostas por hélices-α.

Infelizmente a produção de OmpC falhou durante o passo de clonagem enquanto que a obtenção de OmpF falhou durante o passo de purificação. Por outro lado, as duas bacteriorodopsinas foram expressas e purificadas com sucesso. Embora alguns ajustes (principalmente ao nível da His-tag) devam ainda ser realizados para melhorar a expressão e a purificação num futuro próximo, por cada litro de cultura foram purificados 1 mg de *Hm*BRI e 0,1 mg de *Hw*BR.

A proteína *Hm*BRI foi finalmente escolhida como proteína modelo para testar a capacidade de extração e solubilização de vários líquidos iónicos. A sua característica interessante de produzir soluções e *pellets* com cor roxa, assim como a sua absorvência específica a 552 nm, faz da *Hm*BRI uma excelente proteína modelo facilmente monitorizada.Os líquidos iónicos estudados são derivados de catiões imidazólio, fosfónio e colinio. De um modo geral, nenhum líquido iónico foi capaz de extrair *Hm*BRI sem a desnaturar. No entanto, o decanoato de colinio foi capaz de extrair mais proteínas da membrana biológica em comparação com o detergente comercial, decilo maltosideo. Por fim, foram estudadas misturas de decanoato de colina e surfactante comercial para extrair, apesar de os resultados serem semelhantes ao quando utilizando apenas líquido iónico.

Uma vez que a *Hm*BRI é uma boa proteína de fusão para outras proteínas de membrana, foram produzidos anticorpos específicos de aves (policlonais) pela imunização de codornizes, sendo estes anticorpos posteriormente purificados a partir de gema de ovo. Estes anticorpos são muito úteis na investigação de *Hm*BRI como tag fusão.

keywords Membrane Proteins, Ionic Liquids, Solubilization, Bacteriorhodopsins

abstract

The main goal of this work consists on the study of the ability of ionic liquids (ILs) to extract membrane proteins from biological membranes while keeping their integrity in aqeuous solutions. Since typical surfactants are mainly used for this purpose, ILs are here investigated as a new class of extraction agents.
For the evaluation of the ILs solvation ability power, four proteins were selected to be overexpressed in Escherichia coli and to be used as model proteins, namely the Outer Membrane Protein F (OmpF) and Outer Membrane Protein C (OmpC), that are β-barrel proteins, and two bacteriorhodopsins, *Haloarcula marismortui* (*Hm*BRI) and *Haloarcula walsbyi* (*Hw*BR), that α-helix proteins. In this work, the investigations carried out with OmpC failed during cloning and OmpF failed during the purification step. On the other hand, the two bacteriorhodopsins were expressed and purified successfully. Although some adjustments (mainly the His-tag) must be performed to improve the expression and the purification level, 1 mg/L of *Hm*BRI and 0.1 mg/L of *Hw*BR were purified.
The protein *Hm*BRI was then chosen as a model protein to test the extraction ability of several aqeuous solutions of ILs. Its interesting feature of producing solutions and pellets with a purple colour, and its specific absorbance at 552 nm, make of *Hm*BRI an excellent model protein since it can be easily monotired by Vis-spectroscopy and analysis of its colour.
Iimidazolium-, phosphonium-and cholinium-based ILs were investigated in several concentrations in aqueous solutions. None of the ILs studied revelaed to be able to extract *Hm*BRI without denaturation. However, cholinium decanoate was able to extract a higher amount of protein from the biological membrane compared to the commercial detergent decyl maltoside. Mixtures using cholinium decanoate and decyl maltoside were then used to extract the HmBR altough no further improvements on the extraction were observed.
Since *Hm*BRI is reported as a good fusion tag for other membrane proteins, avian specific antibodies (polyclonal) were finally produced by immunizing quails to evaluate the performance of *Hm*BRI as a fusion tag.

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List of abbreviations

[C_{10}mim]Cl	1-Decyl-3-methylimidazolium Chloride
[C_{14}mim]Cl	1-Tetradecyl-3-methylimidazolium Chloride
[$P_{444,14}$]Cl	Tributyltetradecyl phosphonium Chloride
[P_{8888}]Br	Tetraoctyl phosphonium Bromide
A280	Absorbance at 280 nm
A552	Absorbance at 552 nm
aa	amino acids
Agg	Aggregates
ATPS	Aqueous two-phase systems
bp	base pair
[Ch]Ac	Choline acetate
[Ch]But	Choline butanoate
[Ch]Dec	Choline decanoate
[Ch]Hex	Choline hexanoate
[Ch]Lac	Choline lactate
[Ch]Oct	Choline octanoate
[Ch]Pro	Choline propanoate
CMC	Critical micelle concentration
DDM	Dodecyl-β-D-maltoside
DM	Decyl-β-D-maltoside
DSMO	Dimethyl sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
Ext	Extracted proteins
FT	Flow through
FWD	Forward
GOI	Gene of interest
His-tag	Histidine tag
HLB	Hydrophilic-lipophilic balance
HmBRI	*Haloarcula marismortui* bacteriorhodopsin I
HT	His Trap
HwBR	*Haloarcula walsbyi* bacteriorhodopsin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IL	Ionic liquid
IMAC	Immobilized metal ion affinity chromatography
Inj	Fraction injected on column
IPTG	Isopropyl β-D-1-thiogalactopyranoside

LDAO	Lauryldimethyl amine oxide
MBP	Maltose binding protein
OD	Optical density
OG	Octyl-β-D-glucopyranoside
OM	Outer membrane
OmpC	Outer Membrane Protein C
OmpF	Outer Membrane Protein F
ON	Overnight
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
REV	Reverse
RF	Restriction free
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SLIC	Sequence and ligase independent cloning
TM	Transmembrane domain
UppP	Undecaprenil pyrophosphate phosphatase
W	Wash

1. Objectives

Membrane proteins are extremely difficult to handle; however, the knowledge of their features is tremendously important to develop drugs and therapies to treat inumerous diseases. In the past years, membrane protein studies have advanced significantly [1,2].

Recent studies have shown an enormous potential of Ionic Liquids (ILs) to extract and preserve the nature of several biomolecules, including proteins. ILs can be used to stabilize, and to promote the activity, separation, crystallization, detection and extraction of proteins. In the field of membrane proteins, ILs were already investigated to extract, solubilize, and characterize membrane proteomes [3–5].

In this work, the main objective was to evaluate the ability of different ILs to extract and solubilize membrane proteins as well as to evaluate their stability or denaturing behavior. The majority of studies in the lietrature used ILs based on the imidazolium cation [3–5]. So, in this work, we intended to study ILs more biocompatible and from natural sources, still with surfactant activity. In particular, ILs based on thecholinium cation and anions derived from carboxylic acids with long alkyl side chain lengths were investigated [6–8].

To evaluate the applicability of this family of ILs, a straight and simple method using the bacteriorhodopsin from *Haloracula marismortui* (*Hm*BRI D94N) as a model membrane protein (seven transmembranar domains α helix) was developed. Since this protein produces purple pellets and solutions, it makes possible to evaluate the extraction of this protein as well its state of folding, visually or by measuring the absorbance of aqueous solutions at 552 nm [9]. This protein can be used as fusion tag for other membrane proteins, improving the stability and expression levels. So, the production of polyclonal hen antibodies against this protein, was finally evaluated [9,10].

2. Introduction

2.1. Membrane Proteins

Membrane proteins represent about one-third of the genome of several organisms (e.g. human, *Escherichia coli* and *Saccharomyces cerevisae*) and they are responsible for extremely important functions for the proper behaviour of cells. It is known that the malfunction of several membrane proteins lead to diverse physiological disorders that result in several diseases. Interestingly, 50% of the currently available drugs target membrane proteins. Therefore, detailed knowledge of the structure and function of these proteins is critical for the development of more effective treatments, including the design of more effective drugs [2,11].

Biological membranes are a very important barrier surrounding the cell and establish a border between the intra and extracellular environments in which they are. In the case of eukaryotic organisms, these membranes also serve to compartmentalize intracellular organelles such as mitochondria (outer and inner membrane), the endoplasmic reticulum, the Golgi apparatus, the nucleus and also vesicles. Biological membranes are extremely diversified and contain about 100 or more different lipids, which vary in their physicochemical properties such as electric charge and fluidity [1,12]. Generally, they are composed of 50% of lipids and 50% of proteins (in weight), taking as extreme, the myelin membrane, in neurons, with a ratio lipid/protein of 82:12 and, on the other extreme the mitochondria membranes, with numerous proteins responsible for energy production, with a ratio of 25:75 [1,13].

Being so important one would expect that many of these proteins were already well studied, but in fact, there are few membrane proteins with their structure already determined. Of the 101059 proteins deposited in the Protein Data Bank (PDB) until May 2015, only 2768 are membrane proteins (about 2.7%). This proves how difficult it is to manipulate these proteins to obtain good diffracting crystals for atomic structure determination.

Studies on membrane proteins have indeed increased in recent years, by giving particular attention to the development of new strategies and reagents to extract, purify and manipulate these highly hydrophobic proteins [4,5,9,14,15].

2.1.1. Structure of membrane proteins

Membrane proteins may be encoded from a single gene or they may be a complex of proteins. They are classified depending on the interaction with biological membranes, being divided into two large groups, the peripheral membrane proteins and integral membrane proteins (or transmembrane proteins) [12,16].

Peripheral membrane proteins do not interact with the hydrophobic portion of the membrane, being instead directly attached to the membrane via interactions with the polar group of lipids, or indirectly, by interactions with integral membrane proteins [1].

Integral membrane proteins span the membrane one or more times and are divided into type 1, type 2 and multipass proteins (***Figure 1***), and are encoded by 10, 10 and 11-12% of the genome, respectively. Type 1 proteins exhibit a single transmembrane domain (TM) and have the N-terminal facing the extracellular or luminal space, as opposed to the type 2 proteins having N-terminal facing into the cell or organelle. Multipass membrane proteins have several transmembrane domains, and both the N-terminal and C-terminal may be oriented to both the extracellular space/luminal or into the cell. The membrane spanning regions are highly hydrophobic, consisting of 18 to 30 non-polar amino acids, forming α-helices. The higher number of α-helices crossing the membrane result in more hydrophobic proteins [1,16,17].

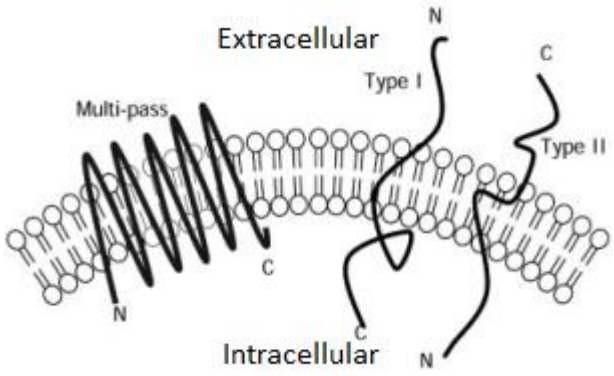


***Figure 1** – Representation of integral membrane proteins and their orientations in the phospholipid bilayer [1].*

Other proteins span the membrane in the form of β-barrels acting as pores. The β-barrels consist on an alternate polar and non-polar amino acids sequence that are facing the channel (aqueous) and the phospholipid bilayer, respectively. For this reason, β-barrel proteins are less hydrophobic than proteins with multiple transmembrane α-helix domains [1,18].

2.1.2. Functional role of membrane proteins

As previously mentioned, membrane proteins are responsible for many important functions for cells survival. These functions depend on the location of membrane proteins. In cytoplasmic membranes, proteins usually act as receptors, providing to cell information from the extracellular environment, which allows them to react to extracellular stimuli. Other proteins act as an anchor to proteins of the cytoskeleton and extracellular matrix, performing a structural support function. In the inner membrane of mitochondria most proteins belong to the respiratory chain, responsible for energy production, but also proteins responsible for cell apoptosis mechanism are present [1,19].

In plants, the proteins found in the membranes of chloroplasts and thylakoids are responsible for transporting and carrying out photosynthesis, respectively. However, there are other functions common to all membranes, as is the case of transporters and channels which are involved in the flow of ions, molecules and other proteins to the interior and exterior of cells or organelles [1].

2.1.3. Difficulties in the study of membrane proteins

Membrane proteins are much more difficult to manipulate than water-soluble proteins because membrane proteins are not by themselves soluble in water and tend to aggregate when removed from the lipid layer. Therefore it is necessary to make use of detergents to solubilize these proteins in aqueous medium. The study of membrane proteins thus becomes challenging due to difficulties inherent to membrane extraction processes, solubilization and subsequent biochemical and biophysical characterization [1,2,20]

Another difficulty of working with membrane proteins is that they are quite heterogeneous, *i.e.*, they have different hydrophobicities, post-translational modifications and sizes. For example, integral membrane proteins may have one to fifteen transmembrane domains and these domains can be α-helices or β-sheets. These variations change the characteristics of proteins, including hydrophobicity. The ratio between the extracellular loops (hydrophilic areas) and transmembrane domains also varies between membrane proteins [1,2,21].

The lipids making up the phospholipid bilayer, where membrane proteins fit, also vary according to the cell/organelle, cell stages and the extracellular environment [1,2].

By being restricted only to biological membranes, the abundance of these membrane proteins is dependent on the amount of the biological membrane itself, which makes them much less abundant than water-soluble proteins. For this reason, overexpressions of these proteins in different cell systems are usually attempted. However, the overexpression can often be toxic by deregulating cellular homeostasis; overexpression promotes instability and also leads to aggregation of these proteins, with the formation of inclusion bodies. Another problem associated with overexpression of membrane proteins in heterologous systems is that their native folding might be compromised. For these reasons, the development of new methods for obtaining high yields of functional membrane proteins is a demanding task [1,2,13,22,23]. Currently, it is necessary to use detergents to solubilize membrane proteins. However, detergents can lead to protein denaturation, compromising the solubilization yields. Additionally, they may limit subsequent analysis of the protein

sample, due to their hydrophobic character. Therefore. The knowledge of the properties of detergents able to extract and stabilize given membrane proteins is of crucial importance. Unfortunately, is difficult to implement a common protocol for this purpose,since for each membrane protein there is the need of the process optimization [1,2].

2.1.4. Strategies for the expression of membrane proteins

The most widely used host for soluble protein expression is *E. coli*, and the same applied for membrane proteins, since it is the most studied organism which leads to higher yields. Still, it is necessary to take into account the strain to be used. *E. coli* BL21 (DE3) is the most frequently used strain; however, high expression of certain membrane proteins can be lethal to this strain which leads to a yield reduction. To overcome this issue mutant strains derived from BL21 (DE3) have been selected; these include strains such as C41 (DE3) and C43 (DE3) that show better ability to withstand high amounts of membrane proteins thereby achieving higher yields. In some particular cases, toxicity associated with the over-expression of membrane proteins can be controlled. For certain channels, and membrane transporters, for example, ions are added to block flow through the channel pore, helping in the maintenance of the homeostatic balance that overexpression tends to change [15].

Regarding the expression of membrane proteins from eukaryotes, mainly due to the presence of post-translational modifications, eukaryotic hosts are often used. These include *Pichia pastoris* and *Saccharomyces cerevisiae*. However, several eukaryotic membrane proteins have been expressed in prokaryotic hosts [22].

2.1.5. Protein-fusion Tags

One of the most widely used techniques for protein expression consists on the use of fusion tags. These tags can be small peptides, protein domains or even complete and functional proteins, which are inserted in the proteins of interest at the genetic level, fusing the gene encoding the tag with the target-protein gene. They allow: i) the improvement of

target-protein expression, by stabilizing the recombinant form, ii) the purification by selective affinity and downstream characterization and iii) the expression monitoring, operating as reporters to locate the target-protein [17].

Tags can be classified according to their application and biochemical properties, as **Table 1** summarizes, being also common to use multiple tags in the characterization of the target protein [17].

Table 1 *- Types and application of fusion proteins [17].*

FUSION TAGS	APLICATIONS	FEATURES	EXAMPLES
ENZYME TAGS	Topology identification	Large size, may interfere with protein structure and functions	B-galactosidase, β-lactamase, alkaline phosphatase
FLOURESCENT PROTEIN TAGS	Topology identification, displaying protein interactions	Large size, may interfere with protein structure and functions	GFP, CFP, YFP
EPITOPE TAGS	Topology identification, increase the hydrophilic surface, displaying protein subunits	Small size, with lower probability of interfering with protein structure and functions	FLAG, Myc tag, M2 g, V5
AFFINITY TAGS	Affinity purification, displaying protein subunits, expression partner	Variable size, may interfere with protein structure and functions	His tag, MBP, GST, Strep tag
PROTEIN TAGS	Expression partner, protein stabilization	Large size	Ubiquitin, cytochrome b562, thioredoxin
PEPTIDE TAGS	Topology identification, displaying protein interactions, detecting proteins	Small size	S tag, tetracysteine, SBP tag, BTX tag

When working with fusion tags there are some considerations to take into account. The first consideration is the size and biochemical characteristics of the fusion tags. In general, a larger tag will likely interfere with the structure and function of the target protein. Regarding the biochemical properties of the fusion tags, they can influence, for instance, certain purification steps. For example, if MBP (maltose binding protein) is to be used as a fusion tag, the DDM detergent (dodecyl-β-D-maltoside) cannot be used to solubilize the protein and then purify it by affinity chromatography, because DDM is an analogue of maltose and will compete for the maltose-binding site of MBP. Thus, in this case, it is necessary to change the fusion tag or detergent [17].

The second consideration relates to the topology of the target protein. As already mentioned, integral membrane proteins usually consist of α-helices, connected by hydrophilic loops, and the tags cannot be inserted into hydrophobic regions of the target protein, having to be inserted in loops or at the N or C-terminal, if they are outside of the biological membrane. It should be kept in mind that intracellular loops have more positively charged residues than extracellular loops. Thus, tags that are positively charged (histidine tag, His-tag) ideally should not be inserted in the terminal or extracellular loops. On the other hand, strep tag (almost neutral in charge) can be inserted in any terminal or loop [17,24].

The third consideration is the linker sequence between the tag and the target protein (linker). The longer this sequence is, greater is the flexibility of the fusion tag, but also a greater susceptibility of being cleaved by cellular proteases happens. Finally, the fourth consideration is the potential use of multiple tags instead of just one; using two epitope tags allows tandem affinity purification strategies, or even improvement of target-signal monitoring [17,25–27].

2.1.5.1. *Haloarcula marismortui* bacteriorhodopsin as fusion tag

Recently, a fusion tag using a bacteriorhodopsin (*Hm*BRI) abundant in the membranes of halobacterium *Haloarcula marismortui* [9]. This protein has seven transmembrane helices (TM) and incorporates the chromophore retinal, which undergoes an isomerization

process driven by visible light. Indeed, upon absorption of a photon in the visible range, all-trans retinal can isomerize to a 13-cis-retinal configuration, promoting functional changes in the protein - **Figure 2** [28].

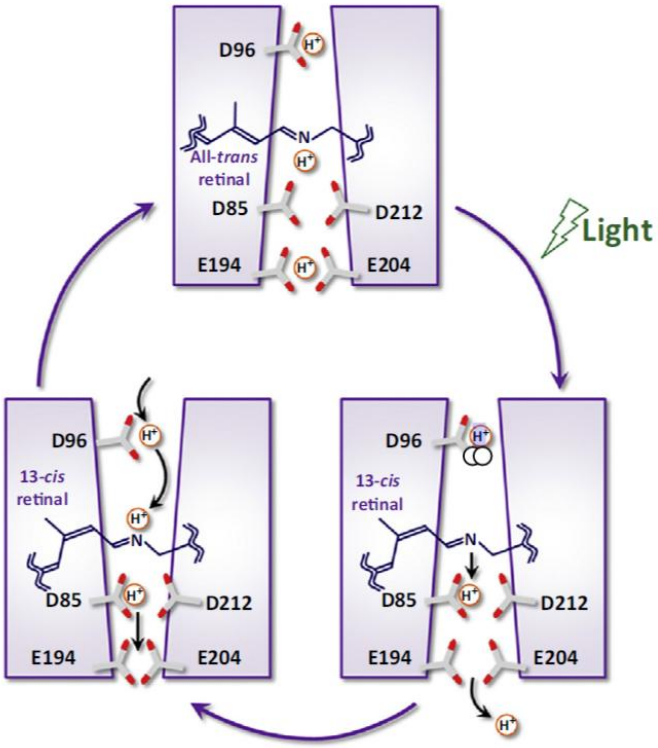


Figure 2 *– Proton transport process in bacteriorodopsinas. Each cycles pumps out a proton from the cell [28].*

*Hm*BRI when used as fusion tag, has proved to be very efficient in promoting the expression and correct folding of target proteins. In addition, the purple colour (with an absorbance peak at 552 nm) associated with the isomerization of retinal, allows a direct monitoring of the folding *Hm*BRI-target protein, making this a unique reporter tag to membrane proteins. The mutant *Hm*BRI/D94N was optimized to achieve higher expression levels, allowing to obtain 40-70 mg of protein *per* litter of culture. The intense purple colour expression pellet reflects the greater stability of mutant compared to the native protein (**Figure 3**) [9,29].

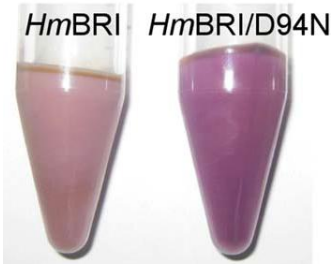


Figure 3 – *Cell expression Pellets of* Hm*BRI and* Hm*BRI/D94N proteins, induction with IPTG and all-trans retinal [9].*

The tag developed [9] has an additional His-tag to facilitate subsequent purification steps, and can only be inserted in the target membrane protein with cytoplasmic N-terminus, for example the UppP (undecaprenil pyrophosphate phosphatase) - **Figure 4**. The bacteriorhodopsin from *Haloarcula walsbyi* also exhibits a similar behaviour to *Hm*BRI, allowing a better stability as a fusion partner, presenting however a much lower expression level. Analysis by molecular size exclusion revealed a single oligomerization state for *Hw*BR, a desirable feature for protein crystallization assays and subsequent structure determination [9]

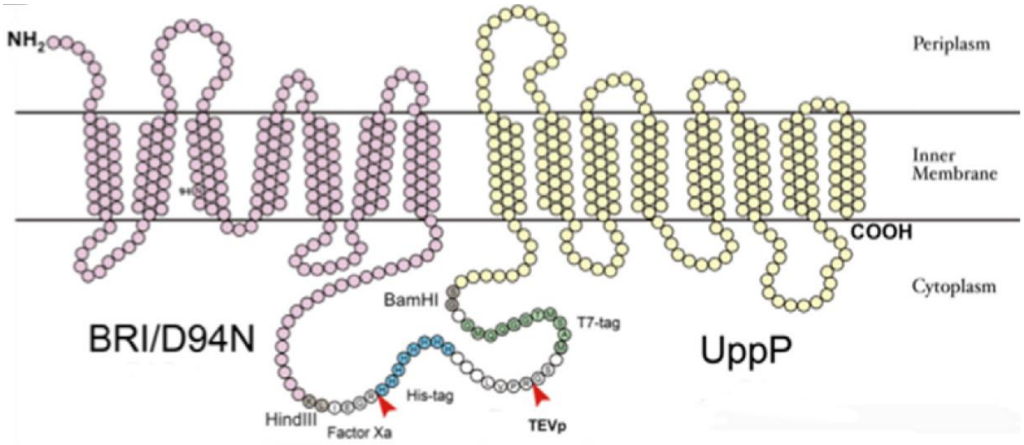


Figure 4 - *Representation of the topology of the membrane protein UppP with BRI/D94N as a fusion protein [9].*

2.1.5.2. Removal of fusion tags

The removal of fusion tags is often a requirement because they may influence analysis and stability of the protein, or following methods, for example crystallization or purification [17].

Tags can either be removed by chemical treatment or enzymatic treatments [30,31]. The enzymatic methods are commonly used because they are more specific. There are two types of tags used to remove proteases, the endoproteases and exopeptidases. The endoproteases require an enzymatic cleavage site between the target protein and the fusion tag. The most commonly used proteases are thrombin, factor Xa, and TEV (tobacco etch virus) PreScission (e.g. HRV 3C). In the case of exopeptidases, carboxilopeptidases or aminopeptidases, eg DAPase, aminopeptidase M (APM), and carboxypeptidase A and B (CPA and CPB) are commonly used. These enzymes will remove amino acids of the N or C terminals until a previously introduced stop site is found [17,32,33].

2.1.6. Sequence and Ligation Independent Cloning (SLIC)

In 2013, Scholz and her co-workers [34] established a simple and fast method to cloning. SLIC cloning enables sequence independent, precise and with minimal changes in the amino acid sequence of the gene of interest by homologous recombination. For that, it is necessary that the vector and the insert with 15-25 bp homologies on both DNA ends. The authors [34] designed vectors with the toxin ccdB gene to remove the vector background from the cloning procedure. This toxin inhibits the DNA gyrase of E. coli, so prevents the bacterial growth [35]. This toxin can be neutralized using the antitoxin ccdA. At downstream of the N-terminal purification or solubility enhancing the HRV 3C recognition site for tag removal was added [36].

Vectors are linearized in order to remove the ccdB gene and leave the HRV 3C recognition site and the 3’ end of ccdB as ends for homologous recombination. With an insert flacked with the same ends, it is possible to SLIC cloning - **Figure 5** [34,37]. At this moment, they have more than 40 different vectors are available (called pCoofy x) with several tags at the N-terminal or C-terminal.

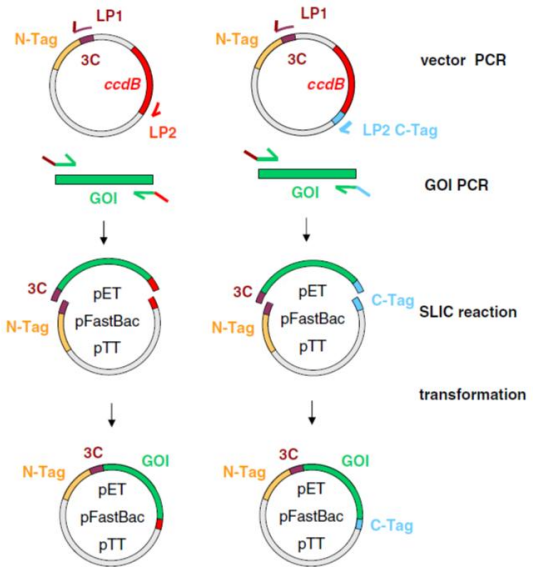


Figure 5 - *Principle of parallel SLIC cloning with neagative ccdB – The vector is PCR linearized with LP1 forward and LP2 reverse primer. The LP1 primer corresponds to PreScission protease site (3C) for tag removal. The LP2 primer is either located at the C-terminus of ccdB or corresponds to a C-terminal tag. In both cases the ccdB gene is deleted upon PCR amplification thereby allowing counterselection of parental empty vector in ccdB sensitive cells. The Gene of Interest (GOI) is PCR amplified with primers composed of 5' and 3' gene specific sequences plus 15 bp – 25 bp extensions complementary to LP1 and LP2 vector primers, respectively [34].*

2.2. Detergents

Detergents have an important role in the extraction, purification and manipulation of membrane proteins. Their amphipathic nature allows them to interact with the hydrophobic regions of these proteins and solubilize them in aqueous media. Unfortunately it is not always possible to keep the proteins in their native conformation, and a detergent used for extraction may not be compatible for the protein purification. Additionally, a suitable detergent for a target membrane protein may not work the same way for other proteins, requiring the selection and optimization of a given detergent for each protein [2,38].

In aqueous solutions, detergents tend to form micelles, thereby protecting the hydrophobic chains, leaving the polar head group facing into the aqueous environment, *i.e.*, the polar groups establish hydrogen bonds with water and the aliphatic tails interact

with each other by dispersive and van der Waals interactions - **Figure 6**. These properties allow the detergents to remain dispersed in water [2,39,40].

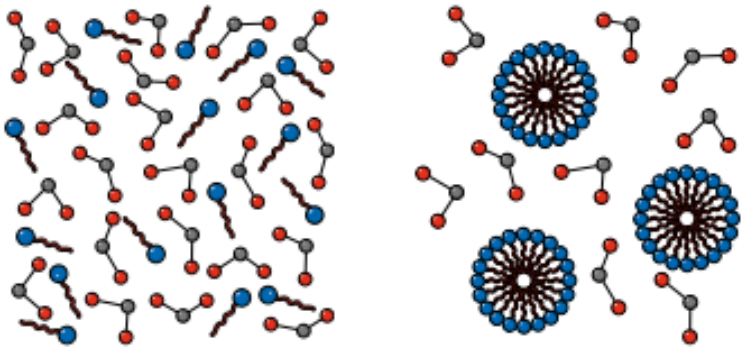


Figure 6 *- Micelle formation in aqueous solution. On the left figure the detergent is monodisperse in solution and on the right figure detergent monomers are aggregated to form micelles.*

2.2.1. Classification of detergents

Detergents can be divided into four major groups according to their structure and chemical features: ionic detergents, non-ionic detergents, zwitterionic detergents and bile acid salts [2].

Ionic detergents may either be anionic or cationic, depending if the the hydrophobic part is located at the anion or the cation. For instance sodium dodecyl sulfate (SDS) is an anionic detergent because contains a negatively charged sulphate group at the end of the long alkyl chain. On the other hand cetyl trimethylammonium bromide (CTAB) is a cationic detergent because it has a positively charged ammonium group attached to the long alkyl chain. Ionic detergents are very effective in solubilizing membrane proteins, but often lead to their denaturation. In some cases, proteins can recover their native form once the detergent is removed [2].

Bile acid salts are also ionic detergents (typically anionic), which differ from SDS by not having a chain of hydrocarbons but rather a steroid group which is much more rigid. In general, bile salts are less denaturing than ionic detergents with straight aliphatic chains [2].

Nonionic detergents have an uncharged hydrophilic head, which can be either a polyoxyethylene group or a glycosidic group. The most common are octyl-β-D-glucopyranoside (OG), decyl-β-D-maltoside (DM) and dodecyl-β-D-maltoside (DDM), in which the polar group is composed of a glycoside. On the other hand, detergents such as Triton X-100 and Triton X-114 are formed by oxyethylene units. Generally, the nonionic detergents are considered nondenaturing, since they tend to break the lipid-lipid interactions, and protein-lipid interactions rather than protein-protein interactions. For this reason, nonionic detergents are frequently used to solubilize membrane proteins. Nonionic detergents with shorter alkyl chains (7 to 10 carbons) are more denaturing that detergents with longer alkyl chains (12 to 14 carbons) [2].

Zwitterionic detergents combine properties of ionic and nonionic detergents, being more denaturing in nature than nonionic detergents, but less than the ionic ones. The most common are lauryldimethyl amine oxide (LDAO), dodecyldimethyl amine oxide (DDAO), CHAPS/CHAPSO and dodecyl phosphocholine (Fos-Choline 12). In **Figure 7** are shown the chemical structures of some detergents used for the solubilization of membrane proteins [2].

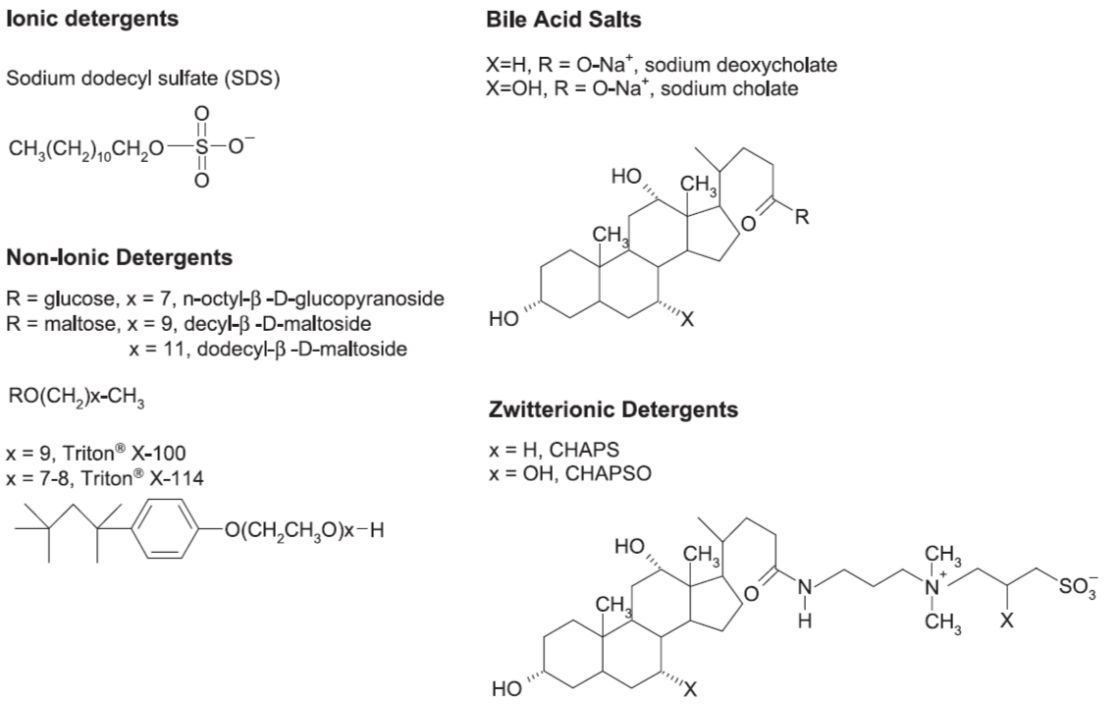


Figure 7 *- Chemical structures of detergents [2].*

2.2.2. Properties of detergents

The selection of the appropriate detergent to solubilize, extract and purify membrane proteins is often achieved through trial and error approches. Still, some properties of detergents can help to decide which detergent should be used for a given protein.

The critical micelle concentration (CMC) is the minimum concentration of detergent necessary for micelle formation. CMC varies when changing the pH, temperature, with the presence of proteins, lipids and other detergents. CMC decreases with increasing the size of the hydrophobic chain and increases with the introduction of double bonds and branching (reason why the bile salts have high values of CMC). A low CMC is often desirable because it allows working with lower amounts of detergent. Moreover, in certain procedures, a high CMC is desirable to be able to remove the detergent by dialysis. **Table 2** presents the main factors influencing the CMC. There are several methods for determining the CMC, the most used are light scattering, surface tension, conductivity and dye binding experiments [2,40,41].

Table 2 - *Factors affecting CMC*

Factors that increase CMC	➢ Carbon-Carbon double bounds ➢ Polar groups within hydrophobic tail ➢ Presence of ionic head groups ➢ Shorter alkyl side chains
Factors that decrease CMC	➢ Increasing of methylene groups in the alkyl chain ➢ Phenyl rings in the alkyl chain ➢ Fluorinated chains ➢ Addition of hydrocarbons to the solution ➢ Longer alkyls side chains

At low temperatures, detergents remain mostly in a crystalline form, which is insoluble in water and, in equilibrium with a small amount of solubilized monomers. As the temperature increases, more monomers will be dissolved until reaching CMC and begin to form micelles. This temperature is called the critical micellar temperature (CMT). The temperature at which the crystalline structure forms, and for which the dissolved

monomers and the micelles exist in equilibrium, is called Kraft point, as shown in **Figure 8** [2,40]**.**

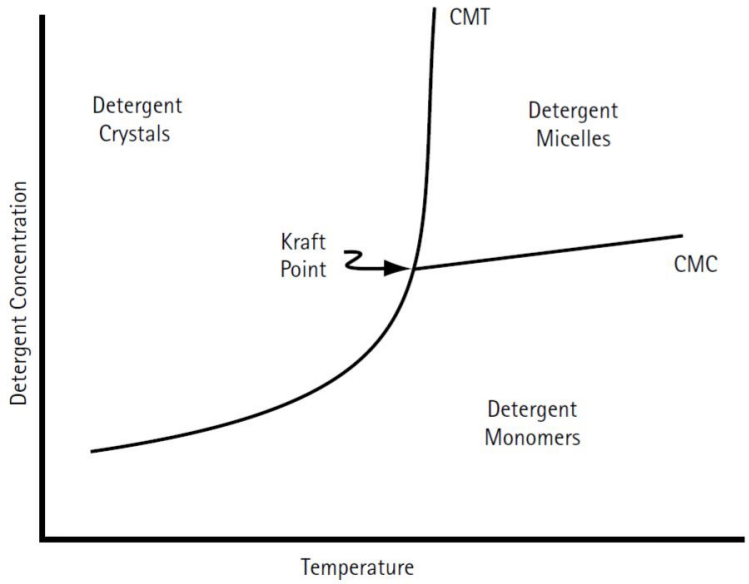


Figure 8 *- Phase diagram for a detergent aqueous solution.*

Above CMT, nonionic detergents become cloudy and separated into two phases, one rich phase in detergent and other phase poor in detergent. This temperature is called the Cloud Point. This feature can be useful for extracting membrane proteins. If the cloud point is low, such as in Triton X-114, about 22 °C, the protein can be solubilized at 0 °C and then brought to a temperature of 30 °C, at which phase separation occurs and the protein will be predominantly enriched in the detergent rich phase. However, few detergents have a Cloud Point below 50 °C and higher temperatures may lead to the denaturation of proteins [2].

The aggregation number refers to the number of detergent monomers contained in a micelle. It is calculated by dividing the molecular weight of the micelle by the molecular mass of the monomer. Most detergents have a number of aggregation between 50 and 100, however, some bile salts have an aggregation number close to 10. In **Table 3** are shown some factors that affect the aggregation number [2,40–42].

Table 3 - *Factors affecting the aggregation number.*

Factors that increase Aggregation Number	➢ Increasing number of methylene groups in the alkyl chain ➢ Addition of counter ions (ionic detergents)
Factors that decrease Aggregation Number	➢ Increasing size of hydrophilic head group ➢ Polar organic additives ➢ Addition of hydrocarbons to solution

Lastly, the HLB (hydrophilic-lipophilic balance) is a measure of the solubilizing ability of detergents. It usually ranges from 0 to 40, and the lower the HLB value is, the more hydrophobic the detergent is. Detergents with HLB between 12 and 20 are the most desirable since they typically solubilize proteins without denaturing them [43,44].

2.2.3. Extraction of membrane proteins

To study membrane proteins in their native and functional conformation, it is necessary to both extract them from biological membranes and to stabilize them in solution with the help of detergents. The amount of detergent to use depends on the amount of material to extract from biological membranes. In this line, the following rules should be considered: 1) working with a detergent concentration several folds above the CMC; 2) working with a detergent:protein ratio of at least 4:1 (in weight); and 3) work with a detergent:lipid ratio of 10:1 (mole:mole). Together, these issues will ensure the solubilization of proteins in enriched detergent micelles instead of mixed micelles [2,41,45].

Dissolution of biological membranes is divided into several steps (**Figure 9**). At low concentrations of detergent (below CMC), detergent monomers bind to the membrane. Once the phospholipid bilayer becomes saturated with detergent monomers, biological membranes tend to destabilize and disintegrate. The lipids of the membranes begin to form mixed micelles with detergent monomers. There is still the formation of protein-detergent micelles, in which the detergent monomers (hydrophobic tail) interact with the hydrophobic regions of the protein. In the last stage there are only lipid-detergent micelles mixed (no protein) and detergent micelles containing ideally a single protein molecule [2,45].

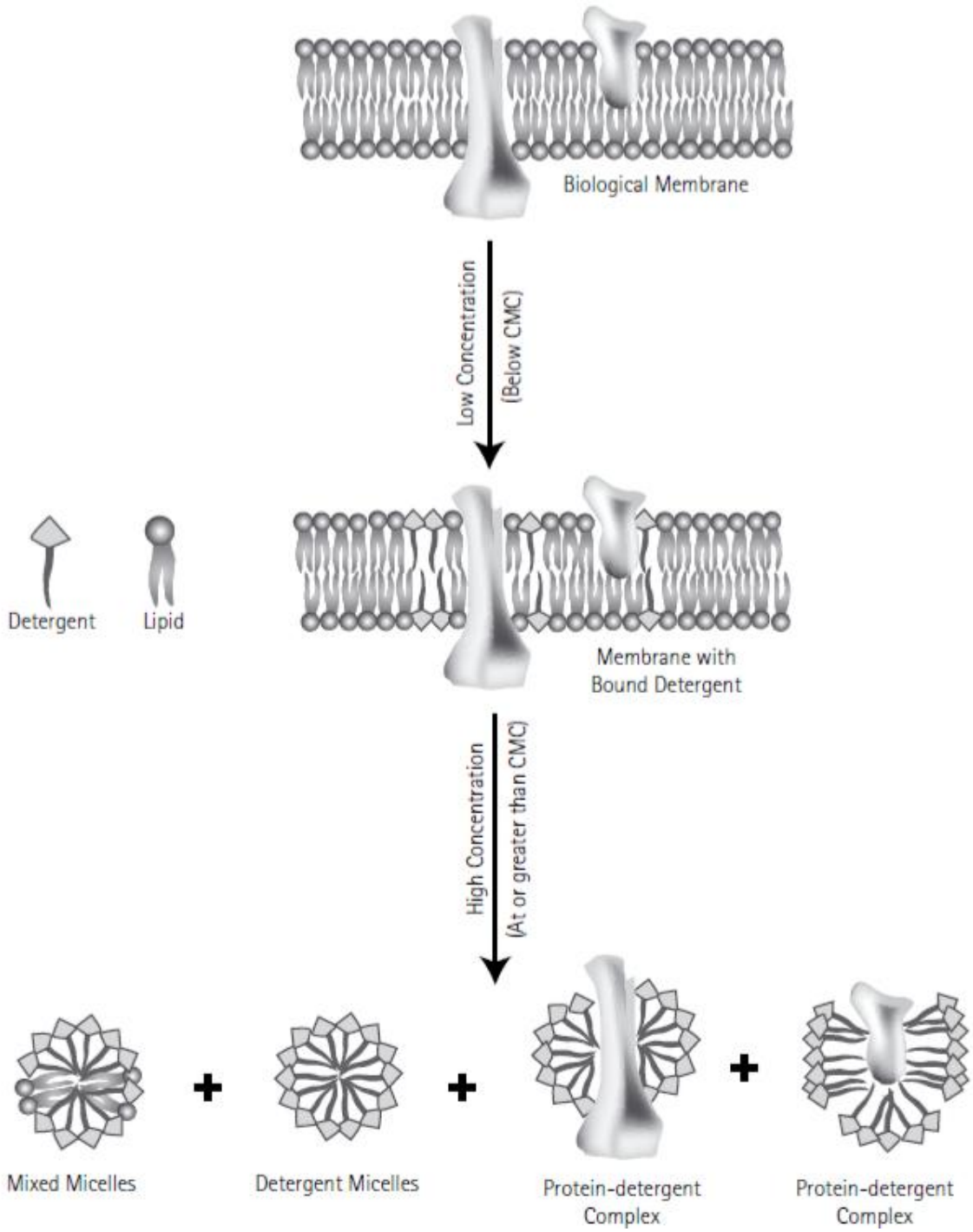


Figure 9 *- Steps in the dissolution of biological membranes with detergents.*

2.2.3.1. Removal of detergents

As mentioned, it is necessary a large amount of detergent to solubilize membrane proteins. In some cases, however the excess of detergent can compromise subsequent steps. Therefore, it is often necessary to remove unbounded detergent after the extraction of membrane proteins, or even change the proteins to another solution containing another detergent. There are several methods that allow the removal or replacement of usingh and manipulating their intrinsic properties such as charge, CMC, hydrophobicity and aggregation number [2].

Detergents can be removed by dialysis, consisting in the dilution of the detergent below the CMC, thereby promoting the disintegration of micelles. As the monomers are considerably smaller than the micelles, this procedure allows diffusion through the dialysis membrane and removal of the detergent. This technique works best in a detergent with high CMC and with small molecule size. On the other hand, with nonionic detergents displaying low CMCs, this technique becomes very limited [2].

The excess of detergent can also be removed using its hydrophobic character. By mixing the solution containing the excess detergent with an insoluble hydrophobic resin or mixing with hydrophobic beads. Detergent tails bind to the resin or bead which can then be separated by centrifugation or filtration. It is a method generally used for detergents with low CMC[2,46].

Molecular size exclusion and ion exchange chromatography are also used methods for the removal of detergent. The first explores the differences in the size of protein-detergent complexes, detergent micelles and the mixed lipid-detergent micelles. To prevent aggregation of the protein, the elution buffer should contain other detergent below its CMC. Since this method depends on the size of the micelles, the factors that change their size should be kept constant (pH, ionic strength, temperature). The second method explore the differences between the charge of protein-detergent complex and detergent micelles. Using nonionic or zwitterionic detergents, the conditions under which the protein detergent complex is retained on the column can be selected. The protein is then eluted by changing the pH or ionic strength, or washing the column with an ionic detergent [2].

Finally, when working with recombinant proteins, fusion tags can allow the immobilization of the protein to affinity resins and subsequent change of detergent [2].

2.2.4. Alternatives to traditional detergents

In order to achieve better results in the extraction and solubilization of membrane proteins, different classes of solubilizing agents have been exploited. Tripod amphiphiles (**Figure 10**) are an example of such agents; these are compounds based on tetrasubstituted carbon atoms, containing three hydrophobic tails and a polar head (usually an amine group). Given the rigidity of their hydrophobic groups tripod amphiphiles seem particularly effective for membrane protein solubilization and crystallization with no apparent denaturing behaviour [2,47,48].

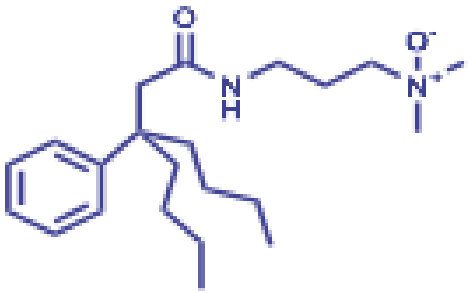


***Figure 10** – TPA structure, a tripod anphiphile.*

Another class of solubilizing agents are amphipols - **Figure 11**. This class is based on polymers having a hydrophilic backbone with multiple hydrophobic side chains. These amphipathic polymers bind to membrane proteins, stabilizing them for long periods of time. As amphipols are fully associated with protein, they produce solutions without "free detergent", which is extremely advantageous for the formation of crystals and NMR analysis [2,49].

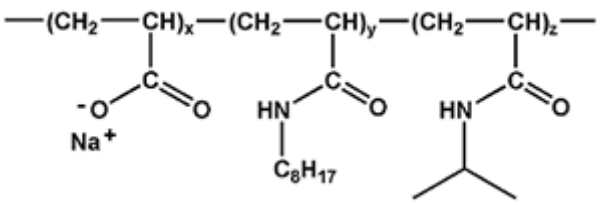


***Figure 11 -** A8-35 structure, an amphipol (x≈0,35; y≈0,25; z≈0,4).*

2.3. Ionic liquids

The emergence of ionic liquids (ILs) dates back to 1914 when Paul Walden [50] synthesized a salt with a 12 °C melting point, ethyl ammonium nitrate. In the last years, numerous papers have been published on the properties and applications of ILs. ILs have gained a large importance as new surfactants agents and, for this reason, they can be used in the extraction and purification of membrane proteins.

By definition, ionic liquids are salts with a melting point below 100 ° C, and so many of them are liquid at room temperature. They have excellent features contributing to their "new green solvents" categorization, namely a negligible vapour pressure and are therefore not volatile in atmospheric conditions, are non-flammable, have a high thermal stability and high ionic conductivity. Due to their tunability and with the proper manipulation of cation/anion design, ILs can cover a wide spectrum of hydrophilicity-hydrophobicity [7,51–53].

Due to their unique properties, ILs are used in various fields of research, such as in organic chemistry, electrochemistry, catalysis and processes of extraction/separation. In particular, ILs have been used for the extraction of biomolecules such as proteins, enzymes, nucleic acids, antibodies, and antibiotics combining non-denaturing properties and the ability to form aqueous two-phase systems (ATPS) [7,52].

There are numerous ILs already synthesized and characterized. The best studied cation family is the imidazolium-based one, followed by others including pyridinium-, tetraalkylammonium- and tetraalkylphosphonium-based. The anions most often used are chloride and bromide, and also the tetrafluoroborate and acetate. It is the combination of cations and anions, in conjunction with the size of the alkyl chain and the presence of functional groups, which makes of the ILs capable of solubilizing a wide variety of molecules, both polar and nonpolar. In **Figure 12** the chemical structures of some ionic liquids are shown.[7,51].

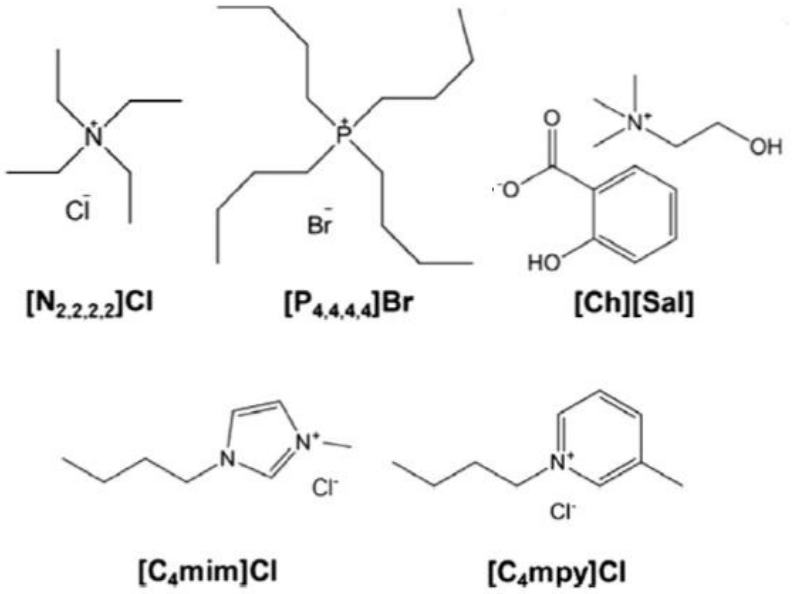


Figure 12 – *Structure of some ionic liquids; $[N_{2,2,2,2}]Cl$ – tetraethylammonium chloride; $[P_{4,4,4,4}]Br$ – tetrabutylphosphonium bromide; [Ch][Sal] – Cholinium salicylate; $[C_4mim]Cl$ – 1-butyl-3-metyhlimidazolium chloride; $[C_4mpy]Cl$ – 1-butyl-3-metyhlpyridinium chloride.*

Some ionic liquids have characteristics similar to traditional surfactants, such as micelle formation, and thus can be used as solubilizing agents. Generally, the surfactant power of ILs are in the cation (long alkyl side chains at the cation) while anions promote the solubilization of ILs in water [8].

As observed with traditional surfactants, by increasing the size of the hydrophobic group (increase of the alkyl chain) decreases the CMC, *i.e.*, promotes the aggregation of ILs and micelle formation. The effect of temperature on the CMC of ILs is negligible, meaning that the CMC is kept constant in a broad range of temperatures [54,55].

2.3.1. Ionic liquids in studies involving proteins

As mention before, ILs can have many distinct characteristics, so they have been used in a large number of studies involving proteins (**Figure 13**) and that increase every year [56].

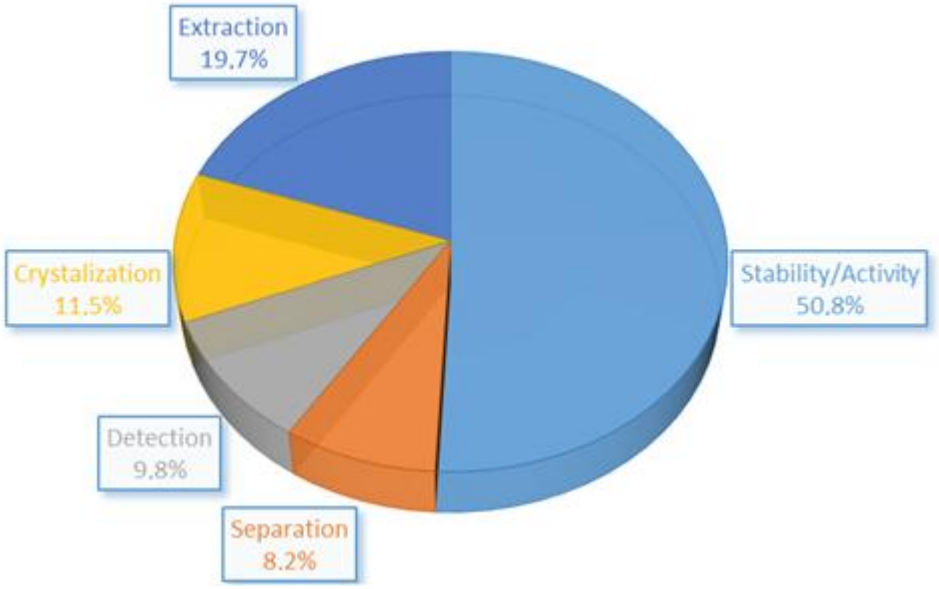


Figure 13 - *Distribution of publications on the use of ionic liquids in studies involving proteins.*

Enhanced ILs improve the stability and activity of proteins since they prevent aggregation and subsequent precipitation, so achieving better yields in the solubilization of proteins in their native state. Thus, ILs are often used as additives to aqueous solutions containing proteins [3,56].

In 1999, Garlitz and co-workers [57] used, for the first time, an IL (ethylammonium nitrate) as a precipitating agent for the crystallization of proteins for subsequent analysis by X-ray. Since then, several studies have been published showing that the use of ILs as precipitating agents or additives often leads to the formation of protein crystals with superior quality when compared to the ones obtained with traditional solvents. ILs allow to solve the problems in the crystal formation rate, crystal size and the tolerance for impurities in the crystallization medium [3,56,58].

Ionic liquids are also used to improve the separation of proteins (*e.g.* in capillary chromatography as running buffers) or in protein detection using ionic liquids as a matrix. SDS-PAGE display a better separation and efficiency in the presence of adequate ILs. The use of ILs in the mobile phase in reversed-phase chromatography revealed to be of great interest because it was possible to keep the native form of the protein. Finally, ILs can also

be used in high-performance liquid chromatography (HPLC) acting as functional groups in the stationary phase [3,56].

ILs may also be used for protein extraction, either directly or as additives. In 2008, the ionic liquid 1-butyl-3-trimethylsilylimidazolium hexafluorophosphate was used to extract Heme-proteins; it was observed that the iron atom of the heme group of hemoglobin interacted with the cation of the ionic liquid. This was the first report of a successful direct extraction of proteins using ILs [56,59]. In the same line,a two-phase aqueous system containing the IL 1,3-dimethylimidazolium chloride ([mim][Cl]) and K_2HPO_4 was successfully used to extract proteins from human fluids, and analyses such as FTIR and UV-vis spectroscopy showed that no changes on the protein structure occurred during extraction [3,60].In 2009, several ionic liquids were tested for direct extraction of yeast proteins [61]. The 3-(dimethylamino)-1-propylaminium formate led to the best results and, after the extraction, it was easily removed by vacuum evaporation.

It was found that ILs with longer alkyl chains have a higher protein denaturing power. It was also noted that in the study of interactions between enzymes and ILs, the combination of kosmotropic anions and chaotropic cations are the best solution [3,62,63].

2.3.2. Solubilization and extraction of membrane proteins with ionic liquids

In 2010, the ionic liquid [C_4mim] BF_4 (1-butyl-3-methyl imidazolium tetrafluoroborate) was used to extract membrane proteins from the brains of mice with subsequent analysis of the proteome by mass spectrometry [5]. Compared with traditional methods using SDS and methanol, the number of identified membrane proteins was three times more using the ionic liquid (with NH_4HCO_3 buffer pH 8.3). It was also possible to identify additional 25% and 80% of membrane proteins compared to the methods using Rapigest and urea, respectively, demonstrating the potential of ILs in the study of membrane proteins [5].

Already in 2014, the same group of scientists decided to follow the same method, but this time using different ILs [4]. The ability of ILs to solubilize the model membrane protein bacteriorhodopsin (seven transmembrane domains, α helices) was initially addressed. Several ionic liquids have been tested with different cations, anions and size of

alkyl side chain. The relative efficacy of bacteriorhodopsin solubilization promoted by different ILs shown in **Figure 14** [4].

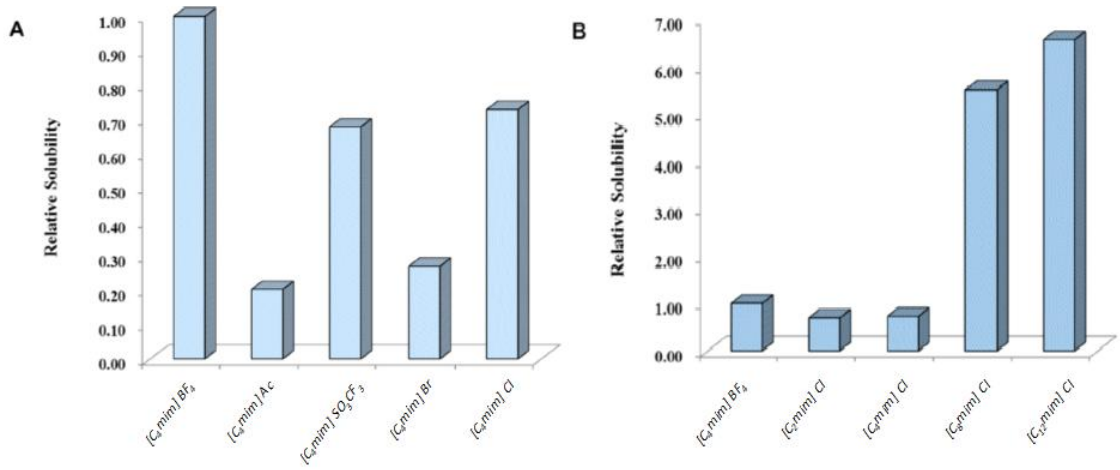


Figure 14 – *Relative solubility of bacteriorhodopsin in different ionic liquids. The Figure A compares the effect of anion and the Figure B the effect of alkyl chain length. C_4mim]BF_4 – 1-butyl-3-methyl imidazolium tetrafluoroborate; [C_4mim]Ac – 1-butyl-3-methyl imidazolium acetate; [C_4mim]SO_3CF_3 – 1-butyl-3-methyl imidazolium trifluoromethanesulfonate; [C_4mim]Br – 1-butyl-3-methyl imidazolium bromide; [C_4mim]Cl – 1-butyl-3-methyl imidazolium chloride; [C_2mim]Cl – 1-ethyl-3-methyl imidazolium chloride; [C_8mim]Cl – 1-octyl-3-methyl imidazolium chloride; [C_{12}mim]Cl – 1-dodecyl-3-methyl imidazolium chloride [4].*

The best IL anion to solubilize the bacteriorhodopsin is tetrafluoroborate, followed by the chloride. Since ILs composed of chloride are more hydrophilic, the researchers focused on this group of ILs and went on to modify the size of the alkyl chains of the imidazolium cation. They concluded that longer alkyl chains have greater solubilizing capacity, for example with [C_8mim]Cl and [C_{12}mim]Cl a solubility of 6-fold and 7-foldrelative to [C_4mim]BF_4 was obtained, respectively. This is because ILs derived from [$C_n$mim]$^+$ begin to aggregate and form micelles when $n \geq 8$, an important factor in solubilization. Ionic liquids based on pyridinium (another type of cation) were also tested, but some incompatibility was found with the further digestion by trypsin. Still with ILs 1-butyl-3-methylpyridinium tetrafluoroborate ([C_4mpy]BF_4) and 1-butyl-3-methylpyridinium chloride ([C_4mpy]Cl) relative solubilities of 0.64 and 6.14 were obtained, respectively [4,64].

The best IL for solubilising bacteriorhodopsin was [C_{12}mim] Cl, which, compared with traditional surfactants such as SDS, SDC (sodium deoxycholate), Rapigest, urea, methanol, CHAPS and Triton X-100, proved to achieve a better membrane protein yield and also to be more compatible with trypsin digestion. Finally, the researchers have obtained a proteome of rat brain and using this IL it was possible to identify more membrane proteins (plus 1.4 times compared with SDS) and more hydrophobic peptides (plus 3.5 times compared with SDS) [4].

In 2010, Merck launched a patent for the use of ILs for the extraction of membrane proteins. Their invention relates to the use of ILs or mixtures containing at least one IL and another solvent to extract membrane proteins from biological samples (tissue, cell culture, fluids, bacteria, fungi, viruses and plants). In this invention, a special attention was given to ILs of the general formula K^+A^-, where the anions A^- are halides, tetrafluorobarate, hexafluorophosphate or imides and the cations K^+ are based on ammonium, phosphonium, uranium, thiuronium, guanidinium or heterocyclic cations [65].

2.3.3. Aqueous two-phase systems

The aqueous two-phase systems (ATPS) normally results when two polymers, a polymer and a salt, or two salts are in an aqueous solution, and where, above a certain concentration, split into two phases. They are used for extraction and purification (liquid-liquid) of numerous biomolecules. The great advantage of these systems is that they are mainly made up of water and non-volatile compounds and are therefore considered "environment friendly" [7,51,52].

In 2003, Rogers and co-workers have proposed the use of ionic liquids to form ATPS [66]. The systems created with ionic liquids have advantages over traditional ATPS not have, such as low viscosity and short time required for phase separation. In a system with an ionic liquid and a salt in water, after phase separation, usually the top phase is rich in ionic liquid and the bottom phase is rich in salt [66].

Generally, the ATPS are ternary systems and can be represented in a triangular phase diagram but, usually the water concentration is omitted and the phase diagrams of ATPS become orthogonal (**Figure 15**). Whenever a given mixture is above the binodal curve (D-

C-B-A) phase separation occurs. For a given mixture M, after phase separation, one phase has the composition indicated by D (phase rich in ionic liquid) and the other phase have the composition B (phase rich in salt), which are terminals of a tie- line (TL). The tie-line length (TLL) is a numerical value that corresponds to the composition difference between the phases. Point C is the critical point of the ternary system, i.e., it is the point at which the two phases have the same composition [7].

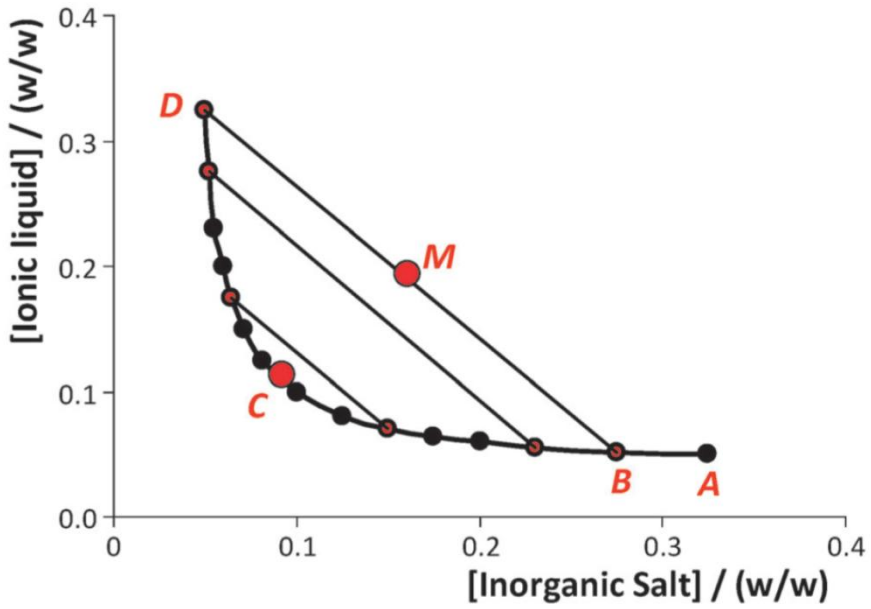


Figure 15 - *Orthogonal phase diagram for a hypothetical ATPS [7].*

There are several factors which influence the phase diagrams and the compositions of each phase determined in ATPS, such as the structure of the ionic liquid, the salt used, the pH and temperature. Regarding the ionic liquid, we have the influence of the cation, the anion and the size of the alkyl chain and/or functional groups. In general, for ATPS formed by ionic liquids and salts, quaternary cations (e.g. $[P_{4,4,4,4}]^+$ and $[_{N4,4,4,4}]^+$) are those that have a greater ability to promote phase separation, followed by ionic liquids derivatives of pyridinium and imidazolium. The larger the size of the alkyl chains of the cation, the greater the hydrophobicity of the ionic liquid and thus the greater the ability to occur phase separation. As regards to the anion, in general, the ability to form ATPS decreases with the ability of the anion to donate electron pairs and establish hydrogen bonds with water [7,51].

Regarding to salts in ATPS, the greater its power to salting-out, the lower the amount of salt needed for phase separation to occur. Finally phase separation occurs more easily at a higher pH and lower temperatures [7,51,67].

2.4. Production of Hen antibodies

The outstanding ability of antibodies to recognize small specific structures, have made them an indispensable tool in several applications in the areas of research, diagnostic and therapy.

The immunization of an individual can be either active or passive. The active immunization is when the individual is exposed to an antigen and then generate specific antibodies in response. This response can take days or weeks. The passive immunization is when individual antibodies (produced in other individual) are provide to protect the second individual. It provides a quick response but give a short-lived protection, lasting from several weeks to four months at most [10,68].

The production of antibodies normally requires the use of model laboratory mammals. Nowadays, common animals used to produce polyclonal and monoclonal antibodies are rabbits and mice, respectively. This procedure involves not only immunization steps, that cause pain and distress to the animal, but also the repeated bleeding [10,69–71].

In 1893, Klemperer demonstrates that a hen transfers the antibodies from the serum to the egg yolk. But only when the animal welfare became an issue, this results gained attention, almost a century after. During the egg formation, IgY is selectively transferred to the yolk while IgA and IgM are transferred to the egg white in order to provide protection to the developing chick - **Figure 16**. So, to recovery the IgY (or the specific IgY, if the hen was previous immunized) it is only necessary to recovery the eggs, and there is no need of invasive methods [68].

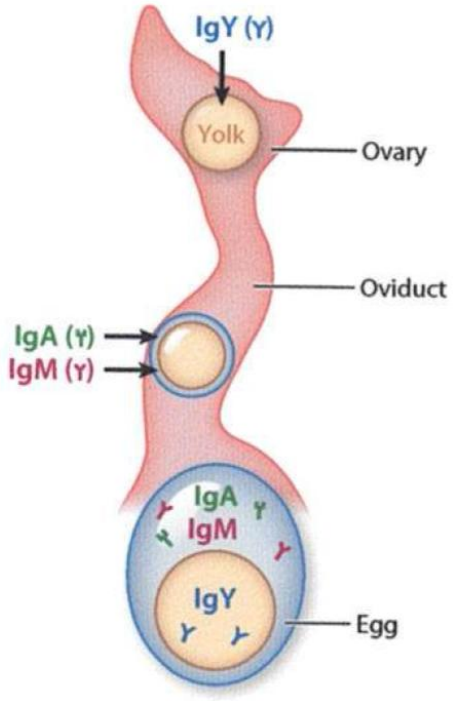


Figure 16 -*Transfer of antibodies during the formation of the egg [68].*

The phylogenetic distance between hens and mammals makes possible the production of antibodies against highly conserved mammalian proteins (impossible to produce in mammals). Other advantage of hen antibodies is the need of less antigen to immunize the hen and the animal care cost is also lower. More advantages are described in **Table 4** [10,70,71].

Table 4 - *Comparisons of rabbit IgG and chicken IgY [10,70,71].*

	Rabbit (IgG)	Chicken (IgY)
Antibody sampling	Invasive	Non-invasive
Antibody amount	200 mg/40mL blood	100-150 mg/egg 5-7 eggs/week
Antibody amount/month	200 mg	1000-2800 mg
Specific antibody yield	≈ 5%	2-10%
Interference with mammalian IgG	Yes	No
Interference with human anti-mouse antibody	Yes	No
Interference with mammalian complement	Yes	No
Protein A/G binding	Yes	No

IgY is composed of two identical heavy (H) and light (L) chains linked by a disulfide bridge having a molecular weight of 180 kDa. The light chain consists of one variable domain (VL) and one constant domain (CL) and the heavy chain is composed of one variable domain (VH) and four constants domains (CH) (one more than in IgG, that only has a molecular weight of 150 kDa – **Figure 17**). IgY has a shorter and a less flexible hinge region (HR) that is a carbohydrate chain that bonds the Fab and Fc fragments [68,69].

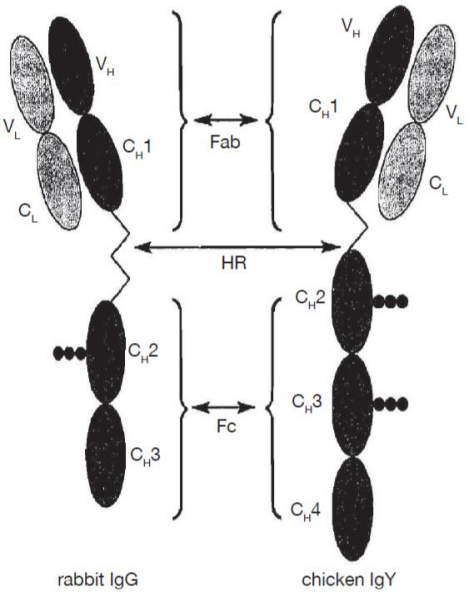


Figure 17 *- Differences between mammal IgG and hen IgY [69].*

IgY has an isoelectric point between 5.7 and 7.6 (lower than IgG – 6.1 to 8.5) and, because of the larger Fc fragment, IgY is more hydrophobic than IgG. Between the pH of 4 and 11 there is no activity loss observed. The IgY is very stable until a temperature of 70 °C, above that occurs loss of activity and then the denaturation of the protein. Freezing and freeze-drying also not affect the activity of the antibody, unless repeated several times [68,69].

As mention before, specific IgY can be achieved by immunizing hens (mature) with the target antigen. Many factors influence the response to the antigen and the IgY production: 1) The antigen – the type of the antigen, the size and the amount injected on the hen; usually, only 10 to 100 μg of protein should be used. 2) The adjuvant – Freud's complete

adjuvant (FCA) is the most used adjuvant, being the most effective too. In mammals, FCA can cause many inflammations in the injection site, however, hens seems to be more resistant to this adjuvant. To overcome this problem, Freud's incomplete adjuvant (FIA) can be used. 3) Route of application – The most common route for antigen injection is intramuscular, preferably in the breast muscle, but can also be in the leg however this type of route can lead to lame. Subcutaneous injection can also be performed, but leads to a longer response and is more distressful to the hen. 4) Immunization frequency and interval between immunizations – it depends on the type of antigen, but it is always necessary two immunizations steps. After 14 days from the last immunization, the titter should be checked and if it is in decay another boost must be given. Normally, the interval between immunizations is between two and four weeks [69,70].

The fists step on IgY recovery and purification consists on the egg yolk from the egg white - **Figure 18**. Although IgY is the only immunoglobulin in egg yolk there is lipids and other proteins too. Several methods can be used to purify IgY from the yolk 1) centrifugation – separates the soluble fraction (IgY) of proteins from the insoluble ones; 2) Water dilution method – results in the aggregation of yolk lipoproteins (better results at pH 5.0, 6-fold water dilution); 3) precipitation of lipoproteins – using polyethylene glycol (PEG) or anionic polysaccharide; 4) removal of lipoproteins – using organic solvents [68–70]. However, the choice of the most adequate method to purify IgY depends on several factors, such as, the desirable purity or activity of IgY, the scale of application (laboratory or industrial), cost effectiveness and required technology [69].

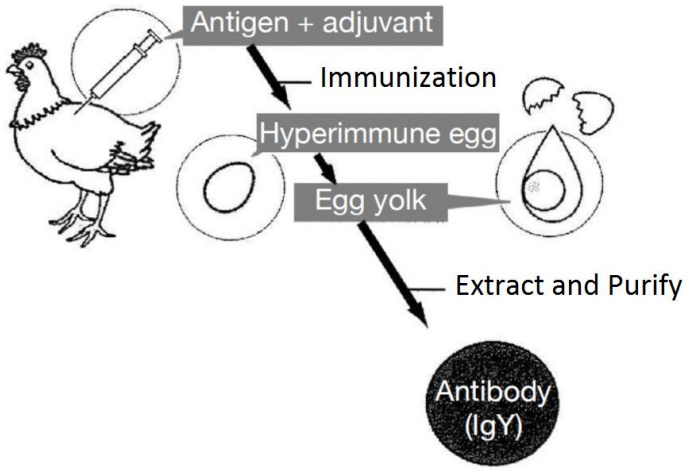


***Figure 18** - Standard protocol for IgY production.*

3. Experimental methods

3.1. Materials

Commercial detergents DM, DDM, OG and LDAO were supplied by Anatrace (SOL-GRADE). *All trans*-retinal was supplied by Sigma-Aldrich (purity, 98%).

3.2. Denaturing gel electrophoresis

The analysis of the proteins profile was made by electrophoresis on polyacrylamide gel under denaturing conditions using sodium dodecyl sulphate. The protein samples were prepared in Loading Buffer 6x [350 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.6 mM DTT, 0.012% (w/v) bromophenol blue] and analysed by 12% PAGE with the following composition: 1.65 mL distilled deionized water, 1.25 mL 1.5M Tris pH 8.8, 2.0 mL 30% acrylamide/bisacrylamide solution – Grisp, 50 µL 10% (w/v) SDS, 50 µL 10% (w/v) ammonium persulfate and 2.5 µL TEMED (N, N, N',N' – Tetramethylethylenediamine – Sigma-Aldrich) and stacking gel composed by 1.05 mL distilled deionized water, 190 µL 1.5M Tris pH 6.8, 250 µL 30% acrylamide/bisacrylamide solution – Grisp, 15 µL 10% (w/v) SDS, 15 µL 10% (w/v) ammonium persulfate and 2.5 µL TEMED. As protein standards Precision Plus Proteins™ Unstained – BIO-RADwas used and the electrophoresis was conducted at 120-180V in running buffer [0.2 M Tris Base, 0.2 M Bicine and 0.1% (w/v) SDS]. At the end of the run, the gel was cleaned with water and stained using PageBlue Protein Staining Solution from Thermo Scientific.

3.3. Western Blot

For the detection of the His-tag a specific antibody (THE™ His Tag Antibody, mAb, Mouse) was used as well a secondary antibody (Anti-Mouse IgG+IgM, whole molecule − Alkaline Phosphatase antibody produced in goat) – GE HealthCare.

After electrophoresis, carried out in the same way to that described in section 3.2. (but using Precision Plus Proteins™ All Blue – BIO-RAD as protein standards), the gel was

incubated in a transfer buffer [25 mM Tris Base, 192 mM Glycine, 0.025% (v/v) methanol] and a nitrocellulose membrane, 0.45 µm – BIO-RAD was pre-soaked in water and then incubated in the transfer buffer. Protein samples were transferred from the gel to the nitrocellulose membrane for 12 min at 25 V using a Trans-Blot®Turbo™ – BIO-RAD unit and a transfer cassette assembled according to the manufacturer.

After the transfer process, the nitrocellulose membrane was incubated in TBS-T [20 mM Tris pH 7.6, 137 mM NaCl, 0.2% (v/v) Tween 20] supplemented with 5% (w/v) milk for 1 hour, with constant agitation, to block the membrane. The primary antibody was diluted (1:10000) in TBS-T and 0.5% milk and the membrane transferred to this solution and incubated for 1 hour. Then, three washing cycles were performed with TBS-T and 0.5% milk (10 minutes each) to remove the excess of primary antibody. The incubation with the secondary antibody (1:10000) was carried out in TBS-T and 0.5% milk for 1 hour. Then, three additional washes were performed with TBS buffer (no milk). WB development was carried out using ECF substrate (alkaline phosphatase dephosphorylates) a chemifluorescent substrate and evaluated in a fluorescence scanner Molecular Imager FX Pro Plus MultiImager System – BIO-RAD.

3.4. Dot-blots

Dot-blot analyses were performed by pipetting the protein samples (1 to 6 µL) onto a nitrocellulose membrane. After allowing the membrane to dry, the following steps were identical to those described for the western blot experiments.

3.5. Agarose gel electrophoresis

DNA samples were analysed on an agarose gel [0.8 to 1% (w/v) agarose in TAE buffer (40 mM Tris pH 8.0, 1 mM EDTA and 0.11% (v/v) glacial acetic acid)] with 0.3 µg/mL ethidium bromide. The electrophoresis was carried out at 100 V for 25 to 30 minutes in TAE buffer using as molecular marker the GeneRuler 1 kb DNA Ladder – Thermo Scientific and the DNA samples were diluted on Loading Buffer (6x DNA Loading Dye – Thermo Scientific). After the run was completed, DNA was visualized by ethidium fluorescence and image acquired using the Gel Doc™ XR+ system (BIO-RAD).

3.6. Preparation and isolation of plasmid DNA

A single colony was inoculated in 5 to 10 mL of LB medium supplemented with the proper antibiotic and incubated at 37 °C overnight. The DNA was then extracted and purified using the commercial kit NZYMiniprep – NZYTech according to the manufacturer's instructions.

3.7. DNA Gel Extraction

The bands from Agarose Gel Electrophoresis containing the DNA of interest were excised from the gel (minimizing the UV exposer to prevent DNA damage). The DNA was extracted from the gel using the commercial kit NZYGelpure – NZYTech according to the manufacturer's instructions.

Using this kit it is also possible to purify DNA from the PCR.

3.8. Bacterial strains

During this work three *Escherichia coli* strains were used: *E. coli* DH5α, *E. coli* BL21 (DE3) star and *E. coli* C43 (DE3). These strains were cultured in LB medium or LB-Agar medium [1.5% (w/v) Agar-Agar].

3.9. Preparation of *E. coli* competent cells

In order to obtain E. coli DH5α, E. coli BL21 (DE3) star and E. coli C43 (DE3) competent cells, each strain was inoculated from a glycerol stock in 5 mL LB medium and grown at 37°C overnight with shaking. After the growth, 2.5 mL of this culture were inoculated on 250 mL R-LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.04% (w/v) NaOH and 0.24% (w/v) $MgSO_4$] and allowed to grow under the same conditions until the Optical Density (OD) at 600 nm reach 0.4 - 0.6. The cells were collected by centrifugation at 3000 rpm for 5 minutes and the supernatant discarded. The

pellet was resuspended in 100 mL of TFB I [30 mM $C_2H_3KO_2$, 100 mM RbCl, 10 mM CaCl2, 50 mM MnCl2, 15% (v/v) glycerol, pH 5.8] and incubated on ice for 5 minutes. The cell suspension as centrifuged again at 3000 rpm for 5 minutes and then, the cells were resuspended in 10 mL TFB II [10 mM MOPS, 75 mM CaCl2, 10 mM RbCl, 15% (v/v) glycerol, pH 6.5]. The cell suspension was incubated on ice for 30 minutes and then divided into aliquots of 130 µL. The aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

3.10. Transformation of *E. coli* competent cells

The transformation of cells was carried out using the heat shock method. At one aliquot of competent cells (130 µL) were added 1 µL of plasmid DNA or 10 µL of PCR product and the mixture incubated on ice for 30 minutes. The heat shock was induced by transferring the cells rapidly to 42 °C during 60 seconds and then incubated on ice for 3 more minutes. To recovery the cells, 1 mL of pre warmed LB medium was added to the mixture and tubes placed at 37 °C for one hour. Finally, the cells were collected by centrifugation at 16100*g* for 30 seconds and then 900 µL of the supernatant were removed. The cells were resuspended in the remaining solution and spread on LB-Agar supplemented with the proper antibiotic. The petri dishes were placed at 37 °C overnight for colony growth.

3.11. pCoofy linearization

In order to proceed with SLIC cloning, the selected cloning vector [pCoofy 1 (6xHis – N-terminal) was linearized using the LP1 (3C) and LP2 (ccdB) primers. The components and conditions of the PCR reaction are summarized in **Table 5** and **Table 6**.

Table 5 *- PCR reaction used for linearization of pCoofy 1.*

Reagents	Amount
LP1 (3C) primer	1 µM
LP1 (ccdB) primer	1 µM
dNTP-Mix	400 µM
pCoofy	25 ng
5x GC buffer	1x

Phusion Polymerase (2 U/µL)	1 U
Sterile distilled water	Up to 50 µL

Table 6 *- Conditions for PCR reaction.*

Step		Temperature / Duration
Initial denaturing		94 °C / 3 min
30 Cycles	Denaturing	94 °C / 30 s
	Annealing/Extension	72 °C / 90 s
Final elongation		72 °C / 10 min

The PCR product was treated with DpnI to digest the template DNA (vector non-linearized) and purified using the commercial kit NZYGelpure – NZYTech according to the manufacturer's instructions.

3.12. SLIC reaction

In order to clone the gene of interest in the selected expression vector a SLIC reaction strategy [34] was used (described in section 2.1.6.). In the case of pCoofy 1, the insert must have on 5' end a 3C site and on 3' end a ccdB site. The reaction is mediated by RecA enzyme performed as described in **Table 7**.

Table 7 *- SLIC reaction for cloning.*

Reagent	Amount
pCoofy linearized	100 ng
Insert	Molar ratio vector:insert = 1:3
RecA (2 µg/mL)	2 ng
RecA buffer 10x	1x
Sterile distilled water	Up to 10 µL

The reaction was incubated at 37 °C for 30 minutes and then used to transform E. coli DH5α competent cells.

3.13. Insert amplification

The genes of interest (GOI) were amplified using specific primers in order to flank them with adequate nucleotide sequence for proper recombination reaction. PCR reaction and conditions used are described in **Table 8** and **Table 9**. Genomic DNA was obtained by boiling bacterial cell extracts for 5 to 10 minutes.

Table 8 - *PCR reaction used for amplification of GOIs.*

Reagent	Amount
DNA Template	1 to 5 µL
Primer 1	0.5 µM
Primer 2	0.5 µM
dNTP-Mix	200 µM
5x GC buffer	1x
Phusion Polymerase	1 U
Sterile distilled water	Up to 50 µL

Table 9 - *Conditions for PCR reaction.*

Step		Temperature / Duration
Initial denaturing		98 °C / 5 min
25 Cycles	Denaturing	98 °C / 30-50 s
	Annealing	50-72 °C / 30-50 s
	Extension	72 °C / 50-60 s
Final elongation		72 °C / 5-10 min

3.14. Primers

All primers - **Table 10** (Integrated DNA Technologies) were designed respecting the following criteria: Tm = 55-65 °C, %GC > 40% and at least one G or C on 3’ end.

Table 10 *- Primers used for cloning strategies and colony PCR.*

Primer	Sequence (5'-3')
OmpF FWD	AAGTTCTGTTCCAGGGGCCCGCAGAAATCTATAACAAAGATGGCAACAAAGTAGATCTGTACG
OmpF REV	CCCCAGAACATCAGGTTAATGGCGTTAGAACTGGTAAACGATACCCACAGC
OmpC FWD	AAGTTCTGTTCCAGGGGCCCGCTGAAGTTTACAACAAAGACGGCAAC
OmpC REV	CCCCAGAACATCAGGTTAATGGCGTTAGAACTGGTAAACCAGACCCAGAGC
HmBRI FWD	AAGTTCTGTTCCAGGGGCCCATGCCAGCACCAGGGAGCG
HmBRI REV	CCCCAGAACATCAGGTTAATGGCGTTAGTCGTCTGCAGGCGTTGC
HmBRII FWD	AAGTTCTGTTCCAGGGGCCCATGTTGCCACTCCAGGTGTCAAG
HmBRII REV	CCCCAGAACATCAGGTTAATGGCGCTAGTCAGCGGCTTCAGCGG
T7 Promoter	TAATACGACTCACTATAGGG
T7 Terminator	GCTAGTTATTGCTCAGCGG

3.15. Restriction free (RF) cloning

With RF cloning [72] there is no need of restriction enzymes. The insert act as mega-primer (the ends are the primers) and the plasmid act as template - **Figure 19**.

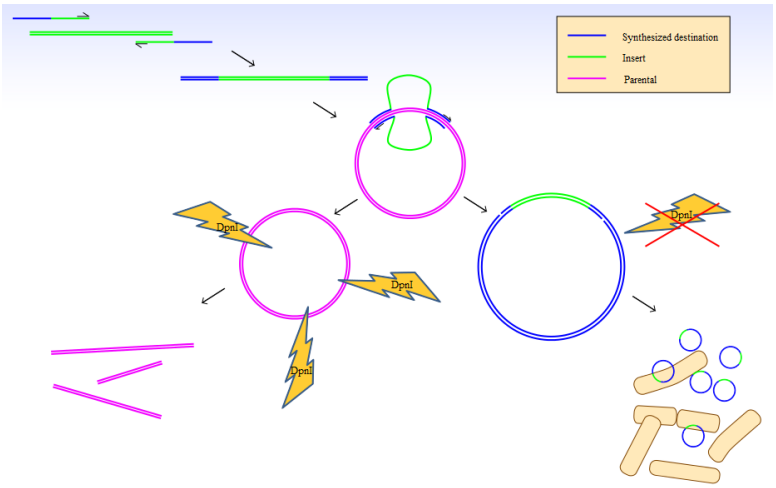


Figure 19 *– Schematic of a RF cloning protocol [72].*

The strategy adopted follows the reaction and conditions described in **Table 11** and **Table 12**. At the end, PCR reaction is treated with Dpn1 for digestion of the template (plasmid without insert) and then used to transform *E. coli* DH5 α.

***Table 11** – PCR reaction for RF cloning*.

Reagent	Amount
Circular vector	20 ng
Insert	100 ng
5x GC buffer	1x
dNTP-mix	200 µM
DSMO	0-3%
Phusion Polymerase	1 U
Sterile distilled water	Up to 50 µL

***Table 12** - Conditions of PCR reaction.*

Step		Temperature / Duration
Initial denaturing		98 °C / 5 min
5 Cycles	Denaturing	98 °C / 30 s
	Annealing	55 °C / 50 s
	Extension	72 °C / 7 min
25 Cycles	Denaturing	98 °C / 30 s
	Annealing	63 °C / 50 s
	Extension	72 °C / 7 min
Final elongation		72 °C / 10 min

3.16. Colony PCR

To check for positive clones, meaning clones harbouring the gene of interest, a colony PCR strategy was used. This is a high-throughput strategy for determining the presence or absence of inserted DNA in the plasmid construct. This strategy uses the T7 primers or the specific primers of gene amplification. Each colony to analyse was resuspended in 20 µL sterile distilled water and 3 to 5 µL used on the PCR reaction - **Table 13**. In the first step of the reaction occurs the cell lysate - **Table 14**. The PCR reaction was analysed on an agarose gel.

Table 13 - *PCR reaction for colony PCR.*

Reagent	Amount
DNA	3-5 µL
Primer 1	0.2 µM
Primer 2	0.2 µM
10x Dream Taq buffer	1x
dNTP-mix	200 µM
Dream Taq Polymerase	1 U
Sterile distilled water	Up to 25 µL

Table 14 - *Conditions for PCR reaction.*

Step		Temperature / Duration
Initial denaturing		94 °C / 5 min
18 Cycles	Denaturing	94 °C / 30 s
	Annealing	55 °C* / 30 s
	Extension	72 °C / 60 s per kb
Final elongation		72 °C / 10 min

*When specific gene primers were, the annealing temperatures were adapted in accordance.

3.17. OmpF Expression

E. coli BL21 Star (DE3) cells transformed with pCoofy1-OmpF plasmid were grown at 37 °C until OD 600 nm reach 1.0 and were then subjected to a cold-shock of 30 min on ice prior to expression induction with 1 mM IPTG (Isopropyl-β-D-1-thiogalactopyranoside), 2% (v/v) ethanol. The culture was further incubated at 37 °C for 4h (alternatively, at 18 °C overnight) to allow OmpF overexpression. The cells were harvested by centrifugation at 4000 rpm for 15 minutes and pellets stored at -20 °C or further processed.

3.18. Bacteriorhodopsins expression

E. coli C43 (DE3) cells transformed with pCoofy1-*Hw*BR plasmid or pCoofy1-*Hm*BRI/D94N plasmid were grown until OD 600 nm reach 1.0 and then induced the expression with 0.5 mM IPTG and 1 µM all trans-retinal at 37 °C for 5h. The cells were harvested by centrifugation at 4000 rpm for 15 minutes and the pellet stored at 4 °C.

3.19. Extraction and purification of bacteriorhodopsins

The bacterial pellet was resuspended in buffer A [150 mM NaCl, 50 mM Tris-HCl, pH 7.5] supplemented with proteases inhibitors [1 µg/mL leupeptin, 1 µg/mL pepstatin A, 0.1 mM PMSF (phenylmethylsulfonyl fluoride)] and cells were disrupted on a cell Emulsiflex-C3 homogenizer (3 cycles, 10000-15000 psi). The lysate was treated on a hot bath at 50 °C for 30 minutes and centrifuged at 20000 rpm for 30 minutes to remove the soluble fraction. The membrane pellet was resuspended in buffer A supplemented with 2% DM formembrane protein extraction overnight at 4 °C with gentle agitation. In the next day solubilized membrane protein fraction was separated by centrifugation from non-solubilized proteins and cell membrane debris.

The lysate containing detergent-solubilized membrane proteins was loaded on a 5 mL HisTrap™ HP column – GE Helthcare (or alternatively, 1 mL Bio-Scale™ Mini Profinity™ IMAC Cartridge column – BIO-RAD, or, Chelating Sepharose Fast Flow – GE Helthcare) equilibrated with buffer A supplemented with 0.2% DM using a peristaltic pump (BIO-RAD) or the AKTA Start – GE Healthcare. The elution was carried out with steps of imidazole solution (50mM, 150 mM and 500 mM) or with linear gradient (0-200 mM). The fractions of interest were concentrated (if necessary) until 1 mL or 500 µL using a concentrator Vivaspin 2 mL – Sartorius and further purifiedby size exclusion chromatography on a Superdex™ 200 10/300 GL column – GE Healthcare using buffer A with 0.2 % DM as the running buffer.. The equipments used were an AKTA Start and AKTA pure – GE Healthcare. All protein fractions were analysed using the NanoDrop® ND-1000 for total protein quantification.

For tag removal, after the affinity chromatography, the fractions of interest were pooled together and 3C HRV protease was added at 1:100 mass ratio and incubated at 4 °C overnight.

3.20. UV-Vis spectrometry

To obtain the UV-Vis spectrum of the bacteriorhodopsins an UV-Visible spectrophotometer CARY 100 Bio – Varian was used. To evaluate the extraction ability of the surfactants, the monitored relative absorbance variations between samples at 280 and 552 nm, using the NanoDrop® ND-1000 (1mm path), we monitored.

3.21. Surfactant Screening

To evaluate the extraction ability of the surfactants, the resuspended membrane pellet (obtained as previously described) was separated in 1mL aliquots in 2mL Eppendorf tubes. Then, a calculated amount of surfactant was added and extraction was allowed to occur overnight at 4 °C with gentle agitation. In the next day the tubes were centrifuged at 14000 rpm for 20 minutes and the supernatant was evaluated by UV-vis spectrometry both at 280nm and 552 nm). An extraction was performed with DM and all extractions were performed in duplicate.

3.22. Production of Anti-HmBRI antibodies

Two adult female quails were immunized following a standard protocol implemented in the lab, consisting of 1 immunization followed by 3 injection boosts. Injections were performed every 2 weeks and 50 µg of purified *Hm*BRI immunogen were administered *per* injection *per* bird. Injectable emulsions were prepared with 50% protein solution and 50% of Freud's adjuvant (complete in the first two immunizations, and incomplete on the other two) so that, 150 µL of emulsion contained 50 µg of protein.

3.23. Processing of hyperimmune eggs for isolation of Anti-HmBRI antibodies

During the immunization protocol, quail eggs were collected daily and yolk samples corresponding a two days of posture were prepared (1 sample = yolk pool of two days).

Yolk samples were subsequently processed by precipitation with 3.5% polyethylene glycol, MW 6000 (PEG 6000) (VWR Chemicals) in PBS (Phosphate-buffered saline) to remove major lipid content. Briefly, samples were diluted 20-fold and incubated with 3.5% PEG 6000 for 10 minutes with agitation and centrifuged at 13000 g at 4°C for 20 minutes; the supernatants were collected and an additional spin was performed to remove pellet contaminants. Finally, the total protein concentration of each yolk sample supernatant was measured using NanoDrop® ND-1000 Spectrophotometer and normalized with PBS for downstream ELISA assay.

3.24. ELISA assays

A F96 MaxiSorp Nun – Immuno Plate was coated over weekend at 4 °C with coating buffer (Carbonate buffer 0.05 M pH 9.6) with 2 µg/mL of antigen (100 µL/well) sealed with PCR film (µltraAMp Plate Seal – Sorenson™). The plate as then washed with a buffer [TBS (Tris-buffered saline) and 0.1 % Tween® 20] followed by blocking buffer [TBS, 0.1% Tween® 20 and 1% bovine serum albumin (BSA) – Capricorn Scientific GmbH] for 2 hours at room temperature. After, 3 washes were performed with washing buffer and then incubated with 100 µL of primary antibody (hen antibody) for 2 hours at room temperature. Washed again 3 times and incubated with the secondary antibody (α-chicken IgY (IgG), Peroxidase antibody, rabbit – Sigma-Aldrich) diluted 1:50000 for one hour at room temperature. Finally, the plate was washed and incubated with ABTS substrate (100 µg/well) and respective buffer – Roche Diagnostics GmbH for 30 minutes at 37 °C. The reactivity was analysed on BIO-TEK® PowerWave XS by measuring the absorbance at 405 nm.

4. Results and Discussion

5. The main goal of this work was to test the ability of several surfactants, including ILs, to extract and solubilize membrane proteins from biological membranes. For that, some model proteins were chosen and their overexpression in bacterial hosts was carried out.

5.1. Outer membrane proteins

The first model proteins chosen for overexpression were the Outer Membrane Protein F (OmpF) and the Outer Membrane Protein C (OmpC). Both proteins are porins that represent 60-90% of the total proteins in the outer membrane of *E. coli*, and OmpF in particular is the most abundant of all, being the first membrane protein that was crystallized [73]. The structures of these two proteins were already determined and are very similar (74% similarity) [74,75], composed of 16 antiparallel trans-membrane β-strands forming a β-barrel protein.

5.1.1. Cloning

The first step of the work was the cloning of these proteins to allow overexpression in E. coli. The genes of interest were amplified from the *E. coli* BL21 star (DE3) using the primers OmpF FWD and OmpF REV for amplification of OmpF gene and the primers OmpC FWD and OmpC REV for amplification of OmpC gene (sextion 3.14). This primers also flanks the gene of interest with specific DNA sequence that allows the use of RF cloning strategy or SLIC strategy. The PCR reaction and conditions used are described in section 3.13.

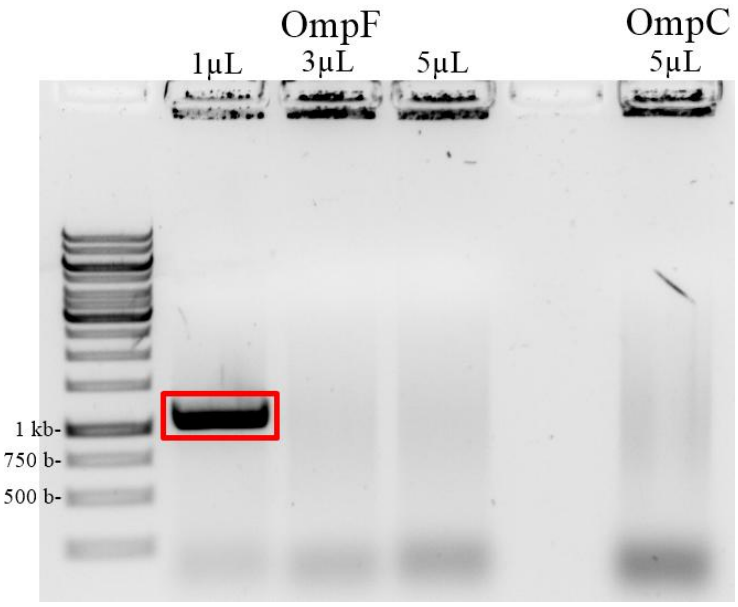


Figure 20 *– Agarose gel from OmpF (1067 bp) and OmpC (1085 bp) gene amplification. For OmpF several amounts of E. coli BL21 star (DE3) boiled cells were used. Apart from the OmpF using only 1 µL of boiled cells, no amplification occurred. The highlighted band was excised from the gel and purified according to section 3.7 yielding 18 ng/µL of insert.*

Figure 20 shows that the increasing of boiled cells seems to influence the PCR reaction. More attempts to amplify the OmpC gene were conducted, using only 1 µL of boiled cells and others temperatures of annealing, but again, no amplification occurred. This can be explained because the OmpC of *E. coli* BL21 star (DE3) as N-terminal truncated compared to other *E. coli* strains [76], so the primers used cannot amplify the OmpC gene from BL21 star (DE3).

Therefore, other gene amplifications were carried out using the same primers and PCR reaction and conditions, but using now the genomic DNA from *E. coli* DH5α (1 µL of boiled cells) – **Figure 21**.

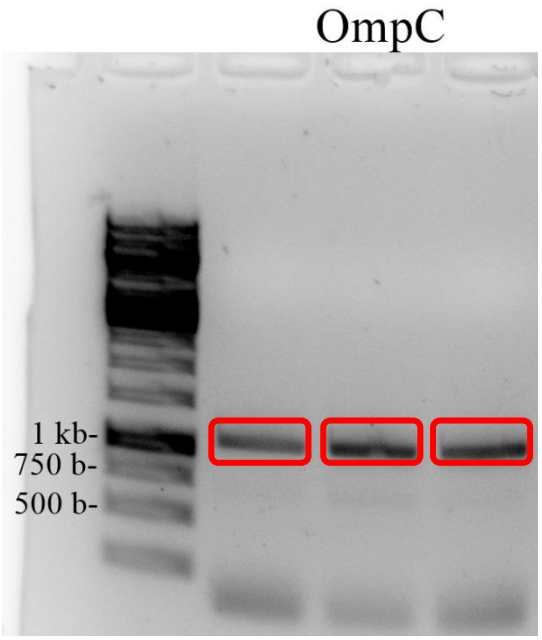


Figure 21 *– Agarose gel from OmpC (1085 bp) gene amplification using 1 µL of boiled E. coli DH5α. The highlighted bands were excised from the gel and purified according to section 3.7 yielding 17.3 ng/µL of insert.*

The expression vector chosen for cloning was the pCoofy1 that adds a six Histidine tag to the N-terminal of the target protein. The first strategy used for cloning was the Restriction-free cloning (section 3.15) using the OmpF and OmpC genes amplified previously as megaprimers (**Figure 21** and **Figure 22**). Two reactions for each cloning were made, one using 3% of DSMO and the other without DSMO. The colonies obtained were screened for gene insertion, but the colony PCR (section 3.16) did not reveal any insertion.

The other strategy for cloning was the SLIC reaction (section 3.12) that requires the previously linearization of the vector (section 3.11)

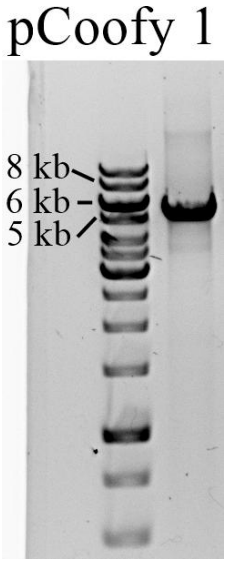


Figure 22 – *Agarose gel from the pCoofy 1 (5737 bp) linearization PCR reaction (5 µL) that removes the ccdB gene. The linearized vector was then purified from the PCR reaction according to section 3.7 obtained a 30.3 ng/µL of linearized vector.*

With a linearized vector and the two inserts amplified the SLIC reaction was conducted (section 3.12), one for each cloning, and six colonies of each reaction were screening for GOI insertion by colony PCR (section 3.16).

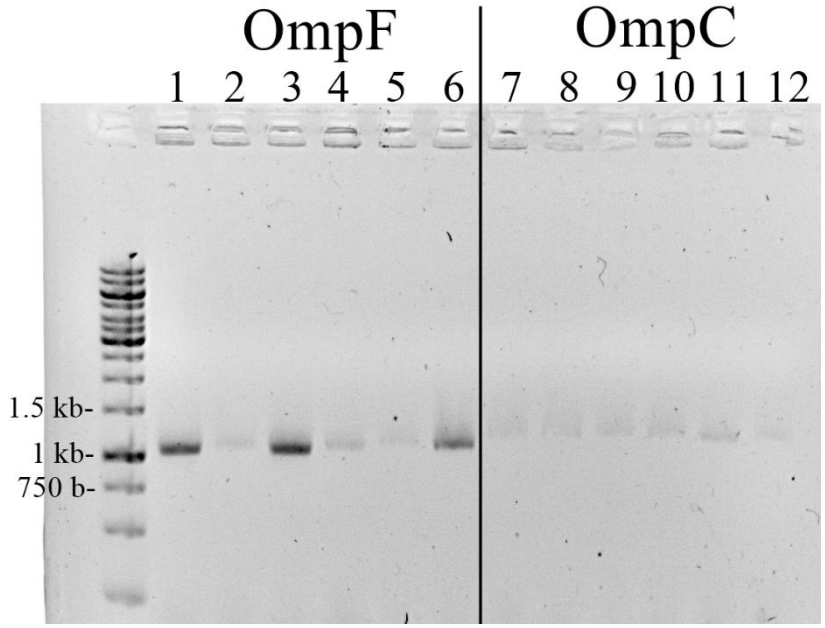


Figure 23 *– Agarose gel from colony PCR reaction using the specific primer for gene amplification; OmpF (1067 bp), OmpC (1085 bp). Although the majority of the bands shows weak intensity, the clones 1 and 3 for OmpF and 9 and 11 for OmpC were selected to perform a MiniPrep (section 3.6).*

The four clones were sent for sequencing but only clone 1 was confirmed. Even though this clone exhibits some conservative mutations, it can be explained because the gene was amplified from *E. coli* BL21 star (DE3) (when compared against the gene from *E. coli* K12). The amount of plasmid (pCoofy1-OmpF) obtained was 75.8 ng/µL.

5.1.2. OmpF Expression

Because only OmpF was successfully cloned, OmpC work was aborted and we proceeded with the optimization of 6His-OmpF (**Figure 24**) expression.

Analysis	Entire Protein
Length	360 aa
Molecular Weight	39372.43
1 microgram =	25.398 pMoles
Molar Extinction coefficient	48500
1 A[280] corr. to	0.81 mg/ml
A[280] of 1 mg/ml	1.23 AU
Isoelectric Point	5.03
Charge at pH 7	-11.61

Figure 24 *– Predicted protein parameters of 6His-OmpF using Vector NTI software.*

In order to find the best conditions to protein expression, two small scale expressions were performed, according to section 3.17; in the first one OmpF was expressed for 4h at 37 °C (**Figure 25**), and in the other, the protein was expressed overnight at 18 °C (**Figure 26**).

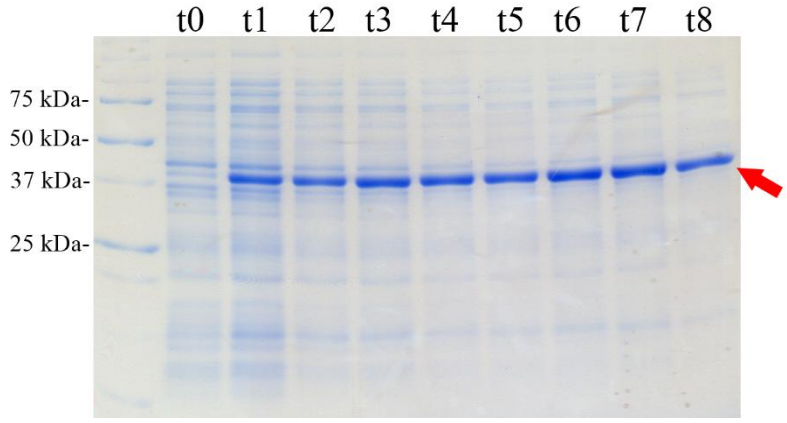


Figure 25 *– Time-course of OmpF expression (4 hours, 37 °C), the samples were taken every half hour and normalized for a same optical density. The red arrow indicates the expected band (around 39 kDa).*

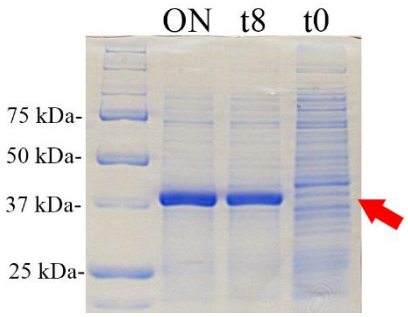


Figure 26 *– Comparison of the two expression trials. ON – overnight expression, t8 – at the end of the four hours expression, t0 – at the moment of induction for overnight expression. The samples were normalized. The red arrow indicates the expected band (around 39 kDa).*

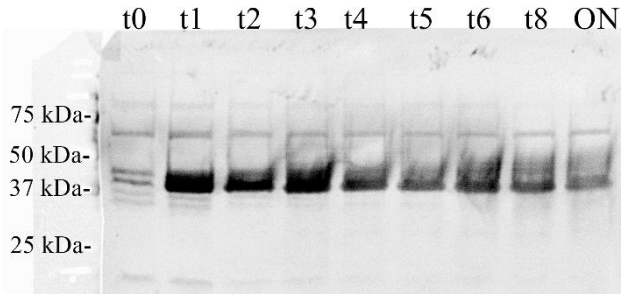


Figure 27 *– Western blot for time-course of OmpF expression, for 4 hours and overnight (ON).*

Both procedures seem to be adequate for overexpression of OmpF. Indeed it only takes half an hour to overexpress OmpF, being easy to detect the OmpF band on an SDS-PAGE protein gel, that was further confirmed by western blot (**Figure 27**).

5.1.3. OmpF purification

After reaching adequate results on the expression of OmpF, the purification was the next step to work on, using a similar method to that described by in Rouslan et al. [77], which in turn was based on Taylor research [78]. This method consists in the isolation of the outer membrane by solubilisation of the inner membrane.

For that, 500 mL of cell culture were expressed (section 3.17). The bacterial pellet obtained was resuspended in 20 mL of 50 mM Tris-HCl, pH 7.5. To 10 mL of this resuspension, protease inhibitors (1 µg/mL leupeptin, 1 µg/mL pepstatin and 0.1 mM PMSF) were added and cells were disrupted by high pressure using the Emulsiflex-C3 homogenizer until the solution becomes clear. A schematic description of the method is presented in **Figure 28**.

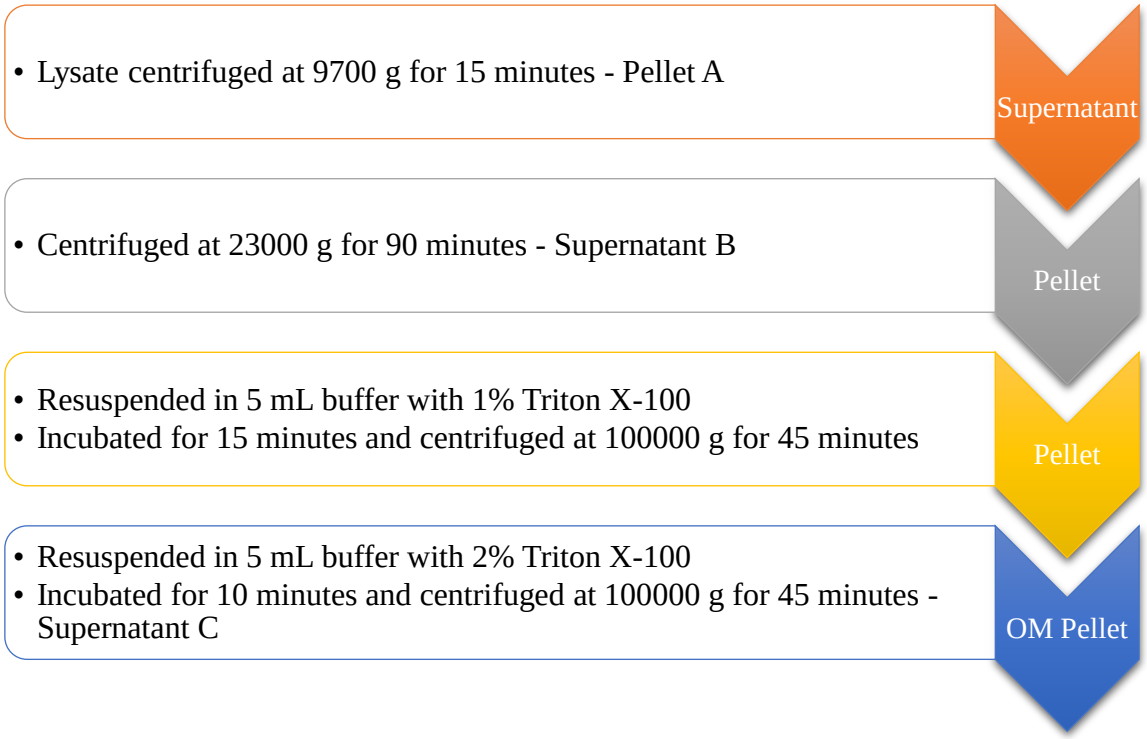


Figure 28 *– Scheme of the method for Outer Membrane isolation. The buffer was 50 mM Tris-HCl, pH 7.5 (adapted from* [77]*).*

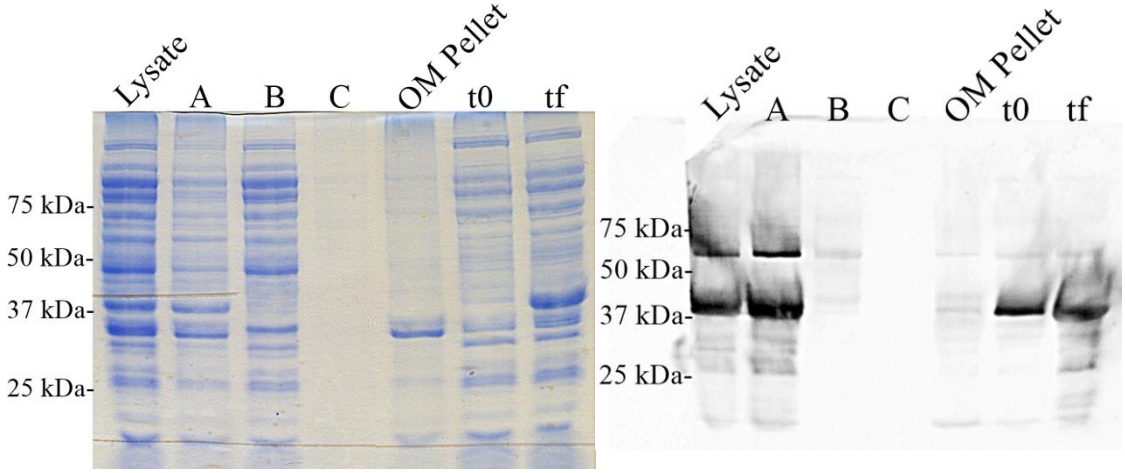


Figure 29 *– Protein gel and respective Western blot from OM isolation. A, B and C represents pellets or supernatants indicated in* ***Figure 28****. t0 and tf are the samples taken at the beginning and at the end of expression, respectively.*

The point of this method was to isolate the Outer Membrane for further extraction of OmpF. Unfortunately, by analysing the protein gel and the respective Western Blot (**Figure 29**) it can be conclude that OmpF is eliminated in the first centrifugation (at 9700 g). Despite that, at this speed, the membranes should not pellet [79]. One reason for this to occur is maybe due to the overexpression that could lead to the aggregation of the protein (inclusion bodies) meaning perhaps that the protein is unfolded.

Due to the failure in the first purification step this type of model protein was left aside and efforts were allocated in other membrane proteins, bacteriorhodopsins, since they are more appealing to characterize the extraction ability of surfactants.

5.2. Bacteriorhodopsins

As explained above (section 2.1.5.1), bacteriorhodopsins are membrane proteins present in the membrane of photoreceptive archaebacteria. Bacteriorhodopsins from *Haloarcula walsbyi* (*Hw*BR) and *Haloarcula marismortui* (*Hm*BRI) have 55% identity (**Figure 30**) and exhibit seven transmembrane domains (α-helix). The molecule *all-trans* retinal can be covalently linked to these proteins and is responsible for the characteristic purple color of bacteriorhodopsin-containing membranes. Since this feature reflects a stable and functional folding of bacteriorhodopsins, we decided to exploit it in our studies with novel surfactants for membrane protein extraction.

Figure 30 *– Sequence alignment of HmBRI and HwBR. The aligned was obtained using Clustal W* [80].

5.2.1. Cloning

Following a similarapproach as described for OmpF and OmpC, the first step on this section was to clone the genes to *Hm*BRI and *Hw*BR in pCoofy 1 vector. At this time, the gene amplification was not necessary since the genes used were synthetic (purchased from Invitrogen) with codon optimization for expression in *E. coli* and already flanked with the sequences required for SLIC strategy.

The SLIC reaction was carried out likewise for OmpF and OmpC, but this time using *Hw*BR and *Hm*BRI synthetic genes.

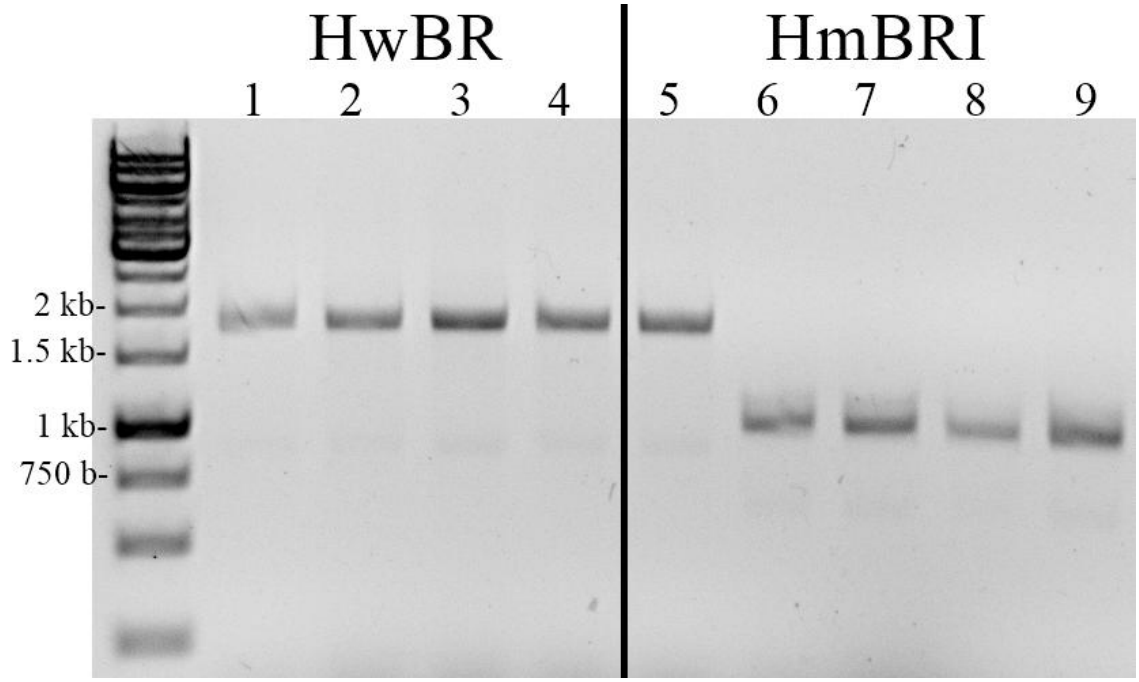


Figure 31 – *Agarose gel of colony PCR from SLIC reaction using T7 primers. Expected molecular size of amplified genes: HwBR (1091 bp); HmBRI (1079 bp).*

The colony PCR - **Figure 31**, shows four positive signals for *Hm*BRI and none for *Hw*BR. The plasmid DNA was isolated from clones 7 (127.7 ng/µL) and 9 (126.1 ng/µL) and sent to sequencing that indicated a sequence entirely correct for both clones.

Since the cloning of *Hw*BR failed, the SLIC reaction was repeated but this time using several ratios of vector:insert to promote the cloning.

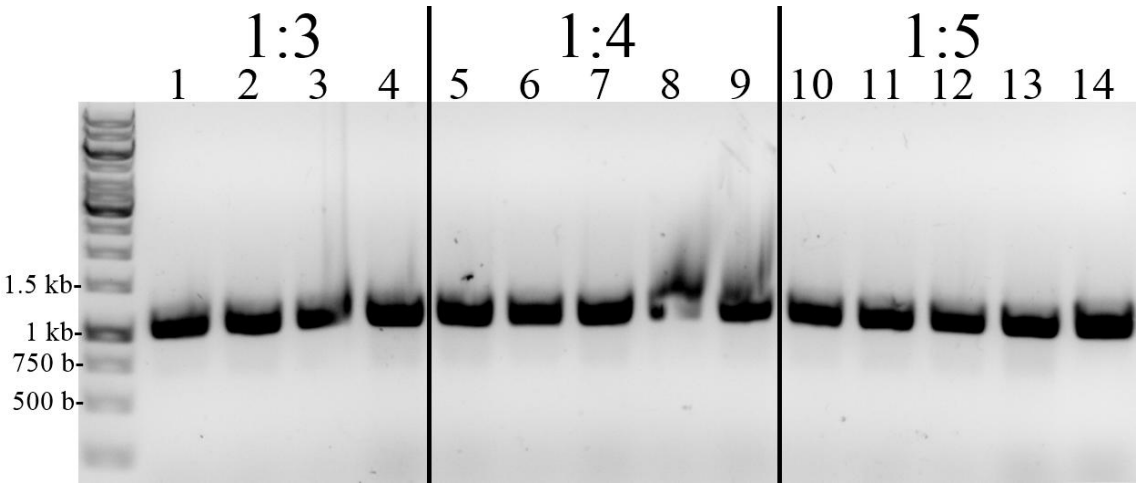


Figure 32 *– Agarose gel of colony PCR from SLIC reaction of HwBR (1091 bp) with several vector:insert ratios. Using T7 primers.*

This time, the cloning presented a 100% effectiveness. Thus, the plasmid DNA of clones 2 (74.9 ng/µL) and 7 (83 ng/µL) – **Figure 32**, was isolated and sent to sequencing The entirely sequence of clone 2 was not covered; yet for clone 7, the sequence was fully confirmed.

5.2.2. *Haloarcula walsbyi* bacteriorhodpsin (HwBR)

The expression and purification of bacteriorhodopsins were based on the work of Min-Feng Hsu [9] and are described in section 3.18 and section 3.19. *E. coli* C43, as well *E. coli* C41 strains, are commonly used to overexpress bacteriorhodopsins [81]. These bacterial strains present uncharaterized mutations that determine an increased resistance tooverexpression of membrane proteins and other toxic proteins.

Analysis	Entire Protein
Length	273 aa
Molecular Weight	29602.50
1 microgram =	33.781 pMoles
Molar Extinction coefficient	46940
1 A[280] corr. to	0.63 mg/ml
A[280] of 1 mg/ml	1.59 AU
Isoelectric Point	4.80
Charge at pH 7	-10.69

Figure 33 *- Predicted analysis of 6His-HwBR from Vector NTI.*

The expression pellet of *Hw*BR (**Figure 33**) did not become purple - fact that can be explained due to the low level of expression expected. Therefore, four litres of culture were expressed to produce more protein. The extracted membrane proteins (obtained according to section 3.19) were load on 1 mL column with Chelating Sepharose Fast Flow charged with Co^{2+} and the proteins eluted by steps (50 mM and 200 mM of imidazole) **Figure 34**.

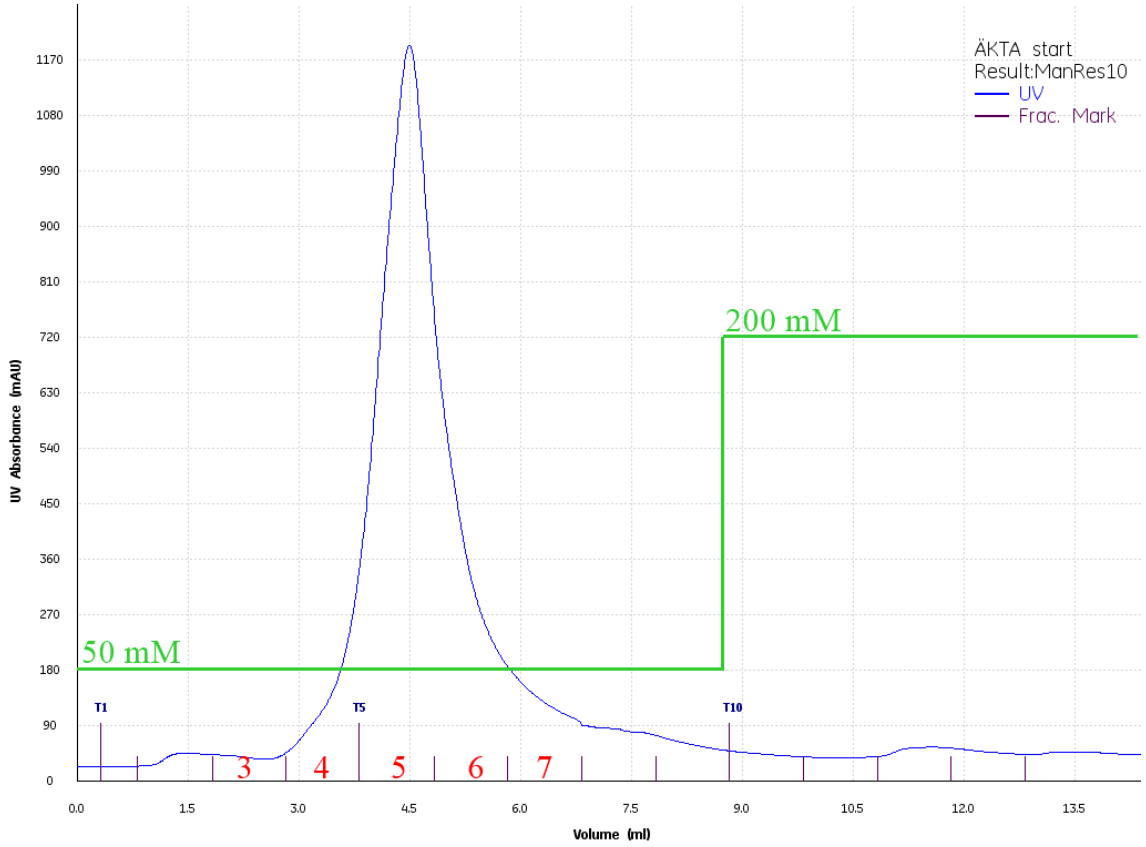


Figure 34 *– Affinity chromatography (IMAC) to purify HwBR. Elution usingsteps of imidazole.*

Table 15 *– Absorbance at 280 nm and 552 nm of the fractions of the affinity chromatography.*

Fraction	A280	A552
4	0.094	0.010
5	0.281	0.021
6	0.076	0.007

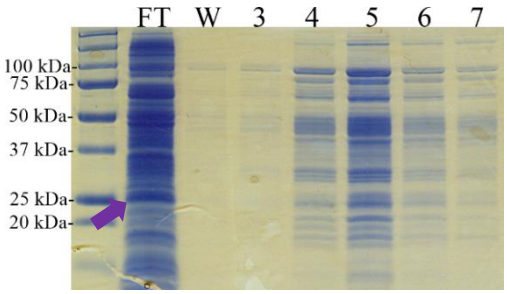


Figure 35 *– Protein gel of the fractions from affinity chromatography. The purple arrow indicates HwBR.*

Despite of fraction 5 was purple (**Table 15** and **Figure 34**), meaning that *Hw*BR was well folded and concentrated on that fraction, the affinity chromatography was not successfully accomplished. *Hw*BR was eluted on the first step of imidazole (50 mM) along with many other proteins, resulting in a fraction not so clean/pure **(Figure 35**). Nevertheless, this led to the conclusion that the protein can be easily tracked by its purple colour or by measuring its absorbance at 552 nm.

In order to get a fraction more pure, by affinity chromatography another batch of four litre of culture was prepared. This time,HwBR was eluted by linear gradient of imidazole – **Figure 36**.

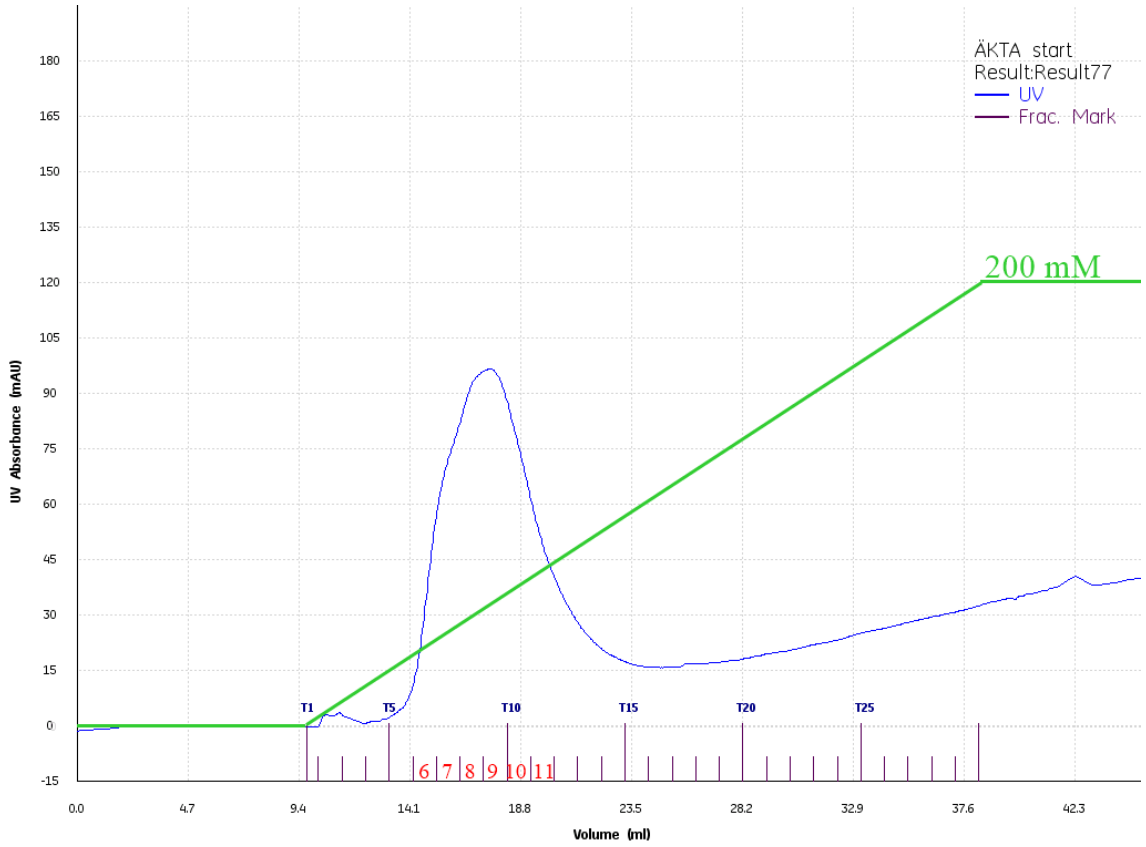


Figure 36 *– Affinity chromatography (IMAC) to purify HwBR. Eluted by linear gradient of imidazole.*

Table 16 *– Absorbance at 280 nm and 552 nm of the fractions of the affinity chromatography.*

Fraction	A280	A552
7	0.036	0.007
8	0.042	0.008
9	0.036	0.012
10	0.035	0.009

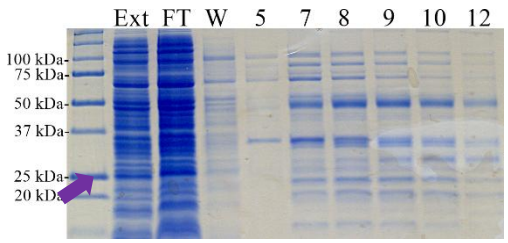


Figure 37 *– Protein gel of the fractions from affinity chromatography. The purple arrow indicates HwBR.*

Once again, the affinity chromatography was not successful. Despite that fractions 7 to 10 showed a purple colour (**Table 16**), the fractions remain very impure (**Figure 37**). The main problem seems to be the low binding capacity of His-tagged HwBR to the column.

However, the fractions that visually contained the *Hw*BR (purple ones) were pooled together and concentrated until a final volume of 500 µL and injected on a gel filtration column. The total protein injected was 0.94 mg with an absorbance at 552 nm of 0.025.

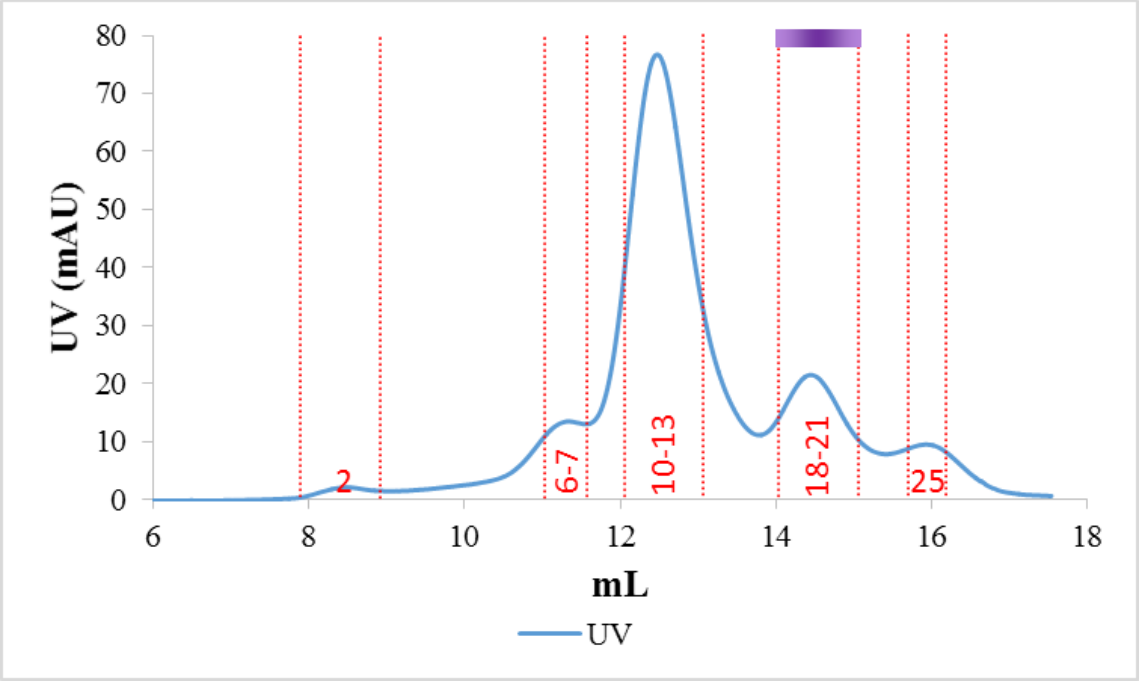


***Figure 38** – SEC profile of HwBR.*

***Table 17** - Absorbance at 280 nm and 552 nm of the fractions of size exclusion chromatography..*

Fraction	A280	A552
10-13	0.048	0.007
18-21	0.010	0.020

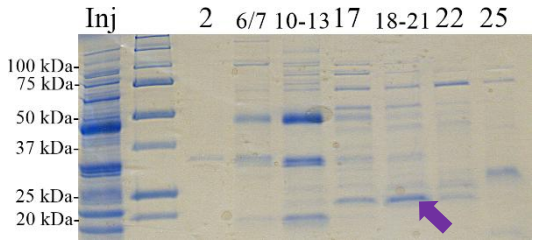


***Figure 39** – Protein gel from fractions of SEC. The purple arrow indicates HwBR.*

The SEC profile is not consistent with the one reported in the literature since this protein should exhibit only one peak [9]. This can be explained due to the poor affinity of the chromatographic column that is not able topurify the protein properly.

However, the fractions 18 to 21 of size exclusion chromatography were purple (Table 17), meaning the presence of *Hw*BR (confirmed by Western blot – **Figure 40**), and this peak is eluted at 14.5 mL as found in the literature [9]. At the end, a pool of the fractions with *Hw*BR was very unclean (**Figure 39**) With only 0.10 mg of protein.

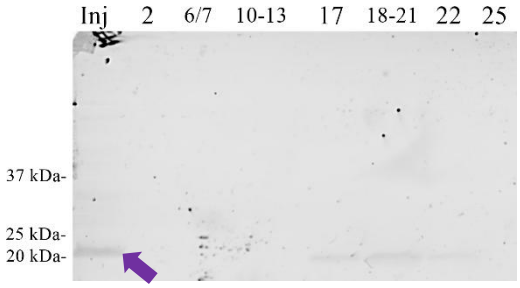


Figure 40 *– Western blot of SEC fractions. The purple arrow indicates HwBR.*

Finally, a last batch of 1 litre of culture was purified, but another column (5 mL HisTrap™ HP column) was used on affinity chromatography keeping the elution strategy (linear gradient) – **Figure 41**.

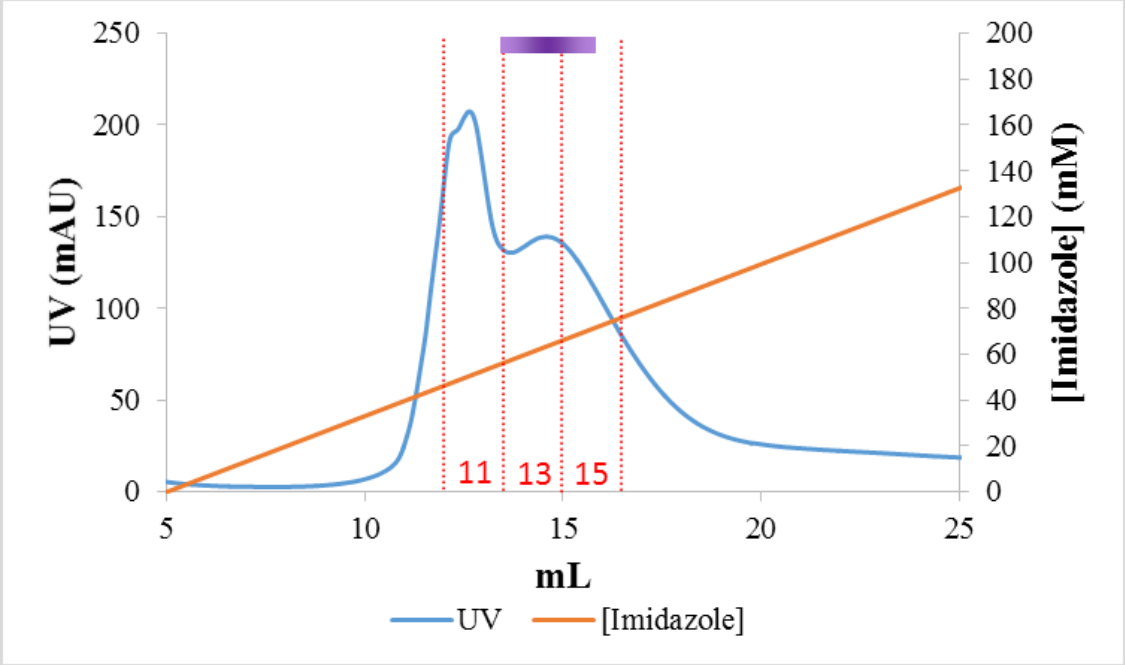


Figure 41 *– Affinity chromatography (IMAC) to purify HwBR.*

***Table 18** – Absorbance at 280 nm and 552 nm of the fractions of the affinity chromatography.*

Fraction	A280	A552
10	0.034	0.005
11	0.075	0.006
13	0.045	0.007
15	0.045	0.007

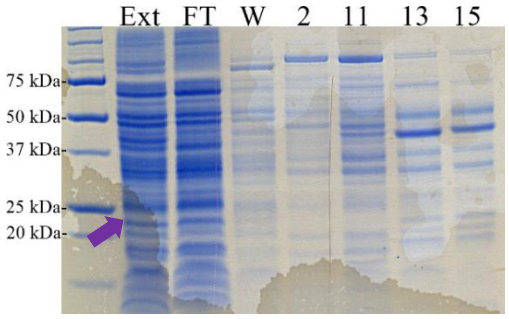


***Figure 42** – Protein gel from affinity chromatography fractions. The purple arrow indicates the HwBR.*

This time, the affinity chromatography performed better with a chromatogram displaying two peaks, so being able to separate some proteins of the fractions with *Hw*BR. Still, some protein is lost in the flow and some contaminants still remai in the fractions where *Hw*BR is eluted (**Figure 42**).

Once again, the purple fractions, 13 and 15 (**Table 18**), were pooled together and concentrated until 500 µL and injected on a gel filtration column – **Figure 43**. The total protein inject was 805 µg with an absorbance at 552nm of 0.022.

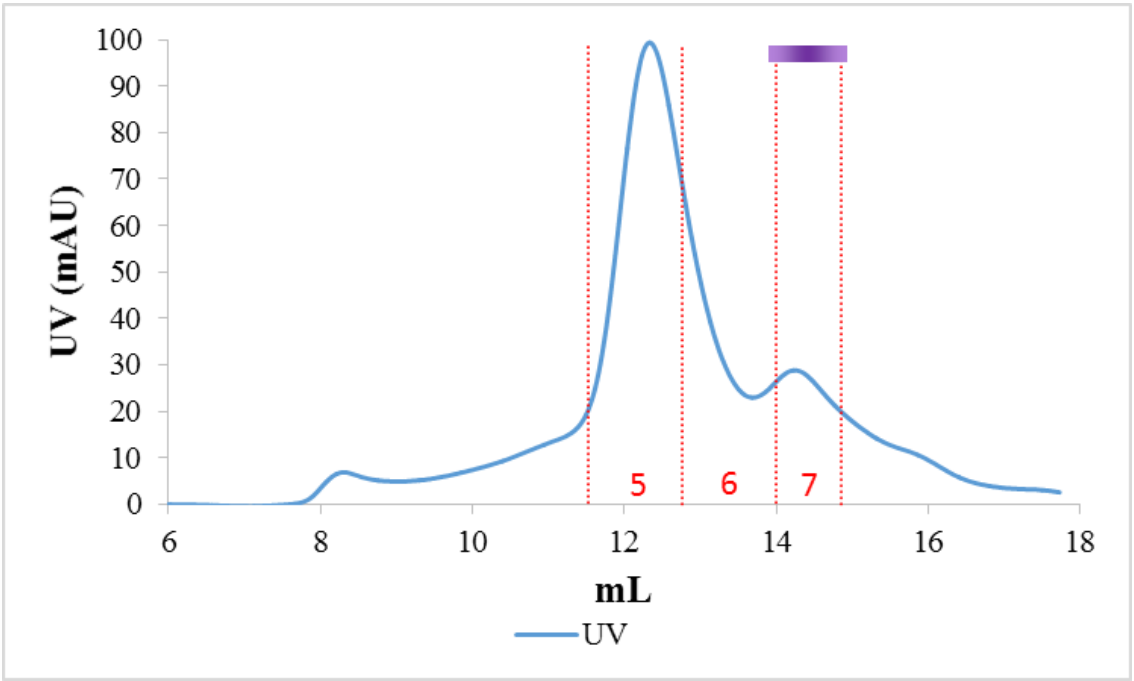


***Figure 43** – SEC profile of HwBR.*

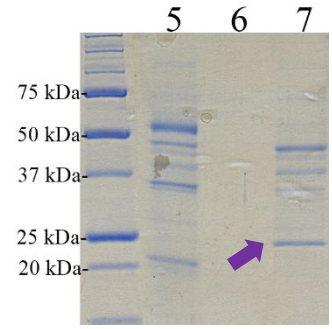


Figure 44 – *Protein gel from SEC fractions. The purple arrow indicates HwBR.*

The SEC profile obtained is very similar to the previous one. Once again the second peak (fraction 7) was purple, but this time the total amount protein obtained in this peak was 0.152 mg, probably due to a better binding of the *Hw*BR on the affinity chromatographic column. Still an improvement of the His-tag should be done because the fractions are very impure – **Figure 44**.

5.2.3. *Haloarcula marismourtui* bacteriorhodopsin (*Hm*BR)

Moving to the expression and purification of 6His-*Hm*BRI – **Figure 45**, expecting a better yield on expression, and as reported by Hsu et al. [9], a first batch of expression as made using 10 µM all-trans retinal. It was expected that the bacterial pellet in the end of expression exhibited a purple colour, albeit the bacterial pellet became yellowish. Because the *all-trans* retinal is yellow, the first thought was that maybe the amount of retinal was too high. So three small scales of expression were performed using different amounts of all-trans retinal (1 µM, 3 µM and 5 µM). The bacterial pellet of the expression with 1 µM of *all-trans* retinal was purple and, with the increase of retinal amount, the bacterial pellet became more yellow and less purple.

Analysis	Entire Protein
Length	269 aa
Molecular Weight	29124.98
1 microgram =	34.335 pMoles
Molar Extinction coefficient	68420
1 A[280] corr. to	0.43 mg/ml
A[280] of 1 mg/ml	2.35 AU
Isoelectric Point	6.04
Charge at pH 7	-3.52

Figure 45 *- Predicted analysis of 6His-HmBRI from Vector NTI.*

A western blot of the time course of expression was made with 1 µM and 5 µM (**Figure 46**). The assay proved that the amount of all-trans retinal does not influence the expression of *Hm*BRI and that, similarly to *Hw*BR, the His-tag seems not to be very accessible. For the next expressions only 1 µM all-trans retinal was used.

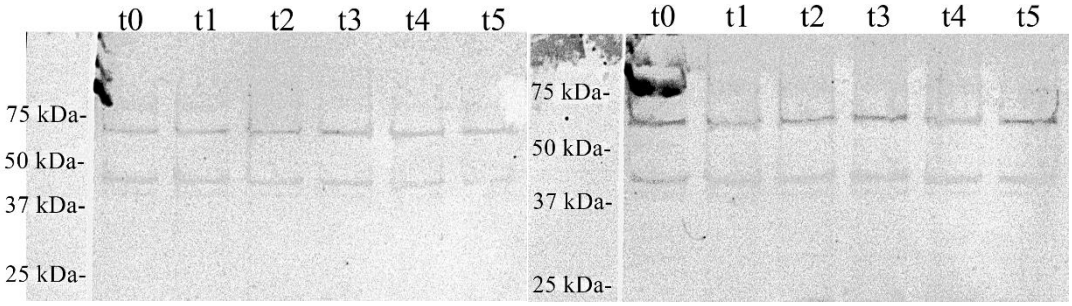


Figure 46 *– Western blots from the time courses of the expression with 1 µM (left) and 5 µM (right) all-trans retinal. The two diferents bands should correspond to a dimer and trimer of HmBRI. Note: the darker bands are contaminations from the protein marker.*

Hsu and co-workers [9] also treated the cell lysate in a 50 °C water bath for 30 minutes, since bacteriorhodopsins are thermostable [82]. To confirm this method, a cell lysate was split into two fractions, and one of the fractions was treated with the 50ºC thermal step. DM detergent was then added to both fractions to extract the *Hm*BRI and the supernatant and pellet were analysed by SDS-Page and Western blot (**Figure 47**).

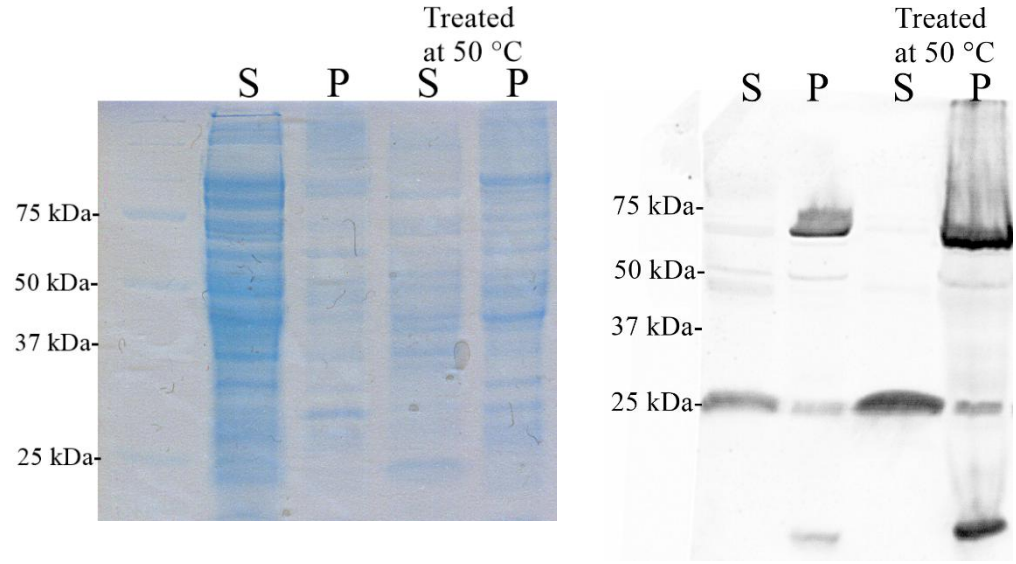


***Figure 47** – Protein gel and respective Western blot from pellet and supernatant after HmBRI extraction using DM, with and without heat treatment.*

Two observations should be emphasized: first the expression is low (when compared with OmpF) because even after extraction it is difficult to see the *Hm*BRI on the SDS-PAGE gel; its presence is only confirmed by Western blot. Second, the supernatant with heat treatment is clearer and Western blot proves that *Hm*BRI is not affected. The supernatant and pellet after extraction of both procedures present the same colour: purple for supernatant and light brown/light purple for the pellet, meaning that *Hm*BRI activity is not affected.

For the first full expression and purification trial of *Hm*BRI (**Figure 48**), the procedure followed the one previoulsy reported by Hsu and co-workers [9] with some adaptations as described in sections 3.18 and 3.19.

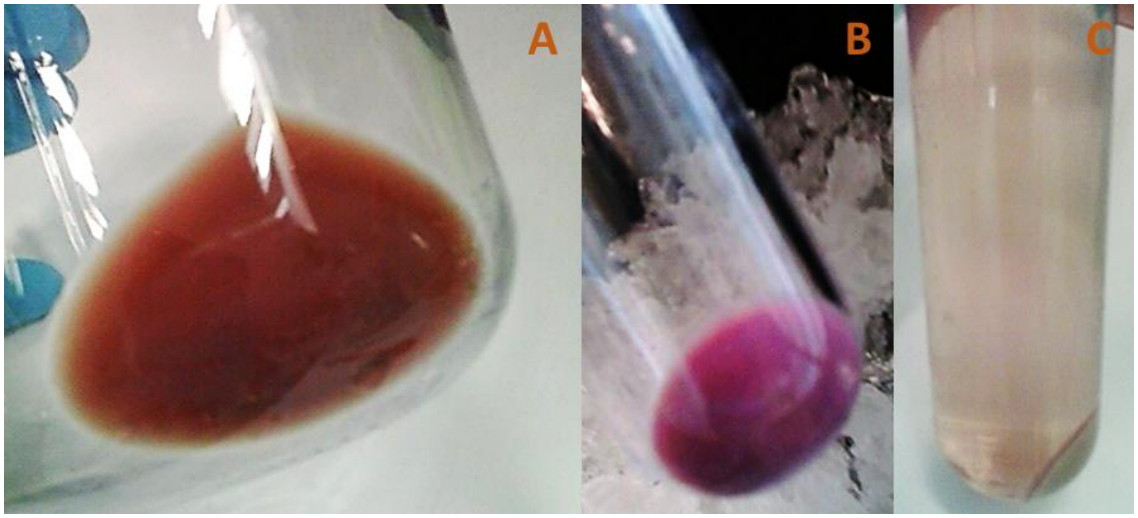


Figure 48 *– Expression and extraction of HmBRI. A- Bacterial pellet, B- Membrane pellet, C- Solubilized proteins after extraction with DM.*

The extracted proteins of one litre of culture were loaded on an affinity column (1 mL Bio-Scale™ Mini Profinity™ IMAC Cartridge column) and *Hm*BRI eluted by steps of imidazole (50 mM, 150 mM and 500 mM) – **Figure 49**.

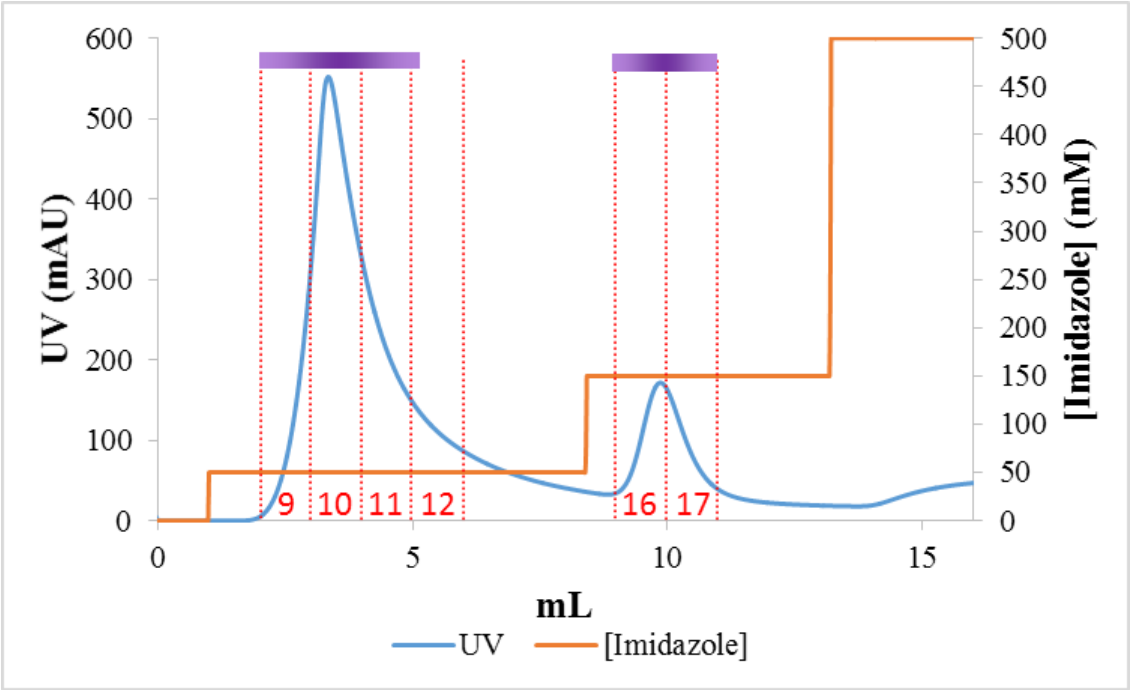


Figure 49 *– Affinity chromatography (IMAC) to purify HmBRI. Eluted by steps of imidazole.*

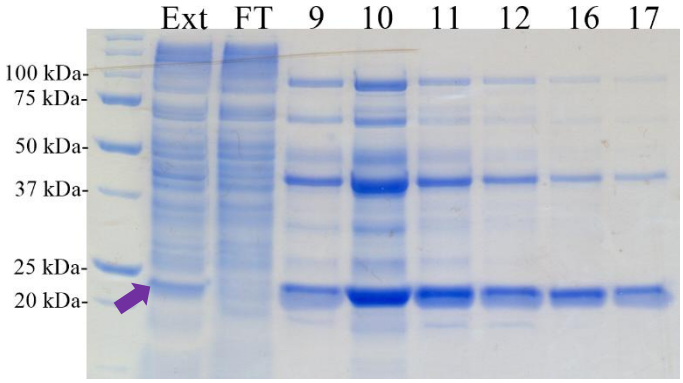


***Figure 50** – Protein gel from affinity chromatography fractions. The purple arrow indicates HmBRI.*

Both peaks on affinity chromatography corresponde to purple fractions. Once again, the majority of *Hm*BRI was eluted with only 50 mM imidazole, as happened with *Hw*BR, indicating *Hm*BRI seems to have the same problem of affinity with the His-tag. Despite the weak binding and the poor purity of the fractions (**Figure 50**), the fractions 9 to 11 (first peak) were pooled together and concentrated until 500 µL and injected on a gel filtration column - **Figure 51**. The total protein injected was 2.95 mg.

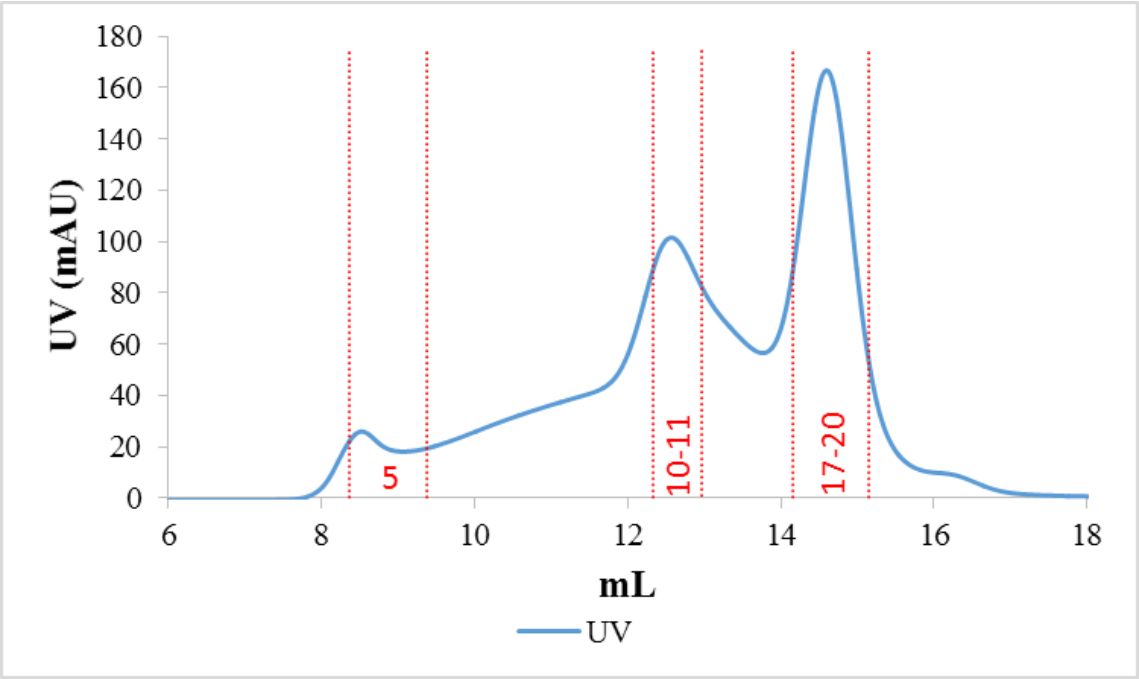


***Figure 51** – SEC profile of HmBR.*

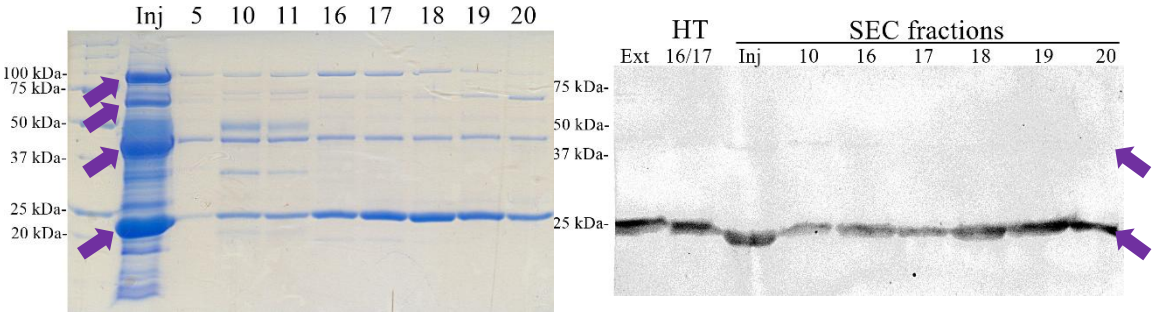


Figure 52 *– Protein gel and Western blot from SEC fractions. The purple arrows indicates HmBRI and its possible oligomers.*

The SEC profile obtained was very close to what is reported in the literature [9], namely concerning elution volumes and number of peaks. However, some aspects are different, such as the much smaller amount of aggregates (eluted at 8.5 mL) and the ratio between the peaks. The first peak (eluted at 12.5 mL) is smaller than the second peak (eluted at 14.5 mL) and this is the opposite to what has been reported [9].

Although only the fractions related to the second peak were purple, the SDS-PAGE and the Western blot (**Figure 52**) showed that *Hm*BRI was present in both peaks. The assays seem to indicatethe presence of oligomers, and at least the dimer is observed by Western blot, despite of the low signal; consistently a*HmBRI* trimer has also been reported during crystalization [83]. This can be explained because larger proteins are more difficult to transfer to the nitrocellulose membrane and, once more, the low accessibility of the antibody to the His-tag. Therefore other bands on SDS-PAGE could correspond to the tetrameric forms of HmBRI (indicated with arrows); yet, the contamination with other proteins due to the weak affinity cannot be discarded.

Analysing the protein gel of the fractions of SEC, the first peak seems to have a lower amount of the *Hm*BRI monomer and more *Hm*BRI oligomers and other unrelated proteins.

Assuming that only the fraction corresponding to the second peak had the functional *Hm*BRI (purple solution), the fractions 17 to 20 were pooled together and the total protein quantified – 785 µg. The protein was stored at 4 °C and the UV-Vis spectrum analysed during several days – Figure 53.

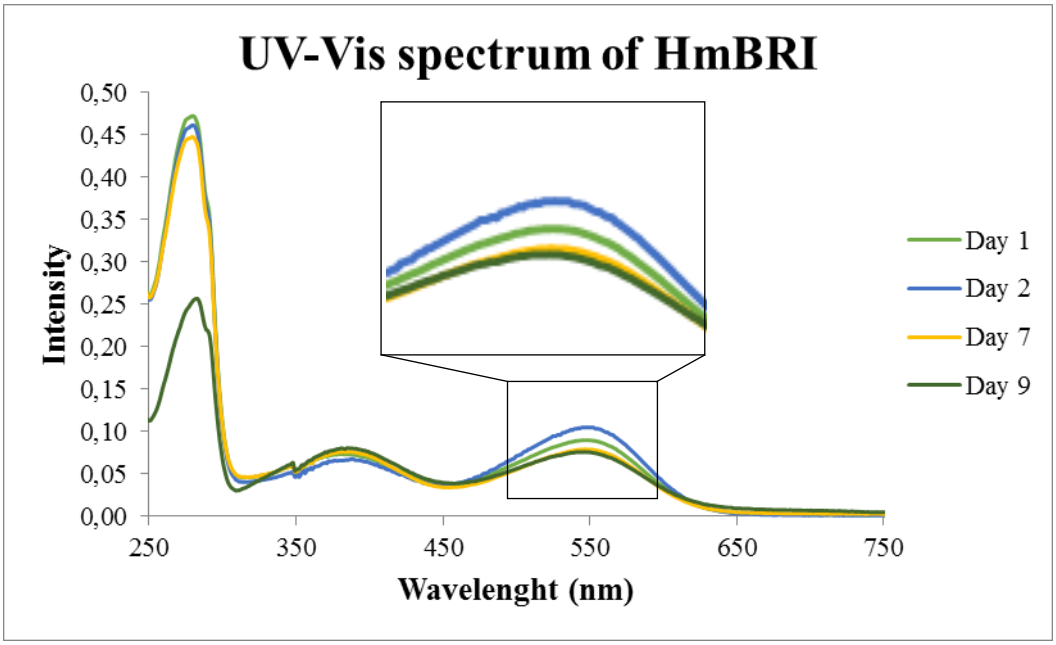


Figure 53 *– UV-Vis spectra scanning of purified HmBRI.*

The UV-Vis spectra show that *Hm*BRI is stable at least one weak at 4 °C. Although, the absorbance at 280 nm drops down, maybe due to the instability of the DM and not because of the loss of *Hm*BRI activity, since the absorbance at 552 nm decreases only slightly.

As in *Hw*BR assays, some modifications to the method of Hsu et al [9] were made to improve the purity and the amount of *Hm*BRI obtained. For that, each assay used two litre of culture, instead of one and, similarly to *Hw*BR assays, the elution method on affinity chromatography was changed to a linear gradient of imidazole (0 mM to 200 mM) and using a 5 mL HisTrap™ HP column – Figure 54.

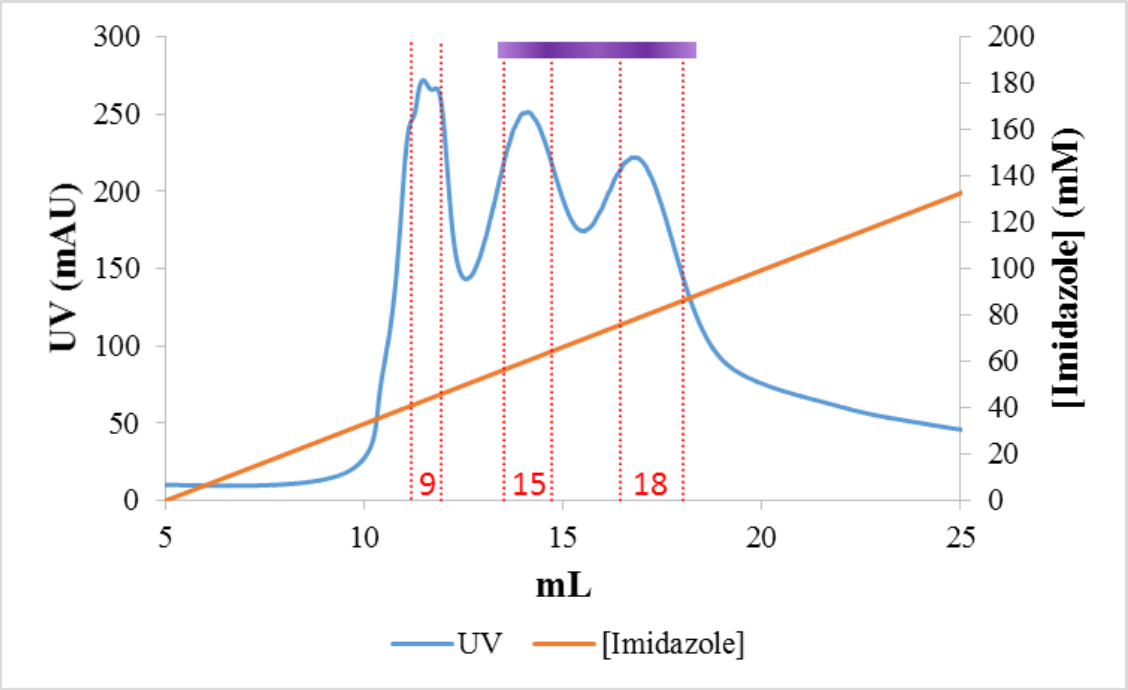


***Figure 54** – Affinity chromatography (IMAC) to purify HmBRI. Eluted by gradient of imidazole.*

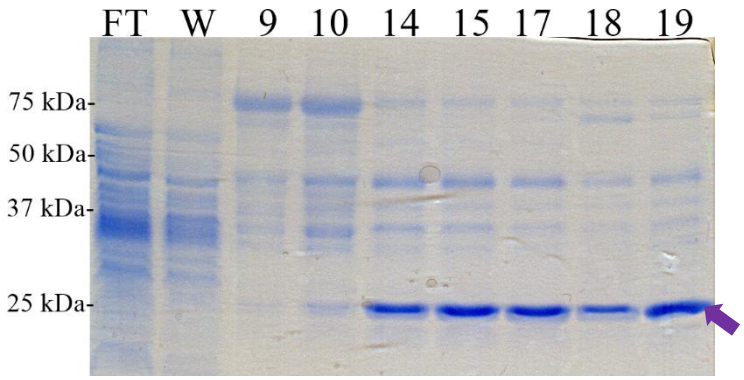


***Figure 55** – Protein gel from affinity chromatography fractions. The purple arrow indicates the HmBRI.*

This affinity chromatography presents three peaks, where the last two peaks correspond to fractions where the solution has purple colour, indicating the presence of *Hm*BRI (**Figure 55**). As happened with *Hw*BR, using a 5 mL affinity column and a linear gradient of imidazole allows separation of *Hm*BR from other proteins that are eluted earlier. Unlike to *Hw*BR, *Hm*BRI is eluted in two different fractions/peaks, as happened when using elution by steps.

To evaluate the differences between the two peaks of the affinity chromatography, both peaks were injected on a gel filtration column (**Figure 56**), fraction 15 for the first purple peak and fraction 18 for the second purple peak. Both fractions were concentrated until 500 µL in order to inject all the fraction on the size exclusion column.

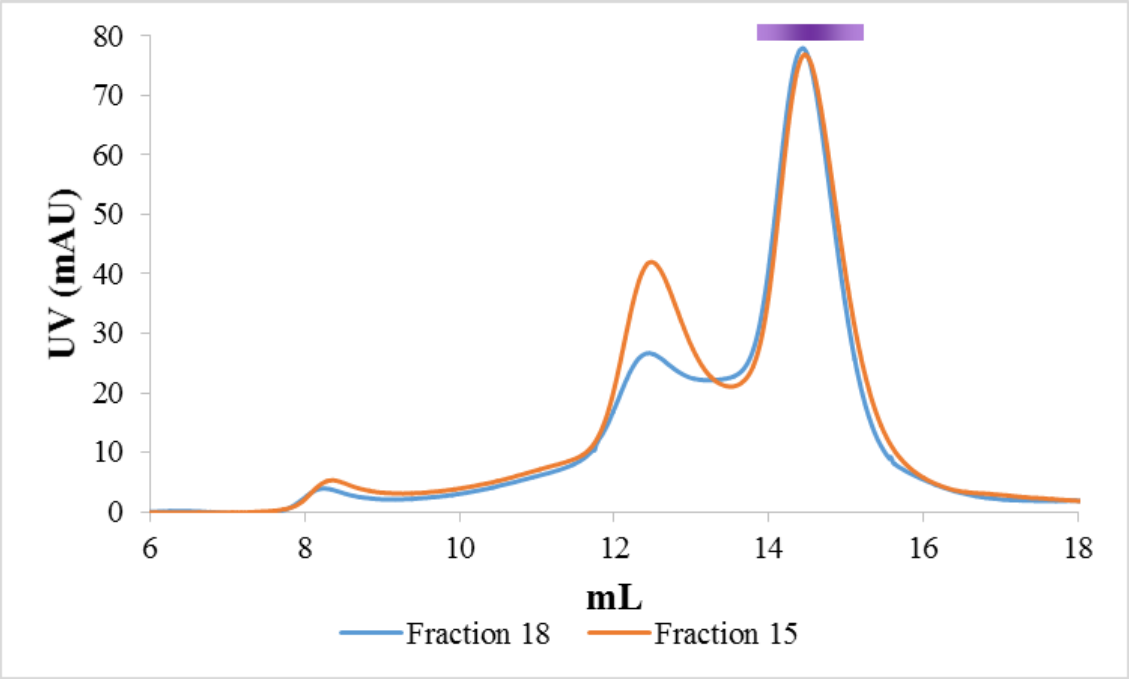


***Figure 56** – SEC profile of both peaks of affinity chromatography.*

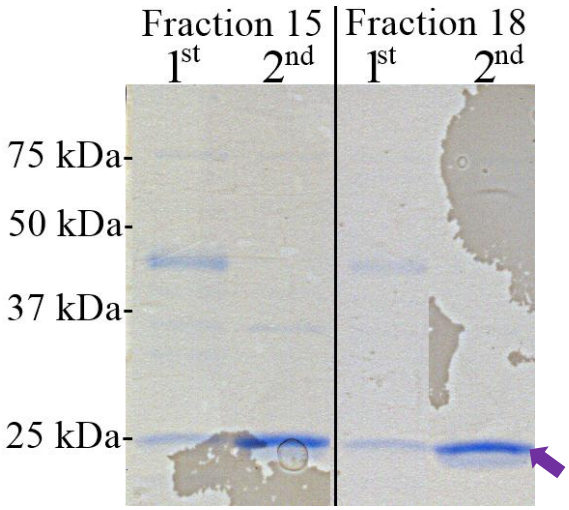


***Figure 57** – Protein gel from the peaks of SEC profiles. The purple arrow indicates HmBRI.*

The SEC profiles and the protein gel (shown in **Figure 57**) reveals that the first peak has more oligomers than the second. Both profiles have exactly the same elution volumes, the only difference is that the third peak of affinity chromatography (fraction 18) has less oligomers than the second peak (fraction 15). Despite of these differences both peaks have similar amount of *Hm*BRI as monomer, as happened before, but only the second peak presented a purple colour. The reason for this peaks eluting at different concentrations of imidazole is maybe due to the presence of slightly differnt proteins with the His-tag less or more exposed.

A new batch of two litres of culture was then purified to ascertain if there is an equilibrium between the first and second peak of the gel filtration chromatography. For that, the first (colourless) and second (purple) peak of SEC were reinjected on the gel filtration column – **Figure 58**.

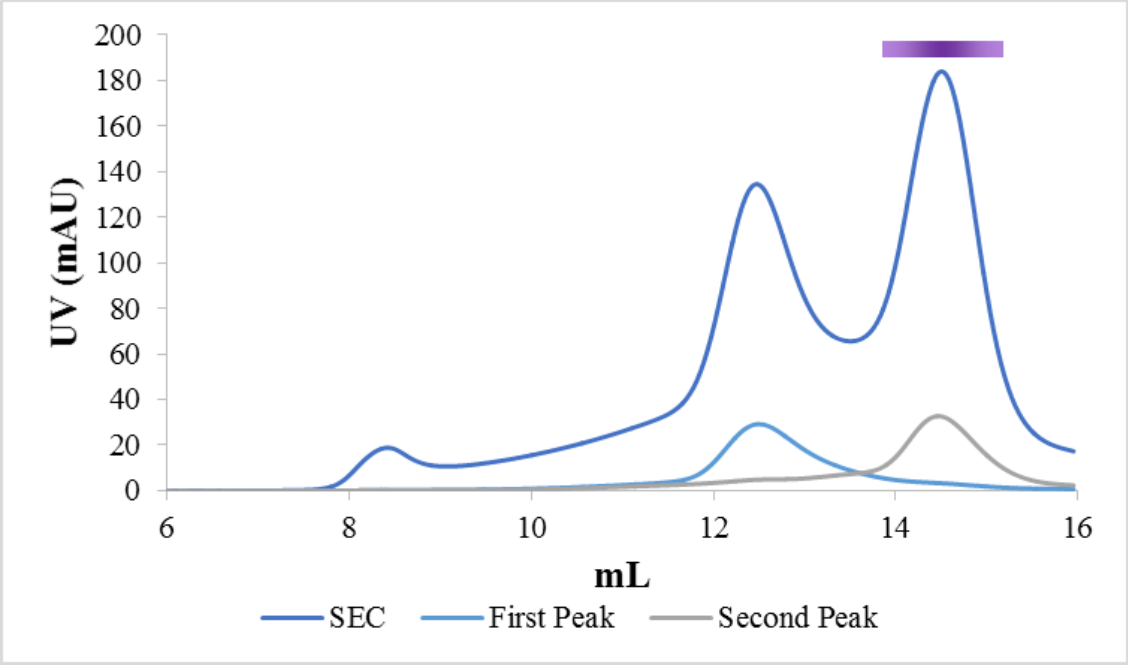


***Figure 58** – SEC profiles of HmBRI and respective reinjections of the two major peaks described in the text.*

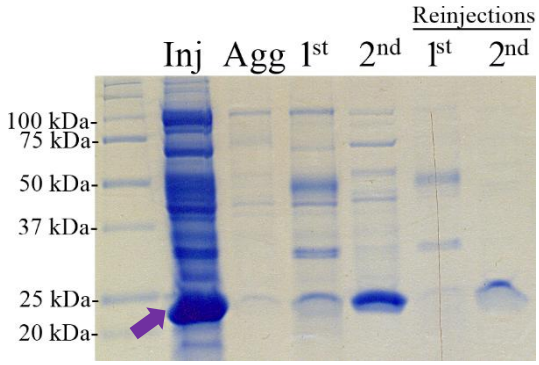


Figure 59 *– Protein gel from the peaks of SEC profiles. The purple arrow indicates HmBRI.1st corresponds to the peaks eluted at 12.5 mL and 2nd corresponds to the peaks eluted at 14.5 mL.*

By analysing the reinjections SEC profiles, it can be concluded that there is an equilibrium between the two species of *Hm*BRI initially separated.

For bird immunizations, *Hm*BRI must be treated with 3C HRV protease to remove the His-tag in order to produce specific antibodies only against *Hm*BRI protein. This procedure was made between the affinity chromatography (**Figure 60**) and the size exclusion chromatography (**Figure 62**), keeping unaltered the remaining procedure.

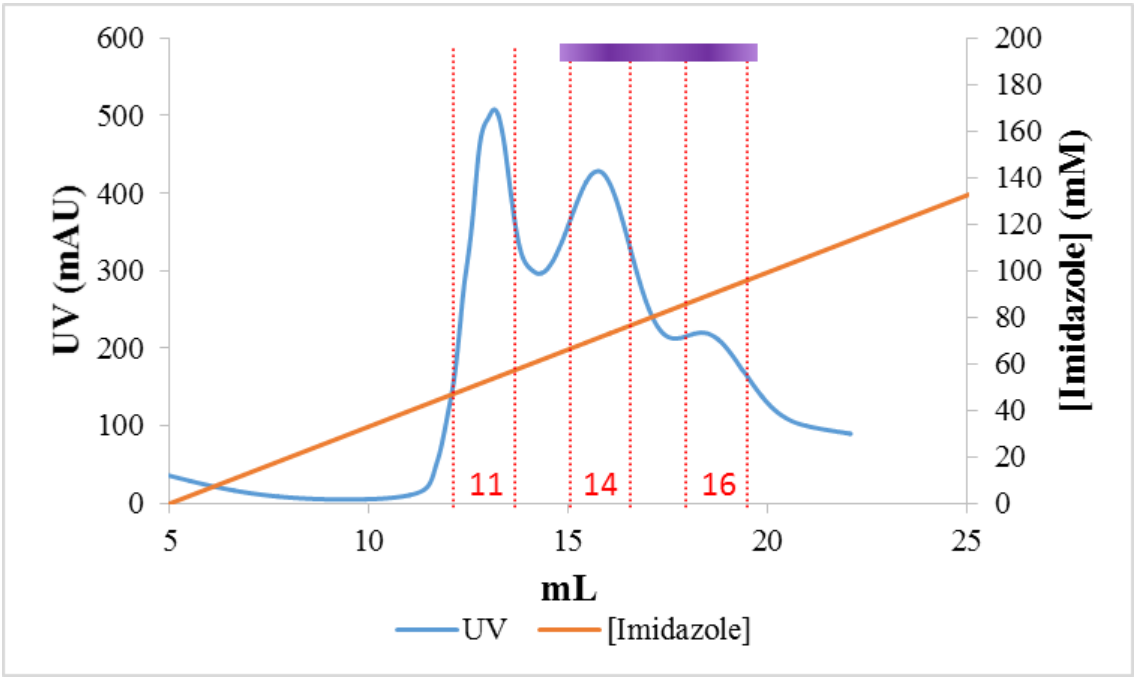


Figure 60 *– Affinity chromatography to purify HmBRI.*

Table 19 *– Absorbance at 280 nm and 552 nm of the fractions of the affinity chromatography.*

Fraction	A280	A552
11	0.203	0.012
12	0.182	0.016
13	0.192	0.037
14	0.210	0.050
15	0.147	0.035
16	0.127	0.035
17	0.091	0.019

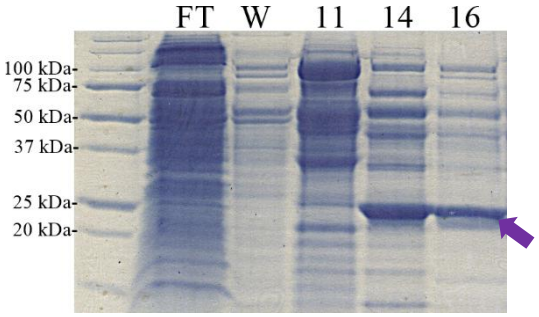


Figure 61 *– Protein gel from fraction of the affinity chromatography. The purple arrow indicates HmBRI.*

As show in **Figure 61** , the two last peaks have purple colour, indicating the presence of the *Hm*BR.. The fractions related to these two peaks, fractions 13 to 16 (**Table 19**), were pooled together and the total protein quantified (6.96 mg) for following 3C HRV protease digestion overnight to remove the His-tag.

After digestion, the pool was concentrated until a volume of 1 mL and injected on a gel filtration column (**Figure 62**).

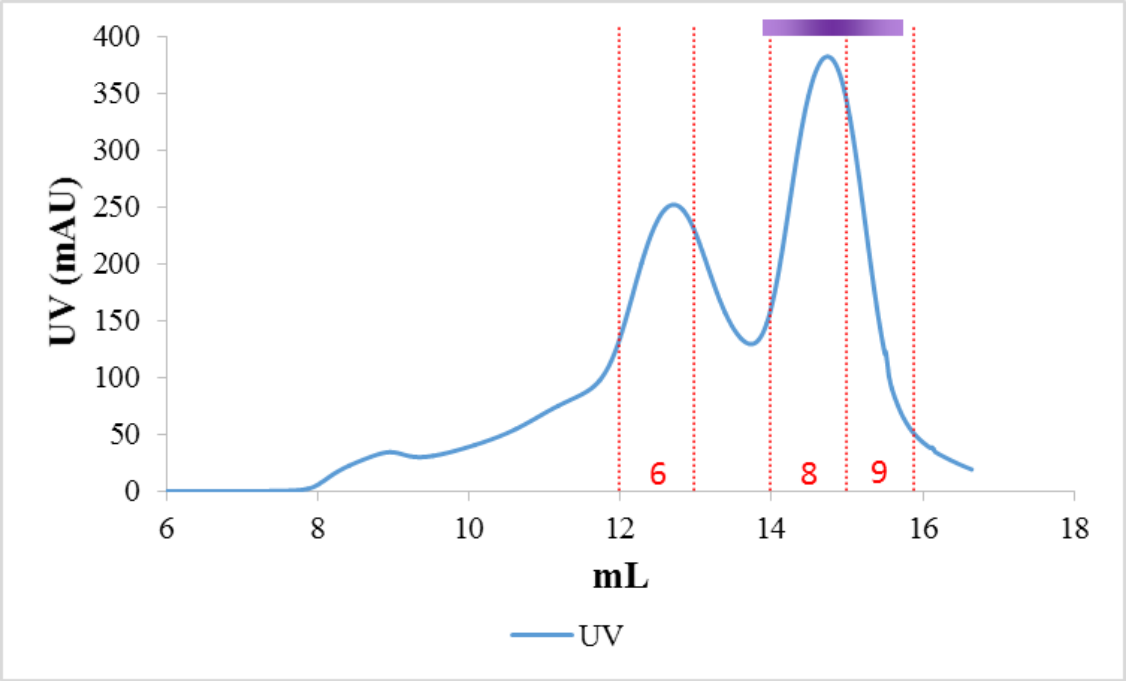


Figure 62 *- SEC profile of HmBRI without His-tag.*

***Table 20** – Absorbance at 280 nm and 552nm of the fractions of SEC.*

Fraction	A280	A552
6	0.101	0.005
8	0.142	0.049
9	0.074	0.028

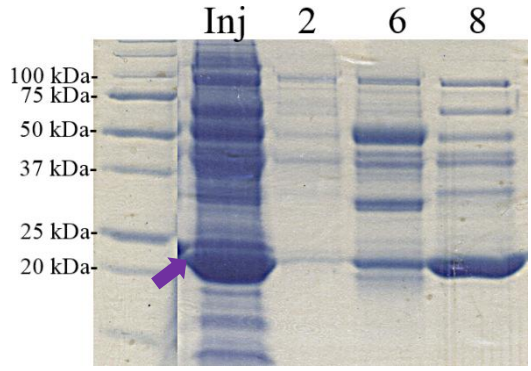


***Figure 63** – Protein gel from fractions of SEC. The purple arrow indicates HmBRI.*

As expected, the fact of the *Hm*BRI loses the His-tag, does not affect the SEC profile. Once again, only the second peak had the purple colour (**Table 20** and **Figure 63**). The total protein purified in its functional state was 2.05 mg related with the fraction 8 and 9.

After several batches of *Hm*BRI production some considerations have arisen. The most important is that, as happened to *Hw*BR, there is a need on improving the His-tag to promote a better purification step when using the affinity chromatography procudure. Increase the tag length to 10 histidine or change the tag to the C-terminal of *Hm*BRI may solve this critical issue.

Other consideration is that Hsu and co-workers [9] showed two peaks in the SEC profile with functional *Hm*BRI, since the fractions were purple. In this work, only the second peak had the *Hm*BRI in its active state. The authors inserted His-tag at the C-terminal of *Hm*BRI, and that seems to be an important difference, since somehow the His-tag on N-terminal could destabilize the protein and promote the formation of oligomers with activity [17]. The authors used the N-terminal version because there is no vector designed in order to proceed with the cloning startegy chosen [9].

Another important aspect is related with the to optimization of the detergent used for HmBRI extraction. In the first purification steps of bacteriorhodopsins the membrane pellet (of one litre of culture) was resuspended in 35 mL of buffer and 0.7 g of DM added. To reduce the amount of DM used, the membrane pellet was resuspended in each time using less buffer. The minimum amount of buffer need to resuspended the membrane pellet was 7.5 mL (per litre of culture), without compromising the extraction. This allow to reduce the amount of DM used per litre of culture to 0.15 g. In general, 1 mg of *Hm*BRI *per* litre of

culture was purified, achieving better yields than that obtained with *Hw*BR (around 0.1 mg/L of culture).

5.3. Surfactant screening: *Hm*BRI model

With the method for expression and purification of the membrane protein *Hm*BRI established, the next step was to evaluate the extraction ability of surfactants, including ionic liquids. The objective was to develop a simple method to correctly evaluate and quantify the extraction capability of different ILs. The following method was implemented: membrane pellets of *Hm*BRI expression were ressuspended in 7.5-10 mL of extraction buffer (*per* L culture) and then splited in aliquots of 1 mL in 2 mL Eppendorf tubes that enable better agitation during extraction, unlike 1.5 mL ones. To each tube, the desirable amount of surfactant to screen was added and the sample was left in contact at 4 °C overnight. At the end of extraction, the tubes were centrifuged at 14000 rpm during 20 minutes, and the extraction ability evaluated.

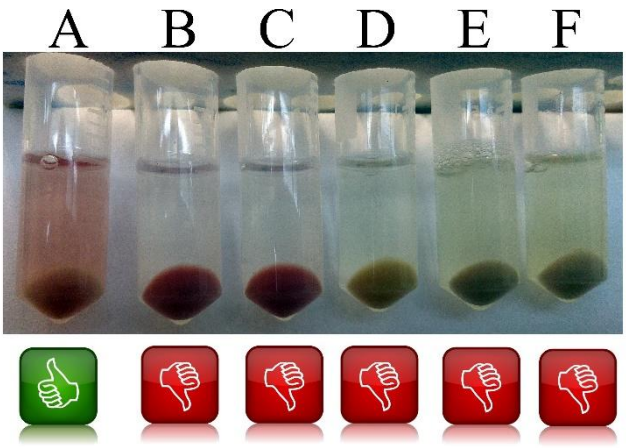


Figure 64 *- Extraction tubes at the end of extraction, for surfactant screening. Solution with purple colour means that the extraction was carried aout with success, while, solutions and pellets with yellow colour means that the HmBR suffered denaturation.*

Visually, and due to the specific purple colour of active *Hm*BRI, it is really simple to identify what is the effect of each surfactant in *Hm*BRI and if it can be used for its extraction from the respective membrane or not. From **Figure 64**, surfactant A is capable of extracting *Hm*BRI from the membrane without denaturing it, producing a purple

solution. Surfactant B and C do not seem to be able to extract *Hm*BRI; similarly, surfactant D does not extract either, and additionally promotes denaturation of *Hm*BRI, as reflected by the yellow pellet produced (due to the free retinal). Surfactant E and F can extract *Hm*BRI from the membrane but not in its active form, producing also a yellow supernatant.Although by this screening visual method it is possible to know if the surfactant is capable or not of extracting this model membrane protein, it is still impossible to quantify their extraction ability or if the surfactant is extracting other (contaminant) proteins from the membrane. For this reason, the supernatants of each sample/surfactant were analyzed by SDS-PAGE. However, the charged nature of ILs comprised the runs and no conclusions could be obtained – **Figure 65**. Thus, SDS-PAGE analysis was not further optimized for quantification purposes at this stage.

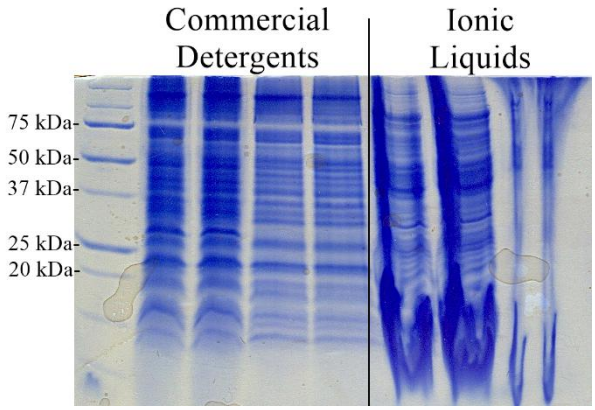


Figure 65 – *Protein gel from supernatants after extraction using commercial detergents (non-ionic) and ILs.*

Another strategy tryied was to analyse the extraction behavior by Dot blot, making use of the His-tag in *Hm*BRI. However this assay was also not conclusive since the results were not quite consistent with the visual observations in the assays described above– **Figure 66**.

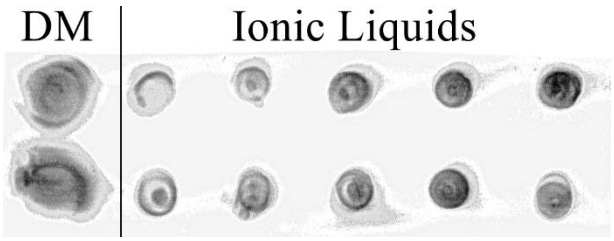


Figure 66 *– Dot blot from the supernatants after extraction using DM and one ILs in several concentrations.*

Finally, we decided to evaluate the extraction ablity by reading the absorbance at 280 nm (for total protein extracted) and at 552 nm (for *Hm*BRI extracted).

To test this method of evaluation, three distinct expressions were made, 1) *Hm*BRI, 2) OmpF, and 3) vector pET 28a with noinsert. The three expressions were carried out all as if they were *Hm*BRI expression, and then DM was used the for extraction. The supernatants were analysed by SDS-Page and by measuring the absorbances of the supernatant at 280nm and 552nm absorbance (**Figure 67** and **Figure 68**).

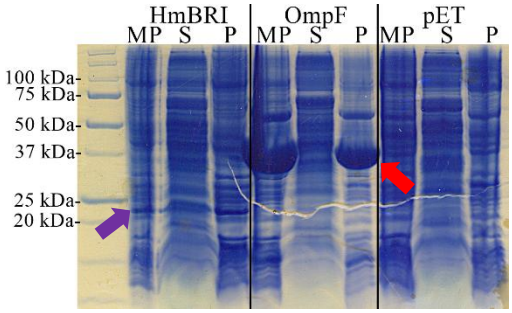


Figure 67 – *Protein gel from the membrane pellet and supernatant and pellet after extraction with DM. The purple arrow indicates HmBRI and the red arrow indicates OmpF.*

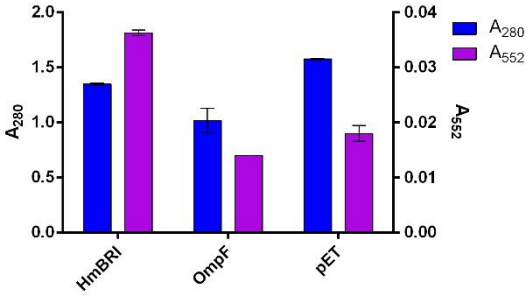
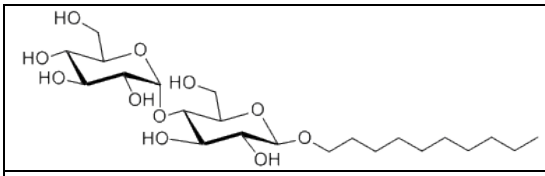
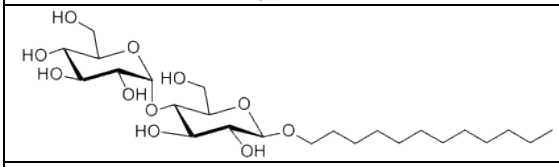
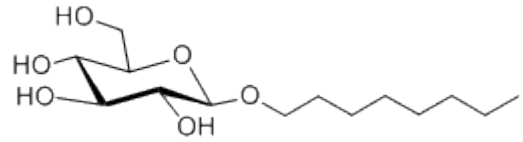
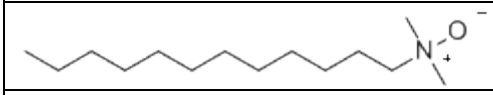
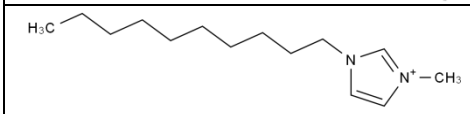
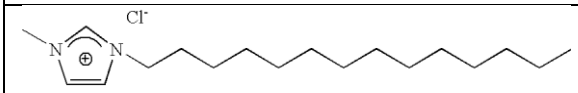
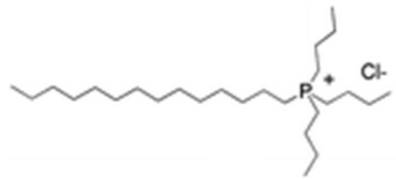


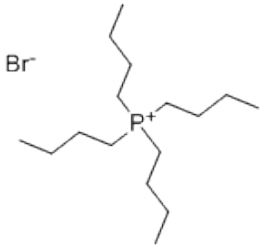
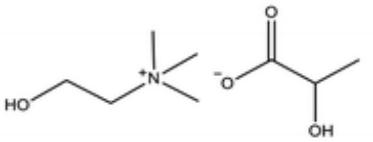
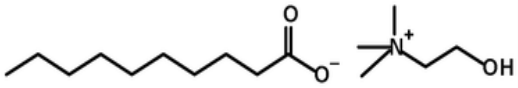
Figure 68 *– Absorbance at 280 nm and 552 nm from the supernatant after DM extraction.*

The most important aspect of this assay was to see what is the background signal at 552 nm. Because it was observed a signal at 552 nm with an absorbance value of 0.02 for OmpF and pET28a (proteins that should not absorb at 552 nm) it was empirically assumed and established for the downstream assays this threshold as the experimental background. The low absorbance at 280 nm for OmpF is probably because this protein was not extracted by DM and remained on the membrane pellet as verified on SDS-PAGE, even

though a large amount of other contaminant proteins should be contributing to the absrobance at 280 nm.

Table 21 – *Chemical structures of the surfactant used.*

	Decyl Maltoside	DM
	Dodecyl Maltoside	DDM
	Octyl Glucoside	OG
	Lauryldimethylamine-N-Oxide	LDAO
	1-Decyl-3-methylimidazolium Chloride	$[C_{10}mim]Cl$
	1-Tetradecyl-3-methylimidazolium Chloride	$[C_{14}mim]Cl$
	Tributyltetradecyl phosphonium Chloride	$[P_{444,14}]Cl$

	Tetraoctyl phosphonium Bromide	$[P_{8888}]Br$
	Cholinium Lactate	[Ch]Lac
	Cholinium Decanoate	[Ch]Dec

The first test was to evaluate the performance of some commonly used commercial detergents, data shown in - **Figure 69** using our HmBRI extraction assay. Three anionic detergents (DM, DDM and OG) and one ionic detergent (LDAO) at a concentration of 2% (w/v) were tested.

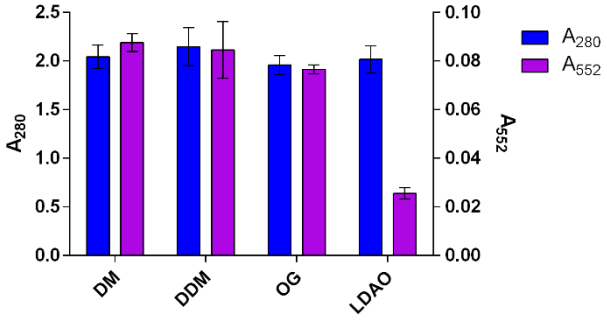


Figure 69 *– Commercial detergents screening for the extraction of HmBRI.*

All four detergents have approximately the same capacity to extract *Hm*BRI from the membrane (similar absorbance at 280 nm). However the LDAO differs from the other detergents in ehat regards the absorbance at 552 nm, meaning that this detergent denatures the *Hm*BRI, and this was confirmed visually since the supernatant displayed yellow colour and the other detergents had purple colour.

The anionic detergents vary from each other in the length of the alkyl chain (OG – 8 carbons, DM – 10 carbons, DDM – 12 carbons) and on the hydrophilic head group (OG – glucoside, DM and DDM – maltoside) and they can extract successfully *Hm*BRI from the

membrane without denaturing it. The ionic detergent, LDAO, also has a 12-carbons hydrophobic chain, such as DDM, but leads to the denaturation protein. Since the hydrophilic head group is the only difference when compared with DDM, this feature seems to be responsible for LDAO denaturing capacity towards *Hm*BRI.

The ionic liquids based on imidazolium are the class of ILs most studied, while [C_{12}mim]Cl was already reported as able to solubilize bacteriorhodopsins [5]. Three ILs of this family, presenting different lengths of the alkyl chains, were tested at 5% (w/v), namely [C_{10}mim]Cl and [C_{12}mim]Cl, and at 2% and 4% with [C14mim]Cl. Unfortunately, for the first two ILs, the analysis of the absorbance was not carried out. However, visually it was possible to take some conclusions: both ILs were able to extract *Hm*BRI, but the supernatant turned to yellow colour, meaning that *Hm*BRI does not maintain its integrety in aqeuous solutions of these ILs. For [C_{14}mim]Cl (**Figure 70**) the results are similar. The extraction was observed along with HmBRI denaturation. This IL seems to have the same behaviour than LDAO, presenting however a less extraction capacity since a lower signal for the total protein was observed (lower absorbance at 280 compared to DM).

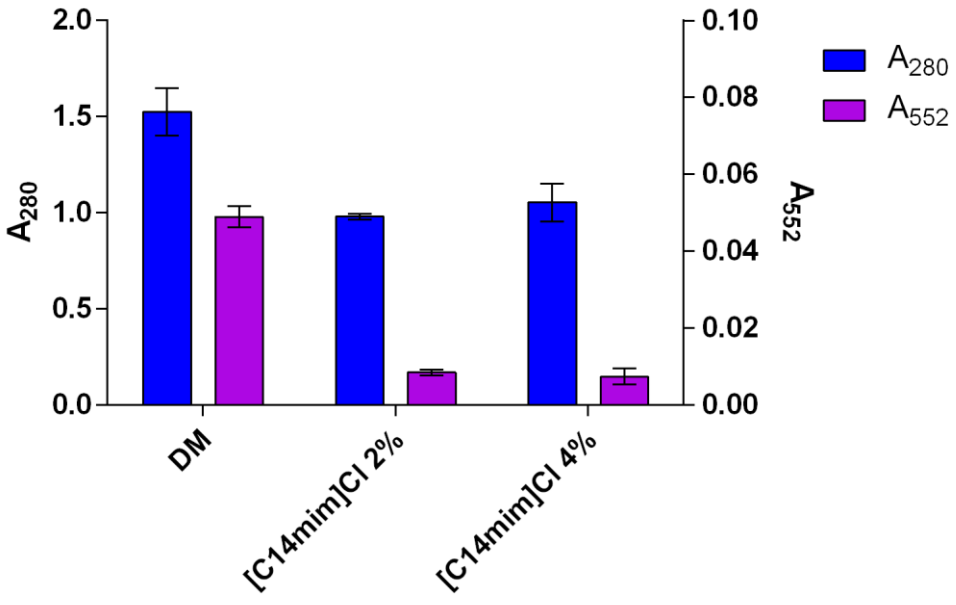


***Figure 70** – Imidazoliu- based ILs screening for extraction of HmBRI.*

Figure 71 depicts the results obtained for ILs comprising phosphonium-based cations, namely [P_{8888}]Br and [$P_{444,14}$]Cl, at concentrations of 1% and 2% (w/v).

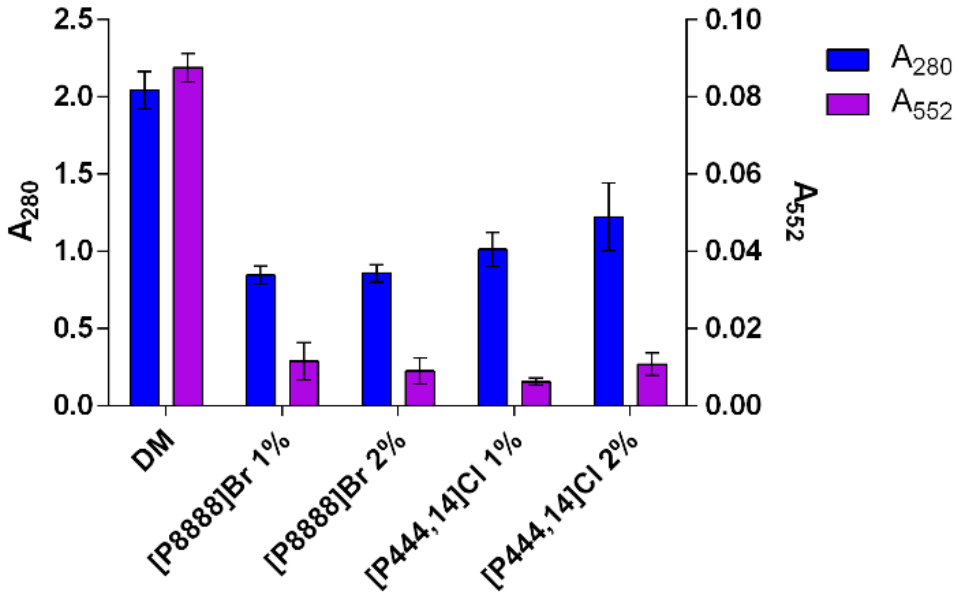


***Figure 71** – Phosphonium based ILs screening for the extraction of HmBRI.*

None of the phosphonium-based ILs, tested at different concentrations, was able to extract the HmBRI from the membrane, since upon extraction the membrane pellet remained with a purple colour. Consistently, as shown in Figure 71, the 552nm signal was quite below the established threshold.

Finally, in a last assay, an IL based on the cholinium cation was used. Unlike the remaining ILs, the cholinium-based is an anionic surfactant with a long alkyl side chain at the anion (derived from a carboxylic acid). It has been reported [84] that cholinium-based ILs are only able to form micelles if the carboxylate has 8 or more carbons, an important feature to extract proteins from membranes by a micelle-mediated phenomenon. Below 8 carbons, ILs tend to act as hydrotropes. Even so, cholinium-based ILs with smaller aliphatic chains ([Ch]Lac, Ch But, Ch Ac, Ch Pro) were tested at concentrations of 3% and

10 % (w/v) and as expected, these ILs were not able to extract *Hm*BRI and the membrane pellet remained purple - **Figure 72**.

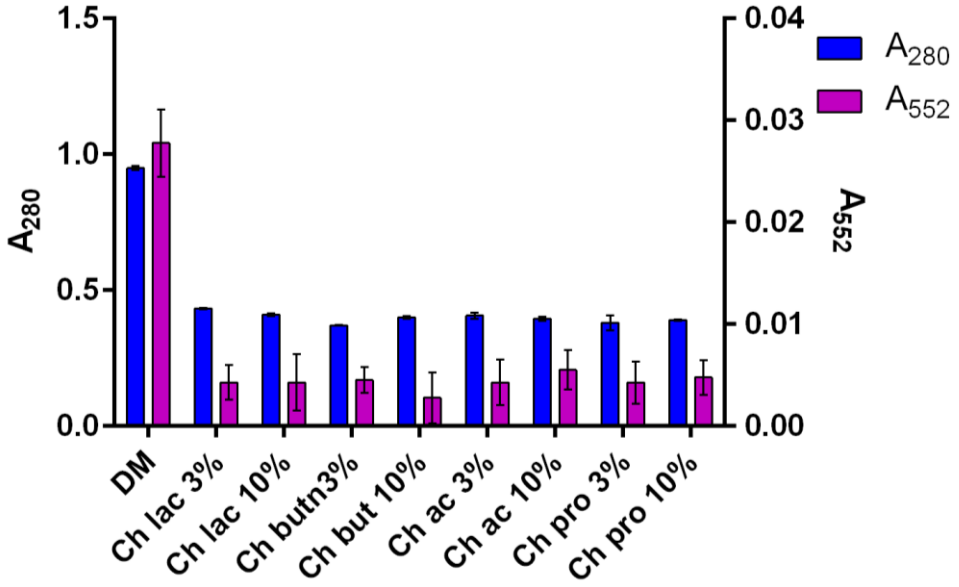


Figure 72 *– Cholinium-based ILs (with small hydrophobic chain) screening for extraction the of HmBRI.*

Finally, cholinium-based ILs with longer alkyl side chains, one of them still not able to form micelles ([Ch]Hex) and the other two ([Ch]Oct and [Ch]Dec) predicted to form micelles, were tested, being the results obtained shown in **Figure 73**. . IL concentrations of 1%, 3%, 5% and 10% (w/v) were assayed. As predicted, [Ch]Hex was not able to extract the *Hm*BRI at any of the concentrations evaluated. On the other hand, with 5% and 10% of [Ch]Oct, as well as with all the concentrations tested of [Ch]Dec, the *Hm*BRI was successfully extracted from the membranes. However, in all these cases, the protein was not active. With 5% and 10% of [Ch]Oct and with 1% of [Ch]Dec the total protein extracted was very similar to DM, but, with 3% or above of [Ch]Dec (all with the same behaviour) other proteins were co-extracted.

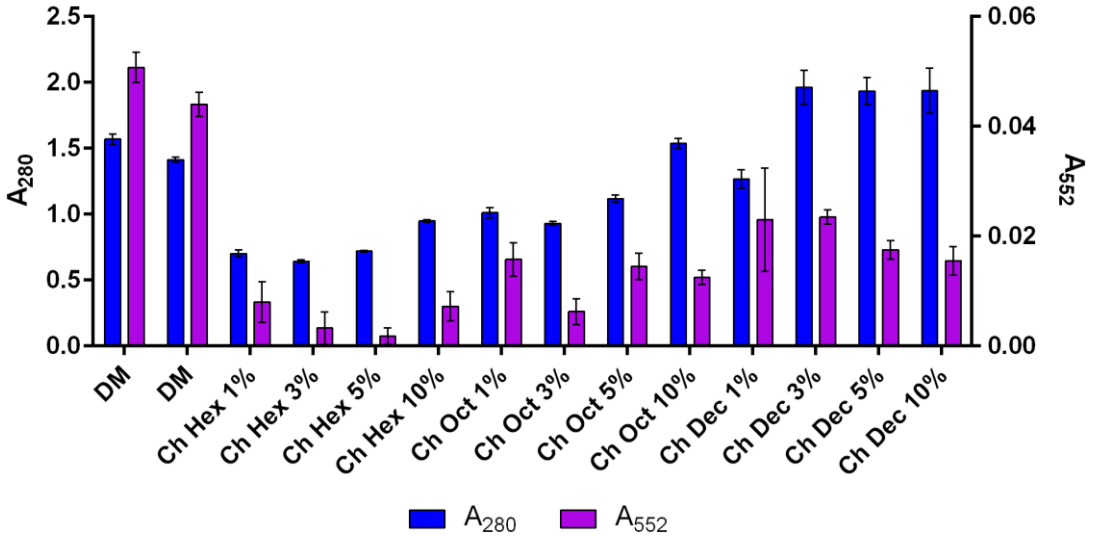


Figure 73 *– Cholinium-based ILs (with long hydrophobic chain) screening for the extraction of HmBRI.*

Since none of the ILs investigated could successfully extract *Hm*BRI and so, no novel surfactants were found to replace the conventional DM, we decided to further test mixtures of IL with DM in order to eventually decrease the amount of DM needed on extractions. The IL chosen was [Ch]Dec since it showed better results in terms of total protein extraction. Therefore, the extractions were preformed using 3% of [Ch]Dec plus increasing amounts of DM. The results obtained are depicted in **Figure 74**.

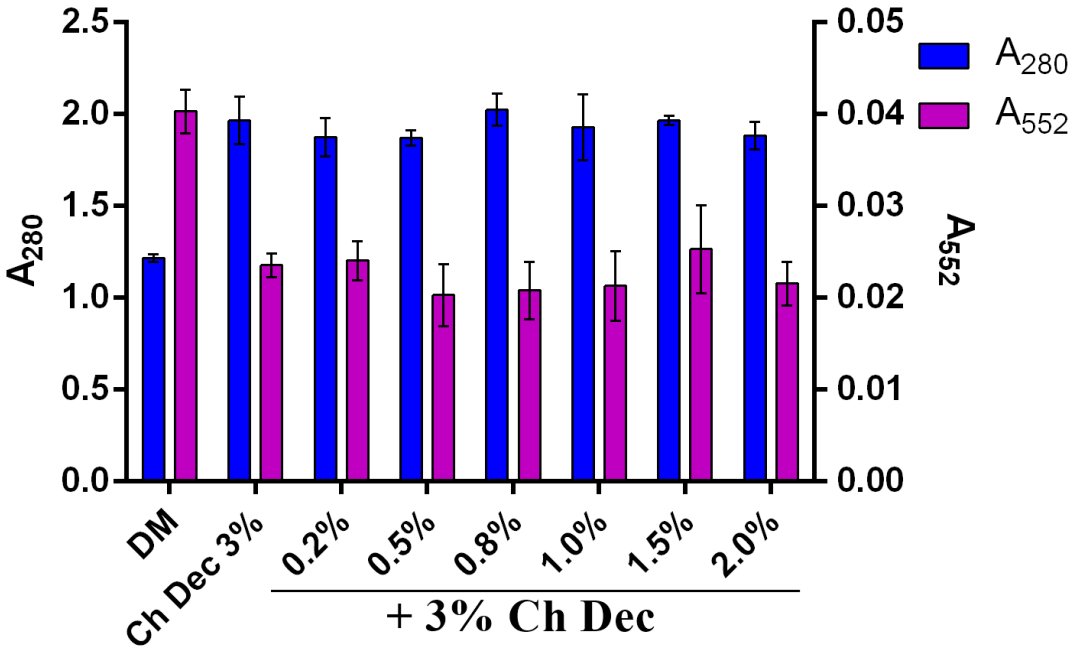


***Figure 74** – Mixtures with DM and [Ch]Dec screening for the extraction of HmBRI.*

All extractions performed with the mixtures, no matter how much DM was added, displayed the same behaviour. At the concentration of 3% of [Ch]Dec it seems to reach a top limit in the extraction of proteins. In order to go further and to infer on the phenomenon, further tests should be attempted using less IL and/or using an IL with a smaller hydrophobic chain, for instance [Ch]Oct or [Ch]Hex. Although the later does not form micelles and cannot extract alone *Hm*BRI, in combination with DM, this IL could help on the solubilisation and extraction of proteins from membranes.

Although, none of the ILs tested were able to extract the *Hm*BRI in its native form, these ILs can successfully extract other membrane proteins. Thus, it is critical to implement a similar method for other model membrane proteins to evaluate such a hyphotesis. The extraction efficiency of novel surfactants will demand a protein-specific charaterization, meaning that a case-by-case evaluation must be performed.

5.4. Production of specific anti-*Hm*BRI

In order to produce specific antibodies against *Hm*BRI, the protein was purified according to the protocol described in sections 3.18 and 3.19. Unfortunately, the purest fraction of HmBRI still had some contaminants thus compromising the specificity of the polyclonal antibody produced. Nevertheless, the production of an anti-*Hm*BRI was carried out. Unlike predicted, the DM used for *Hm*BRI solubilization and present in the protein buffer, did not affect emulsion preparations, as reported for other detergents, such as SDS. Instead, the emulsion retained a light purple colour, indicative of HmBRI stability and resistance to the Freud's adjuvant as well as to the mechanical extrusion treatment. Therefore, until the immunization step, the protein seemed to be maintain its proper fold. It should be noted that the DM-containing buffer did not induce any visible side effects on the quails, such as local inflamations or injuries.

To test the reactivity of the antibodies obtained from the quails' eggs, ELISA assays (section 3.24) were performed. IgY samples were isolated from the egg yolk by PEG precipitation, to remove the maximum of lipo-proteins and lipids. The results obtained are shown in **Figure 75**.

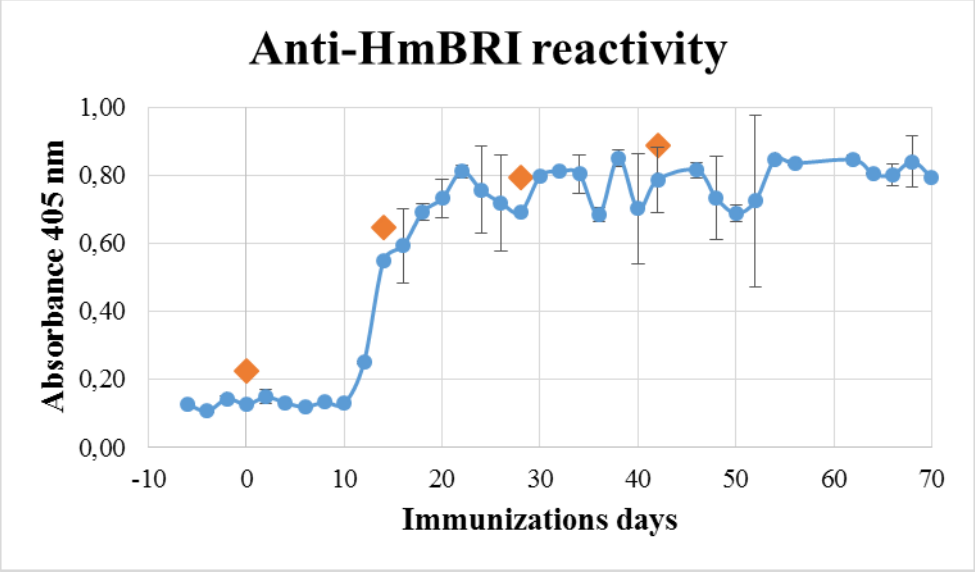


***Figure 75** – Anti-HmBRI reactivity monitor by ELISA. The orange diamonds indicate the days of immunization and subsequent boosts.*

By analysing the ELISA assay results, it can be concluded that birds start to present a anti-*Hm*BRI specific response about 12 days after the first immunization, reaching a maximum titer around day 20. This ELISA profile is consistent with others obtained for similar experiments in our lab, using however soluble recombinant proteins as antigens for antibody production in bird models. Up to this moment, we have not confirmed the specificity of this novel antibody on a western blot assay, and it will be conducted in the near future.

6. Conclusions

One of the main objectives of this work was to develop a strategy to easily screen novel surfactants able to extract and solubilize membrane proteins from biological membranes, while being able to report the folding state of these proteins. To do that, *Haloarcula marismotui* bacteriorhodopsin *Hm*BRI (mutant D94N) was used as a model membrane protein. The fact that this protein presents a purple colour when it is active, makes of this protein very interesting since its extraction and integrity can be evaluated by visual inspection or by measuring the absorbance at 280 nm and 552 nm.*Hm*BRI D94N was expressed and purified successfully, using DM, achieving 1 mg/L of culture of active protein. Nevertheless, the His-tag must be improved to allow better yields.

Since some ILs display surface-active properties, and since they are emerging as novel solvents with enhanced fetures, imidazolium-, phosphonium- and cholinium-based ILs were investigated for the extraction and solubilization of membrane proteins. Although none IL was able to extract the *Hm*BRI without denaturing it, the imidazolium-based ILs and the cholinium-based ILs were able to extract *Hm*BRI from the membranes, with the last family providing better results than the commercial detergent DM. On the other hand, phosphonium-based ILs were not able to extract *Hm*BRI. Based on these overall results, other families of ILs should be explored, as well as some mixtures of ILs.

In this work, anti-*Hm*BRI antibodies were finally produced using the purified *Hm*BRI as a fusion tag. The antibodies were produced in quails and the use of detergents (DM) during the immunization protocol seems not to be a problem.

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