Universidade do Minho
Escola de Engenharia

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Development and optimization of production and purification of the human protein BMP-2 in *Escherichia coli* for biomedical applications

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Dissertation thesis for the Master degree in Biomedical Engineering

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Development and optimization of production and purification of the human protein BMP-2 in *Escherichia coli* for biomedical applications

Abstract

Bone morphogenetic protein 2 (BMP-2) is one of the main representatives osteoinductive protein of a group of the transforming growth factor-β (TGF-β) superfamily of multifunctional cytokines. BMP-2 plays a critical role in cartilage and bone formation during skeletal development and repair. Several clinical studies have proven the clinical benefit of BMP-2 in the treatment of bone defects, being an effective alternative to bone grafts. Nowadays, BMP-2 is one of the two bone morphogenetic proteins approved as biological method to stimulate bone repair in humans. However, these commercial solutions are expensive, the protein purification is a laborious process and the obtained yields are low.

A landmark occurred with the cloning of the human BMP-2 gene and its production by DNA recombinant technology. Production of recombinant human BMP-2, using prokaryotic systems is the preferred method, being *Escherichia coli* one of the most popular systems for protein production. The bacterium *E. coli* is a well studied expression system, which provides a fast and economical production of recombinant proteins. Various studies on the production and purification of BMP-2 protein using *E. coli* as expression system have been reported; however BMP-2 is difficult to express in soluble form and its purification under native conditions remains a challenge.

In this work, the soluble expression of the recombinant BMP-2 protein in *E. coli* was studied using the fusion protein technology, with the novel FH8 tag.

The production of soluble recombinant fusion protein was initially achieved using as backbone the pQE-30FH8 vector, with the study of different culture conditions, in order to determine the conditions that maximize this soluble expression. Then, in order to increase soluble expression of FH8BMP-2, the cloning of *bmp-2* gene was performed in two other different vectors: the pETMFH8 and pStaby1.2FH8, using different *E. coli* strains. Purification strategy of FH8BMP-2 was performed by the following chromatography techniques: using the FH8 as purification tag by hydrophobic interaction (HIC); affinity chromatography with nickel (using the His tag as purification tag) and ion exchange.

The results presented in this work showed that recombinant fusion protein was successfully soluble expressed in the three vectors used. The pETM system was the one that showed the highest soluble expression. In relation to purification, the FH8-HIC technique, when combined with calcium addition to soluble fraction, shows good results in purification profile of target protein and a high yield of purified protein. Calcium ions and the own conformation of FH8 can have an important role on the aggregation of FH8BMP-2 into a dimer.

Overall, the BMP-2 was efficiently expressed in *E. coli* as a soluble protein and an optimized purification strategy was developed to obtain this bone morphogenetic protein under native conditions.

This work may be important for further steps for an *in vitro* production and biomedical application of recombinant BMP-2 protein.

Desenvolvimento e otimização da produção e purificação da proteína humana BMP-2 em *Escherichia coli* para aplicações biomédicas

Sumário

A proteína morfogenética do osso tipo 2 (BMP-2) é uma das proteínas osteoindutoras mais representativas da superfamília do fator de transformação do crescimento β (TGF-β) que agrupa um conjunto de citoquinas multifuncionais. A proteína BMP-2 desempenha um papel crítico na formação e regeneração do osso e cartilagem, durante o desenvolvimento do esqueleto. Vários estudos clínicos têm demonstrado o benefício clínico da BMP-2 no tratamento de defeitos ósseos, sendo uma alternativa eficaz aos enxertos do osso. Atualmente, a BMP-2 é uma das duas proteínas morfogenéticas do osso que se encontra aprovada como método biológico para estimular a reparação óssea em seres humanos. No entanto, estas soluções comerciais são dispendiosas, a purificação da proteína é um processo laborioso e os rendimentos obtidos são baixos.

Um marco importante ocorreu com a clonagem do gene humano da proteína BMP-2 e a sua produção através da tecnologia de DNA recombinante. A produção desta proteína humana recombinante, utilizando os sistemas procarióticos é o método preferido, sendo a bactéria *Escherichia coli* um dos sistemas mais populares de produção proteica. A bactéria *E. coli* é um sistema de expressão bem estudado, que proporciona uma produção rápida e económica de proteínas recombinantes de interesse. Vários estudos têm reportado a produção e purificação da proteína BMP-2, usando como sistema de expressão a *E. coli*; contudo, a proteína BMP-2 é difícil de expressar na forma solúvel e sua purificação em condições nativas continua a ser um desafio.

Neste trabalho, a expressão solúvel da proteína BMP-2 recombinante em *E.coli* foi estudada através da tecnologia de proteínas de fusão, com o novo *tag* de fusão FH8.

A produção solúvel da proteína de fusão recombinante foi conseguida usando como vetor inicial o pQE-30FH8, com o estudo das diferentes condições de cultura, com o objetivo de determinar as condições que maximizam essa expressão solúvel. Em seguida, com a finalidade de aumentar a expressão solúvel da FH8BMP-2, foi realizada a clonagem do gene da BMP-2 em dois vetores diferentes: o pETMFH8 e pStaby1.2FH8, usando diferentes estirpes de *E. coli*. A estratégia de purificação da

proteína FH8BMP-2 envolveu a realização das seguintes técnicas de cromatografia: interação hidrofóbica (HIC), usando o *tag* FH8 como t*ag* de purificação; cromatografia de afinidade com níquel (usando o His *tag* como *tag* de purificação) e troca iónica.

Os resultados apresentados neste trabalho mostraram que a proteína de fusão recombinante foi expressa solúvel com sucesso nos três vetores utilizados. O sistema pETM foi aquele que apresentou um maior nível de expressão solúvel. Em relação à purificação, a técnica de FH8-HIC, quando combinada com a adição de cálcio à fração solúvel, apresenta bons resultados em relação ao perfil de purificação da proteína alvo e um elevado rendimento de proteína purificada foi obtido. Os iões de cálcio e a própria conformação do *tag* FH8 podem ter um importante papel na agregação da FH8BMP-2 na forma dimérica.

Em geral, a BMP-2 foi eficientemente expressa no sistema *E. coli* como proteína solúvel e uma estratégia de purificação otimizada foi delineada para obter esta proteína morfogenética do osso em condições nativas.

Este trabalho pode ser importante para novas etapas de uma produção *in vitro* e aplicação biomédica da proteína BMP-2 recombinante.

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Abbreviations

APS – Ammonium Persulfate;

BMP-2 – Bone Morphogenetic Protein 2;

bp – base pair;

BSA – Bovine Serum Albumin;

CV – Column Volumes;

Da – Dalton;

DNA – deoxyribonucleic acid;

dNTPs – deoxyribonucleoside triphosphates;

IPTG – Isopropyl β-D-1-thiogalactopyranoside;

LB – Luria Broth;

MCS – Multiple Cloning Site;

min – minute(s);

OD – optical density;

PBS – Phosphate Buffer Saline;

pI – isoelectric point;

rpm – revolutions per minute;

RT – Room Temperature;

SDS – sodium dodecyl sulfate;

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis;

TEMED – N,N,N',N'-tetramethylethylenediamine;

TGF-β – Transforming Growth Factor β;

UV – Ultraviolet light.

Motivation and aim of the project

The research on bone regeneration began decades ago with intensive studies on bone growth and healing. Most researches have been conducted to identify proteins capable to induce new bone formation and methods to perform biological applications that can lead to decrease or elimination of bone graft [1,2].

It was in 1965 that a landmark on bone regeneration began with the work developed by Urist [3]. The pioneering work of Urist showed that demineralized bone matrix had the capacity to induce endochondral bone formation and he named the mixture of proteins present in matrix as bone morphogenetic proteins (BMPs) [1,4]. During the decade of 80s and 90s many investigators developed research in this area with the cloning of BMPs genes, demonstrating that these proteins are responsible to initiate a cascade of events, in which stem and mesenchymal cells are differentiated into osteogenic linage capable of producing bone tissue. Since then, several BMPs have been identified and purified from bone of different species, including human, for clinical applications [1,2,4].

Bone morphogenetic protein 2 (BMP-2) is one of the main morphogenetic protein implicated in growth and regeneration of bone and cartilage [5,6]. The expensive production of BMP-2 is a limitation of using this protein in therapeutic applications. A landmark step was made with the use of recombinant gene technology, which allowed the production of large quantities of recombinant human BMPs (rhBMPs) [7,8]. Over the years has been reported the production of recombinant human bone morphogenetic 2 (rhBMP-2) in prokaryotic system. However, to obtain BMP-2 protein in soluble form and in native conditions remains a challenge.

In this work, the expression system chosen for the production of recombinant BMP-2 is the *E. coli* system and for soluble expression, the fusion protein technology with the novel FH8 tag is studied. Beyond the pQE-30 vector, it is used other two different vectors - pETM and pStaby1.2 - to express the fusion protein FH8BMP-2. The main purpose is to get an increase of soluble expression and purity level of target protein. The pET system is a powerful system for the cloning and expression of recombinant proteins in *E. coli*. In relation to pStaby1.2 system, the selection

mechanism is an important feature of this plasmid, because instead of antibiotics, this system uses antidote genes present naturally in plasmids. It is an advantage for commercialization systems, as it is described that contamination of the product by antibiotics is unacceptable by regulatory institutions.

The main objectives of the project are the following:

- Production of soluble recombinant BMP-2 protein fused to the FH8 tag, using the pQE-30 expression system;
- Purification of the fusion protein via the FH8 tag by hydrophobic interaction chromatography and via affinity chromatography with nickel, using the His-tag in the system pQE-30;
- Cloning of the gene coding for BMP-2 and its insertion into two other expression systems (pETM and pStaby1.2) containing the His-FH8 tag;
- Production of soluble recombinant fusion protein in both expression systems;
- Purification of fusion proteins obtained in both systems (pETM and pStaby) by nickel affinity chromatography (His-tag) and hydrophobic interaction chromatography (FH8-tag);

Chapter 1: Review Literature

1.1 Bone morphogenetic proteins

Discovered in 1965, BMPs belong to a group of cytokines from the transforming growth factor-β (TGF-β) superfamily. To date, at least fifteen BMPs have been identified and characterized, and the members of BMPs family have been subdivide into subsets according to their amino acid sequence homology and similarity in protein structure. The main biological functions of BMPs are based especially on bone and cartilage formation [1,9]. BMPs are homo or heterodimers, which chains are connected via disulphide bridges. In general, BMPs are biological active as both homo or heterodimer conformation. The structure of these proteins are based on conserved motif of seven cysteines, that are involved in the formation of six intrachain disulphide bonds and a single interchain bond, to promote the dimer formation [1,4,9]. However, to form active dimer, BMP molecules are dissociated between the propeptide and mature region after proteolytical cleavage (Figure 1). These proteins are produced as large precursor molecules containing a hydrophobic signal sequence, a long and poorly conserved N-terminal pro-region sequence, a mature domain with a highly conserved C-terminal region and an N-terminal region that varies among the different BMPs [1,9]. The molecular signalling of BMPs is based on binding of these proteins to serine-threonine kinase receptors, which are present in the cells surface. This binding promotes important intracellular events that are responsible for activation of gene transcription, leading to cell proliferation and differentiation.

In particular, there are three types of receptors for TGF-β superfamily members, but only receptors type I and II are involved in the signalling of BMPs. That is, the cascade of intracellular events that are involved with BMPs signalling occurs when the binding of these proteins to type I and II receptors (BMPRI, BMPRII) triggers a signal transduction cascade via Smad family proteins. As a consequence, BMP-Smad pathway will activate direct or indirectly target genes, responsible for osteoblast differentiation (Figure 2) [1,4,10].

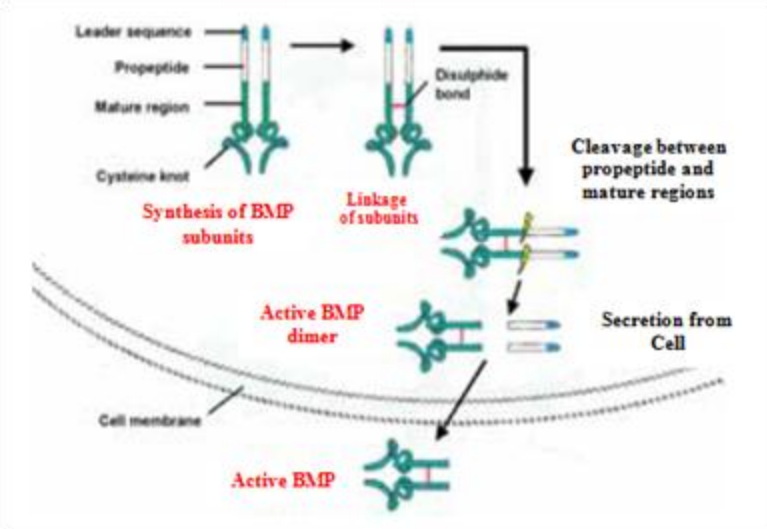

Figure 1: Activation of BMP dimer. Adapted from [9]

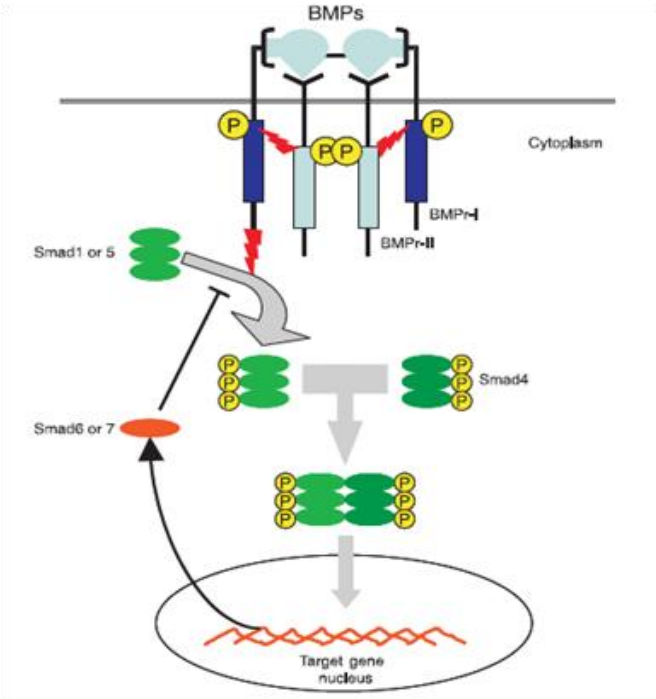

Figure 2: Gene transcription by BMP-Smad activation. Adapted from [4]

1.1.1 Bone morphogenetic protein 2 (BMP-2)

BMP-2 is one of the main BMPs member implicated in growth and regeneration of bone and cartilage, and therefore is one of the most intensely investigated growth

factor. BMP-2 is localized in bone tissue and is released in response to bone damage, stimulating differentiation of mesenchymal cells into osteoblasts and inducing cell proliferation via the Smad pathway demonstrated in Figure 2. In terms of structure, human BMP-2 consists of a long precursor protein of 396 amino acids, which is glycosylated, proteolytically cleaved and dimerized to form the mature homodimeric protein consisting of two 114 residue subunits [11]. Other feature related to the structure of BMP-2 is a heparin-binding domain located in the N-terminal region of the mature polypeptide, which can cause alterations on its biological activity [1]. The surface of the BMP-2 dimer is very hydrophobic causing its low solubility in aqueous solutions. Its osteoinduction properties have been subject of numerous preclinical and clinical experiments, described in literature (McKay and Sandhu (2002,2007); Govender et al. (2002); Raiche and Puleo (2004)), showing various therapeutic applications of BMP-2 [2,5,12–15].

1.2 Commercial BMP2

As a result of its therapeutic potential, BMP-2 has been studied as an alternative to autologous bone grafting in many clinical situations. These include spinal fusion, osteoporosis, treatment of bone defects, non-union fractures and root canal surgery [1,2,16–18]. The approval commercial systems involving this protein in human application occurred in 2002, when the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) have approved the clinical use of BMP-2. Currently, BMP-2 is commercially available under the implant kit name Inductos™ (Medtronic Sofamor Danek and Wyeth Pharmaceuticals). This kit contains the BMP-2 protein as a lyophilized powder, dissolved in sterile water and is applied to an absorbable collagen matrix made of type 1 bovine collagen, for the treatment of acute open tibial fracture. Another similar product is the Infuse™ bone graft (Medtronic Sofamor Danek), indicated to the treatment of degenerative lumbar disc disease [2,5,12–15]. These commercial preparations of BMP-2 are, however, produced abroad and are expensive [1,2,5].

To overcome this bottleneck, during the last years, the investigation has been focused in strategies to increase production of BMP-2 at lower cost taking advantage of the advances in recombinant technology.

1.3 Recombinant production of BMP-2

As with the majority of BMPs, reduced yields are obtained when native BMP-2 is isolated from bone (around 1-2 µg/kg bone). Since the production and purification of this native protein presents difficulties and its clinical applications are limited by the potential health risks associated with its isolation from allogeneic bone donor, researchers have been encouraged to express BMP-2 protein, as well as the other BMPs by DNA recombinant technology [1,4,5,19,20].

Recombinant human BMP-2 (rhBMP-2) was first obtained using mammalian cultures of chinese hamster ovary cells and the recombinant protein promoted ectopic bone and cartilage formation after two weeks of implantation in rats. After this pioneering work, other investigations were performed, to obtain rhBMP-2 using different expression systems (Table 1) [1,2,4].

Table 1: Selected works reporting the production of rhBMP-2 in different expression systems. Adapted from [1]

BMP	Expression system	Novelty	References
BMP-2	CHO cells	Bone formation and characterisation of expressed BMP-2	Wang *et al* – 1990 Israel *et al* - 1992
BMP-2	Insect cells	Alternative expression system	Maruoka *et al* – 1995
BMP-2	*E.coli*	The heparin binding domain reduces ALP and specific *in vitro* biological activity	Ruppert *et al* - 1996
BMP-2	*E.coli*	High density expression in bacteria	Li *et al* - 1998
BMP-2	*E.coli*, pCYTEXP3	Optimisation of refolding conditions	Vallejo *et al* – 2002 Vallejo and Rinas - 2004
BMP-2	*E.coli*	Additional heparin binding domains enhance *in vivo* bone formation	Wurzler *et al* - 2004
BMP-2	*E.coli*, pET-11a	Comparison with BMP-2 propeptide	Hillger *et al* - 2205
BMP-2	*E.coli*, pET-21a	Use of different refolding buffers	Long *et al* – 2006
BMP-2	*E.coli*, pET-25b	Bioactivity in human stem cells	Bessa *et al* - 2008

Nowadays, recombinant human BMPs (rhBMPs) are produced mainly by two expression systems: in mammalian cells or in bacteria. However, with mammalian cells expensive cultivation and poor yields render this procedure cost-intensive, especially at the industrial scale. Production in prokaryotic hosts is usually the preferred method, because these offer important advantages, such as, high yield, low cost cultivation/production and high bio-safety. One of prokaryotic systems widely used for the rapid and economical production of recombinant proteins is *Escherichia coli* (*E. coli*) [1,12,16,17,19].

1.3.1 *E. coli* as a host

Recombinant protein expression has revolutionized all aspects of the biological sciences, namely by the dramatically expansion of the number of proteins that can be studied both biochemically and structurally. In spite of the development of multiple nonbacterial recombinant expression systems over the last three decades (yeast, baculovirus, mammalian cell, cell free systems), *E. coli* is still the preferred host for recombinant protein expression, being widely used in industry and in academic research for this purpose [21–24]. The vast advantages of *E. coli* turn it into a valuable organism for the high-level production of recombinant proteins. Among the important features, one can find: rapid growth, and expression, its well-characterized genetics, high product yields, easy of genetically manipulation, and it is relatively inexpensive to culture. The ability of this bacterial host to accumulate recombinant proteins up to 80% of its dry weight and to survive a variety of environmental conditions represent also important advantages for a wide range of downstream applications . Actually, the expression of human proteins in *E. coli* can reach a high success rate of 75% [21,22,24–26].

However, common drawbacks can occur when using *E. coli* as an expression host. *E. coli* is not capable of producing eukaryotic post-translational modifications, such as glycosylation, which can be critical for the production of folded and active protein. Equally important is the fact that many proteins of biomedical interest have proved difficult to express properly in this host system because they are easily turned into insoluble and instable protein aggregates, identified as inclusion bodies. This aggregation of recombinant proteins in bacterial cells is a result of accumulation of high concentrations of folding intermediates or from inefficient processing by molecular chaperones [24,27]. The main disadvantages of obtaining proteins from inclusion bodies

are related with solubilisation and refolding time-consuming steps and with the use of denaturing agents to extract protein of interest. This can cause problems with protein native conformation and consequently its activity. In the case of BMP-2, it is a protein that is biologically active in homodimeric form, so refolding steps are necessary after recovery from inclusion bodies [5,18,27].

During the last years some investigators have performed important scientific work in this field (in Table 2 is possible to observe some of these publications) in order to produce and purify rhBMP-2 in *E. coli*, from the recovery of inclusion bodies.

To overcome limitations associated to the production of recombinant protein in the form of inclusions bodies, several strategies have been described in literature to achieve soluble protein expression in *E. coli*. In following section it will be described some of these strategies, in which fusion expression technology has an important focus [23,28–30].

Table 2: Studies of the production and purification of rhBMP-2 in *E.coli*.

Author/date	Study
Zhang et al. / 2011	Expression, purification, and refolding of a recombinant rhBMP-2
Zhang et al. / 2010	Optimized procedure for expression and renaturation of rhBMP-2 at high protein concentrations
Sharapova et al. /2011	Production of the rhBMP-2 in *E. coli* and Testing of Its Biological Activity in vitro and in vivo
Bessa et al. / 2007	Osteoinduction in human fat-derived stem cells by rhBMP-2 produced in *E. coli*
Long et al. / 2006	Expression, purification, and renaturation of rhBMP-2 from *E. coli*

1.4 Production of soluble recombinant BMP-2

According to recent studies of structural genomics centres, more than half of all recombinant proteins accumulate in the form of insoluble aggregates when they are

overproduced in *E. coli*. However, in many biomedical applications, the objective is to obtain a product that is soluble, folded and active [21,31].

Many methodologies that have been applied to increase the solubility of recombinant proteins in *E. coli* consist on the manipulation of culture conditions, and altering the temperature at which the target protein is being produced [31,32]. Also, changes in the *E. coli* expression strains, the use of different promoters and co-expression methodology may contribute to improve the solubility of recombinant proteins in *E. coli*.

In relation to BMP-2, there is only a study by *Ihm et al* in which is reported the soluble expression of BMP-2 in *E. coli*, using co-expression of thioredoxin gene in a different expression vector [18].

As referred above, in most cases, strategies previously described do not solve completely the problem and other technologies are been widely explored as, for instance, the use of solubility fusion tags [31,32].

1.4.1 Fusion Protein Technology

Fusion protein technology is widely used for a rapid, efficient and cost-effective protein expression and purification process [33]. Fusion tags are described to be proteins or peptides that are fused to the protein of interest and the main functions of these partners are to increase target protein production yields, promote its solubility and help on its purification [29,31,32]. Over the years several fusion partners had already been described in literature (Table 3), but none of them is ideal with respect to all parameters referred above.

Generally, it is difficult to choose the best fusion system for a specific protein of interest, due to different factors such as the expression system, the target protein itself (its stability or hydrophobicity) and the application of the purified protein. Thus, to determine the “best” tag for a specific target protein remains a challenging.

Additionally, there are some factors related to the fusion tags that can affect the soluble protein expression levels and its purification, for example the placement of the tag, either in N-terminal or C-terminal. On the other hand, as many of affinity tags are large proteins, they can affect important characteristics like the structure of the protein to be studied. Also, biological activity of the recombinant expressed protein can be affected by the presence of a fusion tag and thus, in these cases, it is necessary to

remove the tag after the purification of the fusion protein [21,31,32,34]. The most commonly used solution to remove the tag is to place a protease cleavage site between the solubility tag and the partner protein, allowing an *in vitro* reaction after purification to remove the fusion tag [32]. The commonly proteases used are: enterokinase, tobacco etch virus (TEV), thrombin, and factor Xa. The recovery process of target protein depends on the cleavage efficiency [35].

Table 3: Commonly used solubility fusion tags. Adapted from [32]

Tag	Protein name	Source organism
MBP	Maltose-binding protein	*E. coli*
GST	Glutathione-S-transferase	*Schistosoma japonicum*
Trx	Thioredoxin	*E. coli*
NusA	Utilization substance	*E. coli*
SUMO	Small ubiquitin-modifier	*Homo sapiens*
SET	Solubility-enhancing tag	*Synthetic*
DsbC	Disulfide bond C	*E. coli*
Skp	Seventeen kilodalton protein	*E. coli*
T7PK	Phage T7 protein kinase	*Bacteriophage T7*
GB1	Protein G B1 domain	*Streptococcus sp*
ZZ	Protein A IgG ZZ repeat domain	*Staphylococcus aureus*

1.4.1.1 Fusion system – FH8 tag

The Hitag® fusion system consists of two novel fusion tags, the H and FH8 tags, which have demonstrated to increase protein expression levels in *E. coli* [36,37].

The FH8 tag is an 8 kDa protein with 69 aminoacids and it is a homologous antigen secreted by the parasite *Fasciola hepatica* in the early stages of infection. Previous studies demonstrated that FH8 is a binding calcium protein by the presence of two EF-hands, which contain motifs involved in calcium coordination. Structurally, EF-hands proteins are organized in functional domains which form stable helical bundles. The binding calcium properties of FH8 can cause conformational changes on its structure: when calcium binds to EF-hands domains, a switch from a closed to an open

conformation occurs. This reorientation of the protein conformation leads to the exposure of hydrophobic regions, which act as a target binding surface [36,38]. Figure 3 shows in detail this change of conformation after calcium binding (Figure 3 a)) and also the conformational change of hydrophobic area of FH8, which becomes larger in calcium loaded state (Figure 3 b)).

As other members of the EF-hand family, FH8 tag demonstrates an unusual stability at high temperatures, and in the presence of calcium it is even more stable in other denaturant conditions, such as in the presence of high urea concentrations [38,39].

Another interesting feature as a result of calcium interaction is a dimmerization of FH8 protein [38,39]. A study conducted of FH8 shows the analysis, in non-denaturing conditions, of higher molecular weight band, suggesting the formation of dimers and pentamers. The pentamers are considered the most stable structure of the antigen and its formation is dependent on calcium [38].

Currently, FH8 tag has been tested as solubility and purification tag with proteins difficult to express in *E. coli* [29,37,39,40]. The fusion of these proteins to the FH8 tag resulted in an increase of soluble expression and due to this positive effect, it will be studied the FH8 tag application with BMP-2 protein.

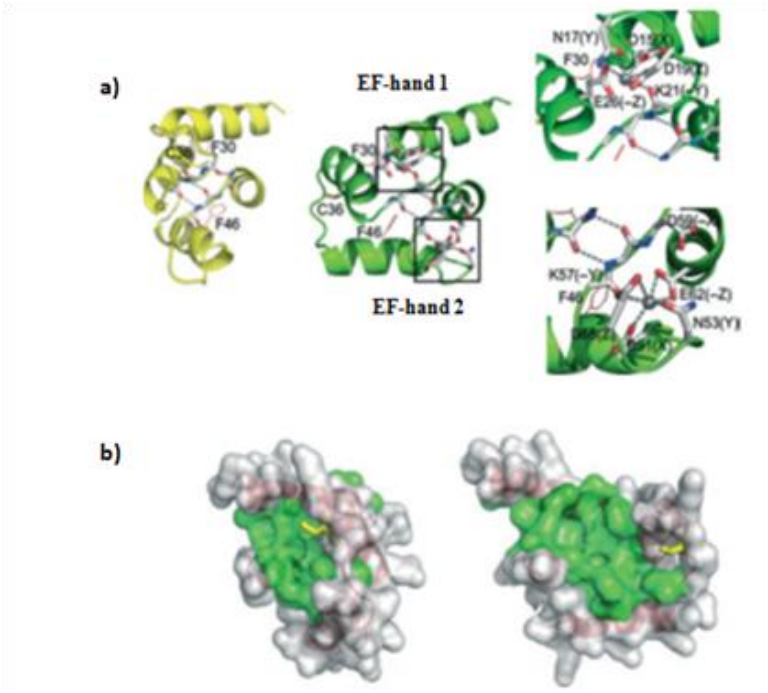

Figure 3: a) Model structure of FH8 in the closed conformation (yellow). After calcium ligation occur a change to open conformation (green) and on the right is possible to see in detail the calcium binding loops, where calcium ions are represented by grey spheres. b) Molecular surface representation of FH8 in closed (left) and open (right) conformations. Adapted from [38]

Chapter 2: Materials and Methods

2.1 Sterilization of material, culture media and solutions

The sterilization of materials, solutions and culture media was carried out by autoclaving at 121°C for 20 minutes. Thermolabile solutions were sterilized by filtration with sterile filters of 0.2 micrometers.

2.2 Chemical products, solutions and reagentes

Composition of all solutions, loading samples and reagents used in this work are described in Table A1 of appendix 1.

2.3 Vectors and Bacterial strains

The plasmid vectors and the corresponding *E. coli* strains used to produce the recombinant fusion protein in this study are presented in Table 4. It is important to note that only the construction of pQE-30/FH8BMP-2 was already available. The other two constructions were conducted in the scope of this work. A more detailed description of each plasmid vector will be presented in this section.

Table 4: Plasmid vectors and bacterial strains used in the present work.

Backbone plasmid	**Constructed plasmid**	***E.coli* strain**	***Phenotype***
pQE-30 (QIAexpress system – Quiagen)	pQE-30/FH8BMP2	M15[pREP4]	NaIS, StrS, RifS, Thi–, Lac–, Ara+, Gal+, Mtl–, F–, RecA+, Uvr+, Lon+
pETM (EMBL)	pETM/FH8BMP2	BL21 CodonPlusRil	B F^- ompT hsdS(rB^- mB^-) dcm^+ Tet^r gal endA Hte [argU ileY leuWCam^r]
		Rosetta 2	F^- ompT $hsdS_B$(rB^-mB^-) gal dcm pRARE23 (Cam^R)
pStaby1.2 (StabyExpress system - Delphi)	pStaby1.2 /FH8BMP2	SE1	gal, dcm, DE3 (lacI, T7 polymerase under the control of the lacUV5 promotor), $ccdB^+$

2.3.1 pQE-30 QIAexpress system

This expression system, distributed by Qiagen, consists of pQE-30 vector and it was used to express BMP-2 protein with and without the FH8 fusion tag. The pQE-30 plasmid, represented in Figure 4, uses a transcription–translation system based in T5 promoter/*lac* operator, which allows high expression levels of recombinant proteins in *E. coli* [41]. This system has other important feature: the presence of a 6xHis-tag codifying sequence in the N-terminal, which when genetically bound to the protein of interest, can facilitate the purification process by affinity chromatography.

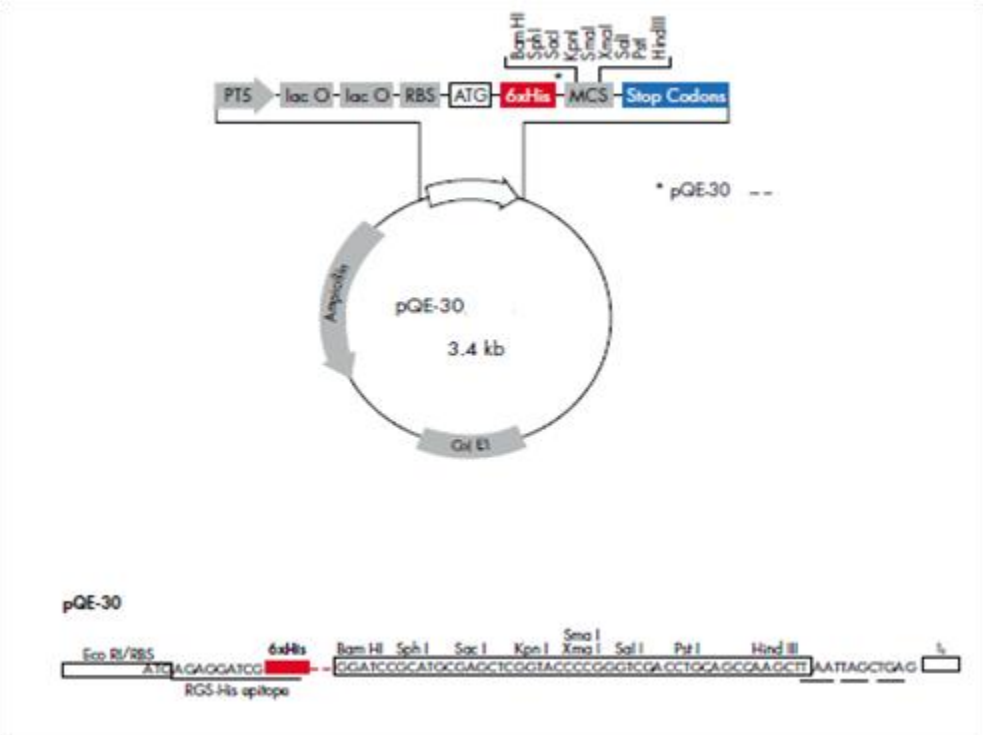

Figure 4: Graphic of plasmid map of pQE-30, Qiagen. Adapted from [41]

2.3.2 pETM vector

The pETM vectors derive from the pET vector series initially developed by Studier and colleagues, which represent nowadays a powerful system to cloning and expression of recombinant proteins in the *E. coli* host. The pETM plasmids use the same transcription-translation system as the pET collection based in T7 promoter to clone the genes of interest. Some important features of pETM vectors are: the presence

of two 6xHis-tags (one before and other after the MCS), a conserved multiple cloning site (MCS) and a TEV protease recognition site.

In this particular study it was used the pETMFH8 plasmid, containing the FH8 tag and a TEV recognition site between the fusion tag and protein. This expression vector has also the presence of a gene responsible to confer kanamycin resistance. The MCS is located between TEV site and the second 6xHis-tags. A schematic diagram of pETMFH8 vector is shown in Figure 5.

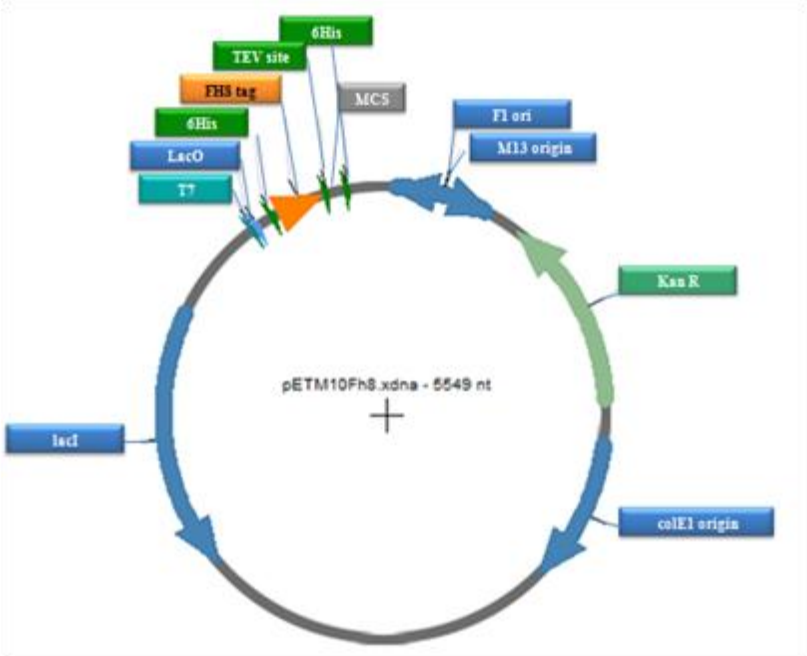

Figure 5: Graphic of plasmid map of pETMFH8, EMBL.

2.3.3 pStaby1.2 Express System

This expression system, commercialized by Delphi Genetics, was also used to clone *bmp-2* gene and produce FH8BMP-2 protein. The plasmid used in this work was the pStaby1.2FH8, which has the following features: T7 promoter, 6xHis-tag at the C-terminal end of the protein; the plasmid is stable without the use of antibiotics. As in the case of pETMFH8 vector, the gene of codifying for BMP-2 was cloned into the pStaby1.2FH8 plasmid, but in this vector FH8 tag and the 6xHis residues are in opposite ends, which may improve the purification process. Plasmid scheme of pStaby1.2FH8 is displayed in Figure 6.

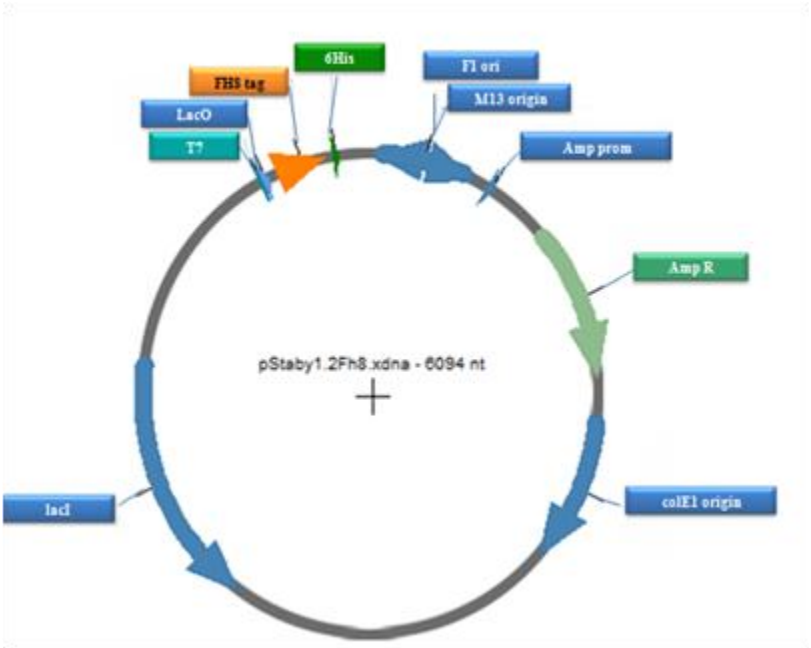

Figure 6: Graphic of plasmid map of pStaby1.2FH8, Delphi.

2.4 Molecular Biological Methods

2.4.1 Extraction of plasmid DNA and template sequence of BMP-2 from *E. coli*

The pQE-30/BMP-2 plasmid, already available in the lab, was used as a template for amplifying the codifying sequence of *bmp-2* gene. *E. coli* M15 pQE-30/BMP-2 cells were incubated at 37 ºC o/n from a plate containing LB/amplicillin/kanamycin and plasmid DNA was extracted. The DNA from bacterial cultures containing the pETMFH8 (LB/kanamycin) and pStaby1.2FH8 (LB/ amplicillin) plasmids were prepared in the same way. The DNA extraction was performed using the NZYMiniprep (Nzytech) kit, according to manufacturer's instructions [42]. At the end of the protocol, fractions of 30-50 µl were recovered, containing DNA of template sequence and plasmid DNA.

2.4.2 PCR of insert fragment – *bmp-2* gene

To obtain the desired constructs, the *bmp-2* gene was amplified by PCR using specific primers accordingly to the destination vector (pETMFH8 or pStaby1.2FH8). The first step before proceeding to the amplification reaction was primer design, in

which specific pairs of primers were constructed, each pair related to the corresponding vector. Template sequence was amplified using the primers presented in Table 5.

Table 5: Sequence of primers used in PCR reaction.

Cloning *bmp-2* gene into	Primer forward – 5'- 3'	Primer reverse – 5'- 3'
pETMFH8	TCTATTCCATGGGATCCACTTTCGGCCACGA TGGTAAAGG	AATAGACTCGAGCTAGCGACA GCCACAACCCTCCACAAC
pStaby1.2FH8	TCTATTGAGCTCGAGAATCTTTATTTTCAGG GCATGACTTTCGGCCACGATGGTAAAG	AATAGACTCGAGGTAGCGACA GCCACAACCCTCCACAAC

The forward primers contain the recognition sequences (nucleotides underlined) for *NcoI* and *SacI* to be used in the pETMFH8 and pStaby1.2FH8 plasmids, respectively; the reverse primers contain the same restriction site for *XhoI*.

PCR was performed in 50 µl of reaction mixture containing 1 µl of DNA template, 1 µl of each primer, 1 µl of dNTP's mixture, 10 µl 5x Phusion HF buffer, 0.5 µl of Phusion DNA Polymerase (Fermentas) and ultrapure water to complete final volume. The amplification reaction was held on a thermal cycler *My CyclerTM Thermal Cycler* (Biorad) with program described in Table 6.

After visualization on agarose gel, the two products of amplification (*bmp-2* for pETMFH8 and *bmp-2* for pStaby1.2FH8) were purified to be further cloned into the final vectors.

Table 6: PCR program.

Step	Temperatute, Time	Cycles
Initial Denaturation	98 ºC, 30 seconds	1
Denaturation	98 ºC, 10 seconds	30-35
Annealing	68 ºC, 30 seconds	
Elongation	72 ºC, 30 seconds	
Final extension	72 ºC, 10 minutes	1
Soak	4 ºC, ∞	1

2.4.3 Agarose Gel Electrophoresis

For DNA analysis, electrophoresis procedure was performed using agarose gel stained with GreenSafe Premium (Nzytech). GreenSafe Premium is a new nucleic acid stain which can be used as a safer alternative to the traditional ethidium bromide stain for detecting nucleic acids in agarose gels [43]. The agarose was dissolved in TAE buffer and the percentages of agarose gels varied between 1% and 1.2 %, according to DNA fragment that is visualized. The DNA samples were mixed with 5x sample loading dye and then were loaded into the gel. The DNA molecular weight used in this project was NZYDNA Ladder III (Nzytech). Electrophoresis running occurred at 90 V in horizontal cells (Biorad) with TAE buffer. The gels photos were taken by a transilluminator *Gel Doc 2000* (BioRad).

2.4.4 DNA purification

After 1% agarose gel electrophoresis (technique described in section 2.4.3), the DNA bands of PCR products were cut off using UV light panel of a transilluminator *Gel Doc 2000* (BioRad) and were treated following standard protocol of QIAEX II Gel Extraction Kit (QIAGEN) [44].

2.4.5 Digestion of DNA with restriction enzymes

For the digestion of DNA fragments, 2 µg of DNA was mixed with corresponding enzymes in a reaction volume of 50 µl, in the presence of the recommended 10x reaction buffer and BSA 100x solution. The reaction mix was incubated at 37 ºC o/n and stopped by incubation at 65ºC for 20 min. The incubation time of reaction mix and concentration of restriction endonuclease were applied according to manufacturer's instructions. Description of restriction enzymes used during this procedure is presented in Table 7.

Table 7: Description of restriction enzymes used.

Restriction Enzyme	Cleavage site	Source
*Nco*I	5'-C^C A T G G-3' 3'-G G T A C^C -5'	New England Biolabs
*Sac*I	5'-G A G C T^C-3' 3'-C^T C G A G-5'	New England Biolabs
*Xho*I	5'-C^T C G A G-3' 3'-G A G C T^C -5'	New England Biolabs

2.4.6 Dephosphorylation of Plasmid DNA

Dephosphorylation of cloning vectors pETMFH8 and pStaby1.2FH8 was carried out with Shrimp Alkaline Phosphatase (SAP) of Fermentas. This enzyme is used to prevent re-circularization and re-ligation of linearized vector DNA. Dephosphorylation procedure was performed in 55 µl of reaction mixture, containing linear DNA, 5.5 µl of 10x reaction buffer, 1 µl of SAP and ultrapure water to complete final volume. The mixture was incubated at 37 ºC, during 1 h and stopped at by incubated at 65ºC for 15 min. After SAP inactivation, plasmid DNA was purified according to the procedure described in section 2.4.4.

2.4.7 DNA quantification

The concentration of nucleic acids was measured by spectrophotometry with NanoDrop™ 1000 (Thermo Scientific), in which 2 µl of DNA sample was used.

2.4.8 Ligation of DNA insert fragment to the vector

After purification of the insert and vector DNAs, it was estimated the amount of insert required at a specific molar ratio of vector:insert for DNA ligation. In this work, for ligation between insert fragment *bmp-2* and pETMFH8 (ligation a) a molar ratio of vector:insert of 1:3 was used; the ligation between insert fragment *bmp-2* and pStaby1.2FH8 (ligation b) involved a molar ratio of vector:insert of 1:2. Apart from insert fragment and vector, ligation reactions implicated the mixture of T4 DNA Ligase (Promega) and the recommended buffer. In this way, ligation reactions of 10 µl and 20 µl to ligation a and b, respectively, were prepared. To ligation a, it was mixed 2 µl of

vector DNA, 2.4 µl of insert DNA, 1 µl of 10x ligase buffer, 0.5 µl of T4 DNA ligase 3u/µl and ultrapure water to complete final volume. Reaction mixture of ligation b containing 15 µl of DNA vector, 0.5 µl of insert DNA, 2 µl of 10x ligase buffer, 1 µl of T4 DNA ligase 3u/µl (Promega) and ultrapure water up to final volume. Both reactions were incubated o/n at 4 ºC.

E. coli DH5 α and *Top 10* cells were transformed with the resulting DNA from ligation of *bmp-2* insert and the corresponding plasmids (methodology described in section 2.4.10).

2.4.9 Preparation of chemical competent *E. coli* cells

A pre culture of 10 ml of *E. coli DH5α* or *Top 10* cells was prepared and incubated at 37 ºC with shaking o/n. Then 5 ml of pre culture was added to 250 ml LB medium with the corresponding antibiotics and cells grew at 37 ºC with vigorous shaking (200-250 rpm) until OD_{600} reaches 0.5. The culture was kept on ice for 10 min and then transferred to sterilized 50 ml falcon tubes, followed by a centrifugation at 4000 g (4 ºC) for 10 min. For each falcon tube, the pellet was resuspended carefully in 20 ml of ice-cold TB solution and kept on ice for another 10 min. Then, the suspension cells were recovered by centrifugation at 4000 g (4 ºC) for 10 min and the pellet was resuspended in equivalent volume of TB solution and DMSO was added for each tube to a final concentration of 7%. The competent cell suspension was dispensed into microtubes (200 µl/tube) and stored at -80 ºC.

2.4.10 Transformation of recombinant DNA to competent *E. coli* cells

2.4.10.1 Transformation of chemically competent *DH5* α *E. coli* cells

This protocol was made to transform DNA of ligation a, according to the following steps: 8 µl of DNA solution were added to 50 µl aliquot of *DH5α* competent cell suspension and this mixture was incubated on ice for 20 min. A heat-shock was made at 42 ºC for 45 seconds and after cell suspension was kept on ice for 2 min. Then, 800 µl of LB medium was added and the mixture was continually incubated at 37 ºC for 1h. At the end, transformed cell suspension was plated onto LB/kanamycin plate.

Separately and following the same procedure, it was prepared a *DH5α* cells control (without ligation reaction) and a negative control (ligation without insert fragment). The plates were incubated at 37 ºC overnight to develop colonies of the transformed cells.

2.4.10.2 Transformation of chemically competent *Top 10 E. coli* cells

To transform DNA of ligation b it was performed a similar transformation protocol to the previous one, but in this case using competent *Top 10 E. coli* cells. In this way, 20 µl of DNA solution were added to 200 µl aliquot of *Top 10* competent cell suspension and this mixture was incubated on ice for 30 min. A heat-shock was made at 42 ºC for 30 seconds and after cell suspension was kept on ice for 10 min. Then, 800 µl of SOC medium was added and the mixture was continually incubated at 37 ºC for 1h. At the end, transformed cell suspension was plated onto LB/ ampicillin plate. Separately and following the same procedure, it was prepared a *Top 10* cells control (without ligation reaction) and a negative control (ligation without insert fragment). The plates were incubated at 37 ℃ overnight to develop colonies of the transformed cells.

2.4.11 Screening of Transformants

To determine if the insert fragment of *bmp-2* gene was successfully cloned into pETMFH8 and pStaby1.2FH8 vectors, colony PCR methodology was used for screening the transformed bacteria. Colony PCR protocol is presented as follows: about half of a isolated colony (from transformed cells plate) was picked using a sterile toothpick and resuspended in a 25 µl amplification reaction, which containing 1 µl of $MgCl_2$ 2 mM, 0.5 µl of dNTP's mixture, 0.5 µl of T7 forward primer, 0.5 µl of T7 reverse primer, 2.5 µl of 10x reaction buffer, 0.5 µl of DNA Taq polymerase 5 u/µl (Nzytech) and ultrapure water up to the final volume. Target DNA was amplified using cycling conditions appropriate for screening primers used (universal T7 forward and T7 reverse primers) and size of amplifier product. The amplification reaction was held on a thermal cycler *My CyclerTM Thermal Cycler* (Biorad) with program described in Table 8. An aliquot of the completed PCR was recovered and analysed by agarose gel electrophoresis to identify the product length, which indicates if the correct insert is present in the clone.

Table 8: Colony PCR program.

Step	Temperatute, Time	Cycles
Initial Denaturation	94 ºC, 7 minutes	1
Denaturation	94 ºC, 30 seconds	30
Annealing	55 ºC, 30 seconds	
Elongation	72 ºC, 2 minutes	
Final extension	72 ºC, 10 minutes	1
Soak	4 ºC, ∞	1

2.4.12 DNA Sequencing

All constructions made with *bmp-2* insertion in pETMFH8 and pStaby1.2FH8 vectors were confirmed by sequencing using the Eurofins MWG Operon (Germany) service.

2.4.13 Transformation of chemically competent BL21 Codon Plus Ril and Rosetta 2 *E. coli* cells

The general protocol followed in this transformation step was the same to the described in section 2.4.10.1.

2.4.14 Transformation of chemically competent SE1 *E. coli* cells

The general protocol followed in this transformation step was the same to the described in section 2.4.10.1.

2.5 Soluble Expression of recombinant FH8BMP-2 protein in *E. coli*

This section will describe the main stages of cell growth, protein induction and recovery of soluble fraction of target protein, FH8BMP2, for the different *E. coli* strains used in this work.

2.5.1 Study of the soluble expression conditions of recombinant FH8BMP-2 protein in *E. coli* M15/pQE-30

These experiments were carried out to evaluate the effect of culture conditions for soluble expression of FH8BMP-2 protein produced in *E. coli* M15/pQE-30.

From a freshly bacterial biomass of *E. coli* M15[pREP4] pQE-30/FH8BMP-2, a pre culture of 25 ml LB/ampicilin/kanamycin was prepared and it was allowed to grow at 37 ºC, o/n with shaking (200 rpm). The next day, four erlenmeyer flasks were prepared as follows: in each one, containing 250 ml LB/amplicilin/kanamycin medium, was inoculated 1/50 ratio of the pre culture. Cell cultures were incubated at 37 ºC and 200-250 rpm until the OD_{600} reach a value between 0.4-0.6. At this point, the expression of FH8BMP-2 was induced by the addition of IPTG to a final concentration of 0.1 mM and incubated at 18-20 ºC at two different induction times: two erlenmeyer flasks were incubated for 16h and the other two were induced for 24h. At the end of induction times, cells were harvested by centrifugation at 4000 g (4 ºC) for 20 min and the resulting cell pellets were stored at -20 ºC.

To study the optimal conditions of soluble expression of FH8BMP-2, cell pellets were resuspended in two different lysis buffers: Buffer A – 50 mM Tris-HCl, 250 mM NaCl, pH 8 and Buffer B – 50 mM Sodium Phosphate, 300 mM NaCl, pH 8. Thus, the four pellets obtained (each one corresponding to 250 ml of culture), were resuspended in 10 ml of buffer A and buffer B with 1 mM PMSF (phenylmethylsulfonyl fluoride (serine protease inhibitor)) as proteases inhibitor, according to their induction time culture, as shown in the scheme of Figure 7.

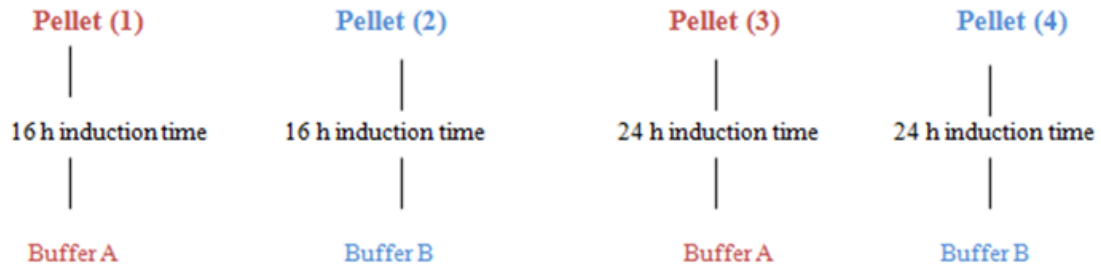

Figure 7: Soluble expression conditions of FH8BMP-2 in pQE system.

Then bacterial cells were incubated at room temperature for 15 min and then transferred to ice and further sonicated (Branson sonifier – 30 seconds on and 30 seconds off for 6

cycles, *Duty cycle* 50 % and *Output control* 5) and soluble fraction was separated from its insoluble part by centrifugation at 16000 g (4 ºC), for 30 min. Samples of soluble fractions and total lysates (the sample of total lysate corresponds to the total extract of *E. coli* immediately after cell lysis) of the four cultures studied were further analyzed by electrophoresis under denaturing conditions: SDS-PAGE (methodology described in section 2.6).

2.5.2 Soluble expression of recombinant FH8BMP-2 protein in *E.coli* M15 /pQE30

After being determined the conditions that maximize the soluble production of recombinant FH8BMP-2, its expression was performed through total cultures of 1L resulting from small cultures of 250 ml. The soluble production of target protein was made according to the process described in section 2.5.1.

2.5.3 Study of the soluble expression conditions of recombinant FH8BMP-2 protein in pETM: *E. coli* BL21 Codon Plus Ril and Rosetta2

In the extent of these experiments, two main goals were established: to determine the most favourable conditions of soluble expression of FH8BMP-2 in pETM system and to select the *E. coli* strain that maximize FH8BMP-2 production. To accomplish these goals, small scale screenings using cultures of 20 ml were performed. 3 ml of LB medium and specific antibiotics (final concentrations of kanamycin: 50 ug/ml and chloroamphenicol: 33 ug/ml) were added to 15 ml falcon tubes and single colonies were picked from corresponding bacterial biomass and inoculated in the medium. Pre-cultures had grown at 37 ºC, o/n with shaking (200 rpm). The next day, 20 ml LB/kanamycin/chloroamphenicol medium was inoculated with 1/50 ratio of the each pre culture (400 µl) using 100 ml erlenmeyer flasks. Cell cultures were incubated at 37 ºC and 200-250 rpm until the OD_{600} reach a value between 0.4-0.6 (2-3h of incubation). At this point, liquid cultures of 20 ml were divided into 2×10 ml cultures in 50 ml falcons to study different inductor concentrations and time of induction. In this way, cultures were placed as follows: the first falcons (2× 50 mL falcons, corresponding to BL21 Codon Plus Ril and Rosetta2 cultures) at 18ºC, o/n with IPTG to a final concentration of 0.2 mM and the second group of falcons (2 × 50 mL falcons,

corresponding to BL21 Codon Plus Ril and Rosetta2 cultures) were incubated at 30ºC for 3h with IPTG 0.5 mM.

After the induction period, cells were harvested by centrifugation at 4000 g, 4 ºC, for 10 min and resulting cell pellets were stored at -20 ºC, o/n.

The next day, bacterial pellets were resuspended in 5 ml of lysis buffer - 50 mM Tris-HCl, 250 mM NaCl, pH 7.4 with 1 mg/ml lysozyme and 1 mM of PMSF; incubated at room temperature for 10 min and then transferred to ice and sonicated (Branson sonifier – 30 seconds on and 30 seconds off for 6 cycles in position 8, *Duty cycle* 50 % and *Output control* 5). Soluble fraction was separated from its insoluble part by centrifugation at 16000 g (4 ºC), for 30 min. Aliquots of soluble fractions and total lysates of all cultures studied were further analyzed by electrophoresis under denaturing conditions: SDS-PAGE (methodology described in section 2.6).

2.5.4 Soluble expression of recombinant FH8BMP-2 protein in *E. coli* BL21 Ril /pETM10

After being determined the conditions that maximize the production of recombinant FH8BMP-2, its expression was performed through total cultures of 1L, as a result of four cultures of 250 ml. The soluble production of target protein was made according to the process described in section 2.5.3.

2.5.5 Conditions study of soluble expression of recombinant FH8BMP-2 protein in *E. coli* SE1/pStaby1.2

The process followed to solubility analysis of FH8BMP-2 protein in pStaby1.2 vector using SE1 *E. coli* strain was identical to that described in section 2.5.3, with the exception of certain aspects: it was studied another induction condition at 37 ºC for 3h and cultures were prepared without antibiotics due to the special feature of pStaby1.2 system, described previously.

2.6 Electrophoresis under denaturing conditions: SDS-PAGE

This methodology was used to analyze all the samples of protein extracts and fractions obtained before, during and after the purification process. This type of electrophoresis is based on Laemmli system [45]. This system uses discontinuous polyacrylamide gel to separate proteins based on molecular weight; an electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive electrode (anode). Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty. The polyacrylamide gel is cast as a separating gel (usually known as resolving or running gel) topped by a stacking gel and secured in an electrophoresis apparatus. In this system, denaturation of proteins occurs in the presence of β-mercaptoethanol to reduce disulfide bonds and SDS reagent, which confers negative charge to molecules making the running uniquely dependent on differences in molecular weight. Ammonium Persulfate (APS) and Tetramethylethylenediamine (TEMED) catalyze the polymerization of acrylamide solutions into gel matrices. SDS-PAGE gels composition is presented in Table 9.

Table 9: SDS-PAGE gels composition.

Reagents	Resolving gel	Stacking gel
40% Acrilamid/Bis	3 ml	0.5 ml
0.5M Tris-HCl, pH 6.8	-	1.25 ml
0.5M Tris-HCl, pH 8.8	2.5 ml	-
10% SDS	100 µl	50 µl
10% APS	50 µl	25.5 µl
TEMED	5 µl	2.5 µl
H_2O	4.35 ml	3.17 ml
Final volume	10 ml	5 ml

Before application into the gel, samples were treated by adding 5x loading sample buffer and by incubating in a bath at 100 ºC for 5-10 min. If samples were not immediately loaded into the gel, they were kept on ice or at -20 ºC. The protein molecular weight marker used in SDS-PAGE electrophoresis was PageRuler™ Unstained Broad Range Protein Ladder (5-250 kDa) from Fermentas.

The electrophoresis run occurred in vertical units with 1x running buffer at constant voltage of 120 V, and at the end gels were stained by *Coomassie Blue* staining method described below.

After gel running, the gel is placed in a box for the following washing steps: distillated water is added to the gel and heated in the microwave for 30 seconds; then the box with the gel is placed on a shaker for 3 min and this washing step is repeated twice with fresh distillated water. After washing steps, enough *Coomassie Blue* solution (its recipe is described in Table A1 of appendix 1) is added to cover the gel in the box and is heated in the microwave for 30 seconds and then the box with the gel is placed on a shaker for finishing the staining during 30 min. At the end, when staining solution is poured off, distilled water is added for destaining of the gel and then gel can be photographed or dried for long term storage.

2.7 Purification of recombinant FH8BMP-2 protein

Purification of soluble fraction of FH8BMP-2 was carried out by the performance of several experiments which embraced the following chromatographic techniques: hydrophobic interaction; immobilized metal ion affinity and ion exchange. The first two purification techniques were executed in small screenings and then in column system. Ion exchange chromatography was used as a second purification step in column system.

2.7.1 Hydrophobic Interaction Chromatography (HIC)

The FH8 tag was used as purification tag through the use of HIC technique to purify the fusion protein. HIC is a liquid chromatography technique, which separates biomolecules based on differences in their surface hydrophobicity. Therefore, hydrophobic amino acids exposed on proteins surface can interact with the hydrophobic surface of HIC medium and since the amount of exposed hydrophobic amino acids varies between proteins it is possible obtain their separation and the specificity of the process.

HIC media contain ligands of alkyl or aryl groups coupled to an inert matrix of spherical particles. The porosity of matrix allows high internal surface area and the ligand plays an important role in the final hydrophobicity of HIC media.

Most HIC experiments are performed in four main stages: equilibration; sample application and wash; elution and regeneration. The first step is the equilibration of stationary phase, which is performed by adding salt to the mobile phase to get the desired start conditions: binding of interest protein in HIC protocol is promoted by moderately high salt concentrations. The second step corresponds to sample load onto the column and when sample loading is completed and the column has been washed so that all non-bound proteins have passed through, conditions are altered to begin elution. Proteins are released from the column by decreasing the salt concentration in the elution buffer. As the level of salt decreases, proteins with the lowest hydrophobicity begin to elute from the column. In the final step of regeneration, all molecules still attached to the column are removed.

In this work, for the small-scale purification of FH8BMP-2 it was used a Phenyl Sepharose™ 6 Fast Flow (high substitution) resin (GE Healthcare). Phenyl Sepharose 6 Fast Flow is composed of cross-linked 6% agarose beads modified with standard aromatic phenyl groups via uncharged, chemically-stable ether linkages.

In larger scale assays a prepacked 5 ml column Phenyl Sepharose™ 6 Fast Flow (high sub) (GE Healthcare) was used.

Tables 10 and 11 present the description of all buffers used in HIC purification protocols performed in this study. The volumes used in each step of HIC experiments and protocols used in small and larger scale assays are described below.

Table 10: Buffers recipe for FH8-HIC small screenings.

HIC - Small scale Screenings							Incubation conditions	
Screening	**Target protein**	**Lysis Buffer**	**Binding Buffer**	**Washing Buffer**	**Elution Buffer**	**Other additives**	**Time**	**°C**
Screening 1	pQE-30/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 8	50 mM Tris, 250 mM NaCl, pH 8	50 mM Tris, 250 mM NaCl, pH 8 (1:2)	50 mM Tris, pH 10		3h	4/ RT
Screening 2	pQE-30/FH8BMP-2	50 mM Naphosphate, 300 mM NaCl, pH 8	50 mM Naphosphate, 300 mM NaCl, pH 8	50 mM Naphosphate, 300 mM NaCl, pH 8 (1:2)	50 mM Tris, pH 10		3h	4/ RT
Screening 3	pQE-30/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 7	50 mM Tris, 250 mM NaCl, pH 7	50 mM Tris, 250 mM NaCl, pH 7 (1:2)	50 mM Tris, pH 10	**5 mM of $CaCl_2$**	1h	RT
Screening 4	pQE-30/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 7.5	50 mM Tris, 250 mM NaCl, pH 7.5	50 mM Tris, 250 mM NaCl, pH 7.5 (1:2)	50 mM Tris, pH 10	**5 mM of $CaCl_2$**	1h	RT

Table 11: Buffers recipe for FH8-HIC 0.5-1L purification assays.

HIC - 0.5-1L Purification Assays						
Purification assay	**Target protein**	**Lysis Buffer**	**Binding Buffer**	**Washing Buffer***	**Elution Buffer**	**Other additives**
Assay 1	pQE-30/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 7	50 mM Tris, 250 mM NaCl, pH 7	50 mM Tris, 250 mM NaCl, pH 7 (1:2)	50 mM Tris, pH 10	**5 mM of $CaCl_2$**
Assay 2	pQE-30/FH8BMP-2	50 mM Naphosphate, 300 mM NaCl, pH 7	50 mM Naphosphate, 300 mM NaCl, pH 7	50 mM Naphosphate, 300 mM NaCl, pH 7 (1:2)	50 mM Tris, pH 10	
Assay 3	pETM/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 7.4	50 mM Tris, NaCl 250 mM, pH 7.4	**W1** – 50 mM Tris, 250 mM NaCl, pH 7.4 (1:2); **W2**- dH2O	50 mM Tris, pH 10	**5 mM of $CaCl_2$**
Assay 4	pETM/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 7.4	50 mM Tris, 250 mM NaCl, pH 7.4	**W1** – 50 mM Tris, 250 mM NaCl, pH 7 .4 (1:2); **W2** – H2O; **W3** – 50 mM Tris, 250 mM NaCl, pH 7.4 + 2.5 M MgCl2; **W4** – 50 mM Tris, 250 mM NaCl, pH 7.4 + 1.5 M Urea + 5% glycerol;	50 mM Tris, pH 10	**5 mM $CaCl_2$**
Assay 5	pETM/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 7.4	50 mM Tris, 250 mM NaCl, pH 7.4	50 mM Tris, 250 mM NaCl, pH 7.4, 3%(w/w) isopropanol	50 mM Tris, pH 10, 10%(w/w) isopropanol	**5 mM $CaCl_2$**

*W1-W4: corresponds to the different washing buffers used during purification assays.

2.7.1.1 Small scale screenings protocol

The procedure below has been used to purify the soluble fraction of FH8BMP-2 in small amounts in order to determine the optimal conditions for HIC purification to subsequently perform larger scale purifications. The stages relative to the recovery of soluble fraction (ressuspension of cell pellet, enzymatic and mechanical cell lysis) are described in section 2.5.1 and 2.5.3.

1. The first step in this small scale purification protocol was the preparation of resin: since it was used 15 ml tubes, the Phenyl Sepharose™ 6 Fast Flow resin was divided equally in these tubes (1-2 ml of resin solution in each tube). Then, each tube was centrifuged for 10 min at 4 ºC to 4000 g to remove the 70% ethanol which is stored with the resin;
2. Resin was washed and equilibrated by adding 2 ml of the correspondent binding buffer and centrifuged for 10 min at 4 ºC to 4000 g;
3. In screenings 3 and 4, calcium ($CaCl_2$) at 5 mM of concentration was added to soluble fraction of FH8BMP-2 prior to sample incubation with the resin;
4. 2.5-5 ml of soluble fraction of FH8BMP-2 was added to the resin and incubated at specific period of time and temperature – time and temperature of incubation (described in Table 10) were two variables to test in screening assays;
5. At the end of incubation time, each tube was centrifuged for 10 min at 4 ºC to 4000 g to collect the flowthrough sample and an aliquot of this sample was stored for SDS-PAGE;
6. Resin was washed with 2 ml of correspondent washing buffer and centrifuged for 10 min at 4 ºC to 4000 g. Washing samples were collected and aliquoted to be analyzed by SDS-PAGE;
7. Finally target protein was eluted three times with 2 ml of correspondent elution buffer and centrifuged for 10 min at 4 ºC to 4000 g. Purified samples were collected and aliquoted to be analyzed by SDS-PAGE;
8. After elution, impurities and proteins that remained attached to the resin were removed by adding urea at 8M concentration. Corresponding sample was collected and aliquoted to be analyzed by SDS-PAGE;

9. Resin was regenerated by washing it with distilled water and 20 % ethanol to be stored and then used for a new purification.

2.7.1.2 0.5-1L Purification Assays protocol

The protocol used in these purification assays applies for the HIC purification technique, but it was adapted to the other chromatographic techniques used in this work to purify FH8BMP-2. The purification protocol was performed by using a peristaltic pump and the different steps were followed by observation of the reaction with Bradford reagent (5 µl Bradford + 200 µl sample).

The stages relative to the recovery of the soluble fraction (ressuspension of cell pellet, enzymatic and mechanical cell lysis) are described in section 2.5.1 and 2.5.3. The detailed HIC protocol is described below and it is important to refer that all the procedure was performed at 4 ºC.

1. The 5 ml prepacked column Phenyl Sepharose™ 6 Fast Flow (high sub) was stored in 20% ethanol at 4 ºC. Prior to usage, the column was washed with water and it was determined the appropriate flow rate to apply during purification – 1 ml/min to equilibration and washing steps; 0.5 ml/min to apply sample to the column and to elute target protein;
2. The column was equilibrated with 5 CV[1] of corresponding binding buffer;
3. Soluble fraction, previously recovered, was applied onto the column (soluble fraction was previously filtered/centrifuged);
4. The flowthrough sample was collected and aliquoted to be analyzed by SDS-PAGE;
5. The column was washed with at least 5 CV of corresponding washing buffer until the Bradford signal is low intense blue to ensure that all unbound material has washed out through the column. Washing samples of 10 ml were collected and aliquoted to be analyzed by SDS-PAGE;
6. Target protein was eluted with corresponding elution buffer, and purified samples of 5 ml were collected in 15 ml tubes and aliquoted to be analyzed by SDS-PAGE;

[1] Buffer volumes used during sample application, washing, elution and re-equilibration are expressed in column volumes (CV). In this project were used columns with a 5ml of bed volume: for example 5 CV= 25 ml.

7. The cleaning procedure of the column after use was performed as following: column was washed with 2 CV of distillated water; impurities were removed with 8 M urea solution or 1 M NaCl; it was again washed with distillated water and finally the column was washed with 20% ethanol (usually 2 CVs);
8. The column was stored at 4 ºC.

2.7.2 Immobilized metal ion Affinity Chromatography (IMAC)

IMAC separates proteins on the basis of a reversible interaction between target protein through histidine residues (or Trp and Cys) present on their surface and divalent metal ions (e.g., Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}) immobilized via a chelating ligand. In the particular case of this study, nickel was used as immobilized ligand, which allows the affinity of histidines tail present in FH8BMP-2 protein. Immobilized metal ion Affinity Chromatography with nickel (IMAC-Ni) purification experiments involves the following steps: equilibration of the column with binding buffer containing a low concentration of imidazole, which is a counter ligand that binds to the immobilized nickel. Then sample is applied to the column, but before is adjusted to the same imidazole concentration; in this stage, proteins with histidines bind while displacing the imidazole counter ligands. The third step is column wash and then elution of the target protein using a higher concentration of imidazole. Instead of imidazole gradient, it is important to note that these experiments can also be performed using a pH gradient. Final step corresponds to column regeneration, when all molecules that still remained are removed.

In this work, for purification of FH8BMP-2 it was used in small scale screenings a nickel Ni-NTA resin (GE Healthcare); in larger scale assays a prepacked 5 ml column HisTrap IMAC HP (GE Healthcare) was used.

Tables 12 and 13 present the description of all buffers used in IMAC purification protocols performed in this study. The volumes used in each step of IMAC experiments are described below for the protocols used in small and larger scale assays.

Table 12: Buffers recipe for IMAC small screenings.

IMAC - Small scale Screenings						Incubation conditions	
Screening	**Target protein**	**Lysis Buffer**	**Binding Buffer**	**Washing Buffer**	**Elution Buffer**	**Time**	**ºC**
Screening 1	pQE-30/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 8	50 mM Tris, 250 mM NaCl, 20 mM imidazole, pH 8	50 mM Tris, 250 mM NaCl, 40 mM imidazole, pH 8	50 mM Tris, 250 mM NaCl, 300 mM imidazole, pH 8	2h	4
Screening 2	pQE-30/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 8	50 mM Tris, 250 mM NaCl, pH 8	50mM Tris, 250mM NaCl, pH 6.5	50 mM Tris, 250 mM NaCl, pH 4.0	2h	4
Screening 3	pETM/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 7.4	50 mM Tris, 250 mM NaCl; 20 mM imidazole, pH 7.4	50 mM Tris, 250 mM NaCl; 40 mM imidazole, pH 7.4	50 mM Tris, 250 mM NaCl; 300 mM imidazole, pH 7.4	30 min	4

Table 13: Buffers recipe for IMAC 0.5-1L purification assays.

IMAC - 0.5-1L Purification Assays					
Purification assay	**Target protein**	**Lysis Buffer**	**Binding Buffer**	**Washing Buffer**	**Elution Buffer**
Assay 1	pETM/FH8BMP-2	50 mM Tris, 250 mM NaCl, pH 7.4	50 mM Tris, 250 mM NaCl; 20 mM imidazole, pH 7.4	50 mM Tris, 250 mM NaCl; 40 mM imidazole, pH 7.4	50 mM Tris, 250 mM NaCl; 300 mM imidazole, pH 7.4
Assay 2	pStaby1.2/FH8BMP-2	50 mM Naphosphate, 300 mM NaCl, pH 7.4	50 mM Naphosphate, 300 mM NaCl, pH 7.4, 20 mM imidazole	50 mM Naphosphate, 300 mM NaCl, pH 7.4, 40 mM imidazole	50 mM Naphosphate, 300 mM NaCl, pH 7.4, 300 mM imidazole

2.7.2.1 Small scale screenings protocol

The general protocol followed in these purification screenings was the same to the described in section 2.7.1.1. In this section it only will be described the main differences registered to the case of IMAC experiments:

- Soluble fraction was adjusted, by adding imidazole at 20 mM of concentration;
- In screening 3, it was used only 300 µl of resin solution, and consequently the buffer volume applied in each step was 300 µl.

2.7.2.2 0.5-1L Purification Assays protocol

As referred before, the HIC purification protocol is similar to the adapted for IMAC assays and so the steps performed in these experiments can be consulted in section 2.7.1.2.

2.7.3 Ion Exchange (IEX) chromatography

Ion exchange (IEX) chromatography can separate molecules, like proteins, on the basis of slight differences in their surface charge. In the specific case of proteins, they are formed by different amino acids containing acid and basic groups and consequently their net surface charge will change according to changes in pH environment [46]. Relationship between net surface charge and pH is unique for a specific protein and a model of this relationship can be visualized in Figure 8.

Considering this feature, in an IEX separation, a reversible interaction between charged molecules and an oppositely charged chromatography media occurs to ensure that the target molecules or proteins bind to the medium while they are loaded onto the column.

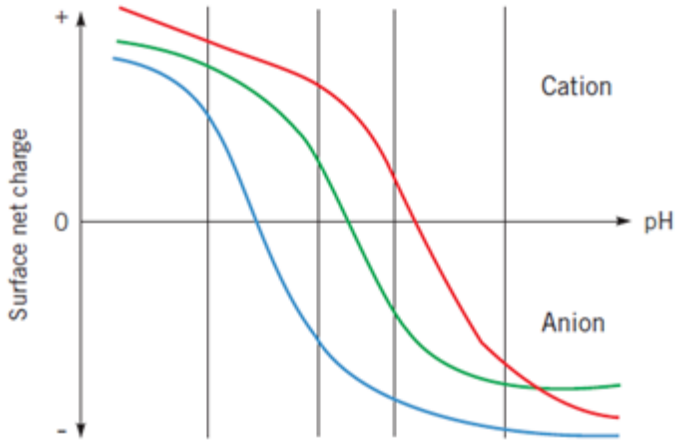

Figure 8: Variation of surface net charge according to pH of the medium [46].

An IEX medium is formed by a matrix of spherical particles alternated with ionic groups that are negatively (cationic exchange) or positively (anionic exchange) charged. Matrix porosity is important to give a high internal surface area. In the first

step of IEX chromatographic technique, which corresponds to column equilibration, the pH and ionic strength of the binding buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the medium and as many impurities as possible do not bind. Then conditions are altered so that proteins are eluted differentially. The elution step can be performed in two different ways: continuous gradient or a stepwise increase in ionic strength. This last type of elution is commonly made using NaCl. On the other hand, conditions can be chosen to maximize the binding of contaminants and in this way target protein is conducted to pass through the column and is recovered in the flowthrough sample without contaminants. This last strategy was performed in this study to recover FH8BMP-2 protein in the flowthrough sample. The removal of contaminants was conducted using a stepwise elution. To perform the IEX technique, an anionic exchanger incorporated in a prepacked 5 ml column HiTrap Q HP (GE Healthcare) was used. The purification protocol was performed by using a peristaltic pump and the different steps were followed by observation of the reaction with Bradford reagent (5 µl Bradford + 200 µl sample). The detailed IEX protocol is described below and all the procedure was performed at 4 ºC. Table 14 presents the composition of buffers used during this procedure:

1. The column was washed with 2 CV of distillated water;
2. The column was equilibrated with 5 CV of corresponding binding buffer at a flow-rate of 1 ml/min;
3. Soluble fraction of FH8BMP-2 protein was applied onto the column (soluble fraction was filtered/centrifuged before being applied to the column) at a flow-rate of 0.5 ml/min;
4. The flowthrough sample was collected and aliquoted for SDS-PAGE;
5. Column was washed with at least 5 CV of binding buffer, at a flow-rate of 1ml/min, until the Bradford signal is low intense blue to ensure that all unbound material has washed through the column. Washing samples of 10 ml were collected and aliquoted to be analyzed by SDS-PAGE;
6. Step gradient elution procedure (at a flow-rate of 0.5 ml/min) was made using NaCl, as described in Table 14;
7. The cleaning procedure of the column after use was performed as following: column was washed with 2 CV of distillated water; impurities were removed

with 1M NaCl; it was again washed with distillated water and finally column was washed with 20% ethanol (usually 2 CVs);

8. Column was stored at 4 ºC.

Table 14: Buffers recipes for IEX chromatography.

Binding buffer	50mM Tris, 100mM NaCl, pH 8
Step gradient elution	**1.** 50 mM Tris, 200 mM NaCl, pH 8 **2.** 50 mM Tris, 400 mM NaCl, pH 8 **3.** 50 mM Tris, 600 mM NaCl, pH 8 **4.** 50 mM Tris, 800 mM NaCl, pH 8 **5.** 50 mM Tris, 1 M NaCl, pH 8

2.8 Protein dialysis

After purification, it was necessary to perform the dialysis of all obtained protein samples, to return to the physiological pH. In this way, protein samples were dialysed in 1x PBS buffer (prepared from 10x PBS concentrated buffer) at pH 7.4. Mostly, the dialysis procedure occurred overnight at 4 ºC.

2.9 Protein quantification

Protein quantification was performed by the Bradford colorimetric method with protein assay dye reagent (Biorad) in microplate. The protocol was done using plates of 96 wells, where 10 µl of total protein sample was loading in each well with 200 µl of BioRad Protein Assay diluted 1:5. After 5-10 min it was measured the absorbance at 595 nm, using the Biotech Synergy HT Microplate Reader. In each quantification test, a calibration curve was obtained measuring the absorbance at 595 nm of known concentrations of bovine serum albumin (BSA).

2.10 Dynamic light scattering (DLS)

DLS measurements were obtained in the Zetasizer Nano ZS (Malvern Instruments). The FH8BMP-2 protein concentration was in the range of 0.5-1 mg/ml and protein samples were centrifuged and filtered before measurements to remove any aggregates.

Chapter 3: Results and Discussion

The present section is divided into four main parts: in the first part, the test of soluble expression and purification of recombinant FH8BMP-2 in *E. coli* M15 using the pQE-30 system (construction already available) is presented and discussed; secondly, the construction of recombinant vectors (pETM/FH8BMP-2 and pStaby1.2/FH8BMP-2) is also discussed, followed by the test of soluble expression of recombinant protein in these new vectors. In the fourth part, the large scale production, purification and quantification of recombinant FH8BMP-2 in pETM and pStaby1.2 systems is presented and discussed.

3.1 Soluble expression and purification of recombinant FH8BMP-2 in *E. coli* using the pQE-30 system

The results of the expression and purification of FH8BMP-2 in pQE-30 vector are here divided into two parts: the main results of soluble test expression of fusion protein, in comparison with BMP-2 without FH8 tag and HBMP-2 (BMP-2 fused to H tag), and the results of purification tests performed with FH8BMP-2, under native conditions.

3.1.1 Soluble test expression of recombinant FH8BMP-2 in *E. coli* M15 using the pQE-30 system

The *bmp-2* gene was previously cloned into the pQE-30, pQEH and pQEFH8 plasmids and these vectors were first used in this study to assess the improvements in the BMP-2 soluble recombinant production in *E. coli*. The direct comparison between the soluble expression of BMP-2 and HBMP-2 proteins is presented in Figure 9. As can be seen, through the box marked in both SDS-PAGE gels of the Figure (corresponding to soluble fractions), there is no expression of BMP-2 in soluble form at the expected molecular weight of 17 kDa (Figure 9.a) and for the case of HBMP-2 there is a minimal

expression in this state, at the expected molecular weight of 18.5 kDa (Figure 9.b). Therefore, the expression of both BMP-2 and HBMP-2 proteins occurs mainly in the insoluble form as observed in samples marked by a brown arrow. This result reflects the difficulty in obtaining BMP-2 protein in soluble form.

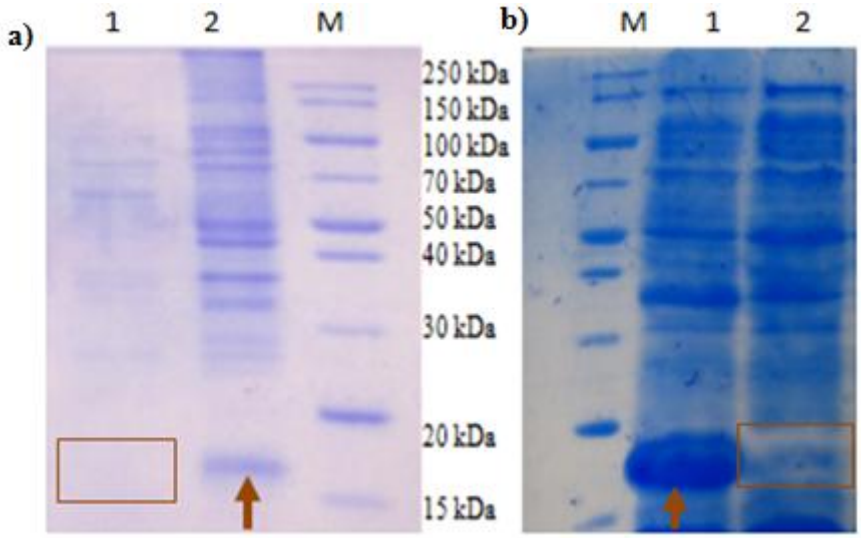

Figure 9: a) 15%-4% SDS-PAGE solubility analysis of BMP-2 in pQE-30 system: 1 – soluble fraction; 2 – insoluble fraction; M - PageRuler™ protein marker (Fermentas).
b) 15%-4% SDS-PAGE solubility analysis of HBMP-2 in pQE-30 system: M - PageRuler™ protein marker (Fermentas); 1 – insoluble fraction; 2 - soluble fraction.

Figures 10 and 11 present, respectively, the SDS-PAGE gels of induction and soluble expression analysis of recombinant protein BMP-2 fused to the FH8 tag, obtained in 250 ml cultures. This analysis allowed to access if the induction process occurred and if the recombinant protein was expressed in soluble form. Taking into account the expression profiles in Figure 10, it is possible to observe after induction the expression of a protein around 25 kDa, which is in agreement with the estimated weight of FH8BMP-2 that corresponds to 25.13 kDa. An optimization of culture conditions for soluble expression of FH8BMP-2 protein was then conducted using four experiments (Table 15), testing different induction times and compositions of lysis buffer. Buffer A corresponds to *Tris* composition and Buffer B to *Sodium Phosphate* composition (detailed composition of buffers are described in section 2.5.1 of materials and methods chapter).

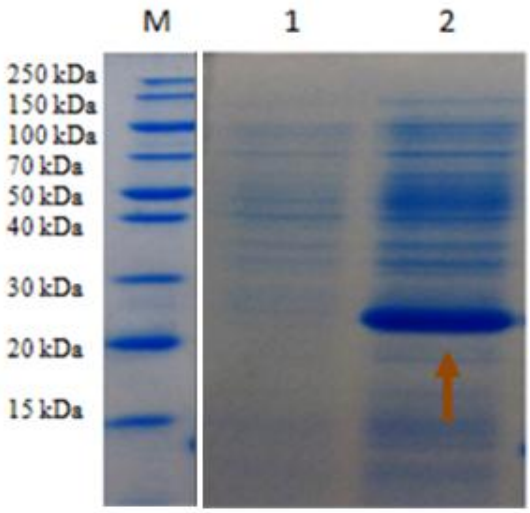

Figure 10: 15%-4% SDS PAGE analysis of expression level of recombinant FH8BMP-2 in *E. coli M15/*pQE-30: M – PageRuler™ protein marker (Fermentas); 1 –uninduced total bacterial protein; 2 – induced total bacterial protein.

Table 15: Studied culture conditions for soluble expression of FH8BMP-2.

Culture conditions	Description
a)	Induction time: 16h Lysis buffer: Buffer B
b)	Induction time: 24h Lysis buffer: Buffer B
c)	Induction time: 16h Lysis buffer: Buffer A
d)	Induction time: 24h Lysis buffer: Buffer A

Figure 11 shows the SDS-PAGE of total lysate and soluble fraction samples obtained in the soluble expression optimization. Looking at the gel, it is possible to observe the expression of soluble FH8BMP-2 (lanes 2, 3, 6 and 7) at the same molecular weight as observed in the induction analysis (around 25 kDa). In general, the soluble expression is very similar in the four conditions tested. However, there is a slightly increase in band intensity for condition c) (Table 15), which is marked in Figure 11 with a red arrow. This result confirms that the FH8 tag solubilises the expression of BMP-2 protein in *E. coli*, in comparison to the results obtained with the protein without tag. This result is in agreement with previous work showing that FH8 is a solubility tag [36,39]. Regarding the induction conditions tested, the 16h was the most favourable condition and so, it was selected to further proceed with large scale production. In relation to the type of buffer, the lysis buffer A shows better results for soluble expression of the fusion protein. Nevertheless, it was decided to proceed with the two

buffer compositions in further experiments to study the conformation behaviour and stability of the fusion protein.

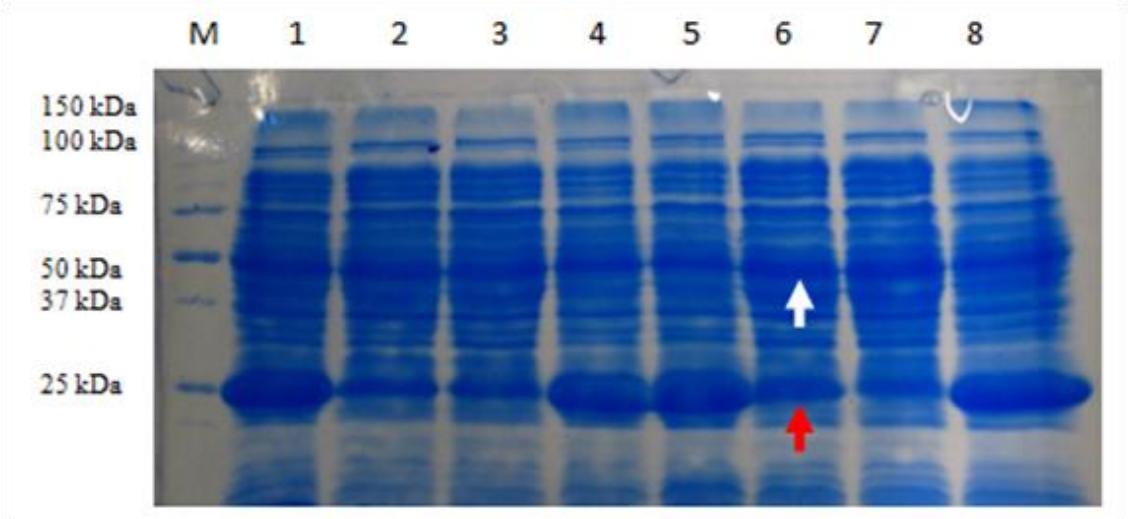

Figure 11: SDS-PAGE Solubility analysis of FH8BMP-2 in pQE-30 system of 250 ml cultures: M – PageRuler™ protein marker (Fermentas); 1 – total lysate of a); 2 – soluble fraction of a); 3 – soluble fraction of b); 4 – total lysate of b); 5 – total lysate of c); 6 – soluble fraction of c): monomer of FH8BMP-2 – red arrow; oligomer of FH8BMP-2 – white arrow; 7 – soluble fraction of d); 8 – total lysate of d).

In Figure 11 it is also observed a gel band in all soluble samples around 50 kDa (indicated by a white arrow). The intensity of this band is relative high comparing to others of crude extract of *E. coli*, and for that reason, it may correspond to an oligomer of FH8BMP-2, which have an estimated molecular weight of approximately 50 kDa. The formation of a oligomeric form can be related to the native conformation of BMP-2 in homodimer [11,16]. Interestingly, comparing this result to the previous one of the HBMP-2 solubility analysis, it is possible to observe a similar band close to 40 kDa for insoluble HBMP-2, which may also correspond to an oligomeric form (estimated at around 37 kDa). This can indicate conformational changes of BMP-2 in the presence of the fusion tag. BMP-2 is a protein that is biologically active in homodimeric form and the presence of the tag, can possibly help to acquire this conformation. However, it is necessary to perform an immunoblotting assay to prove that the protein bands around 50 kDa detected in the gel correspond to BMP-2 protein.

3.1.2 Small screening purification test of soluble recombinant FH8BMP-2 produced in *E. coli* M15 using the pQE-30 system

Small scale purification assays were performed using FH8BMP-2 soluble samples of previous expression experiments. The small screening of purification embraced two purification techniques: HIC and IMAC-Ni. Figure 12 presents the HIC results using the buffers recipe described in section 2.7.1 of materials and methods. In the first two assays (see assays 1 and 2 from Table 10 of that section), which involved *Tris* composition buffer and *Sodium Phosphate* composition buffer at pH 8 (previously tested in solubility analysis), results were not satisfactory because large amount of target protein was lost in the flowthrough and washing samples, but the FH8BMP-2 was still recovered in the elution step, as seen in Figure 12.a (gel band around 25 kDa). The purity level of the obtained fusion protein was low as a large amount of contaminants are present in the eluted sample. These results showed an unspecific interaction of FH8BMP-2 with resin ligands that can be justified by the presence of strong hydrophilic residues of FH8BMP-2 (hidrophobicity analysis is shown in appendix 2). The hydrophilic nature of this protein can promote a weak interaction between protein and hydrophobic resin.

Taking into account the results obtained in assays 1 and 2, two different approaches were tested in order to improve the interaction of FH8BMP-2 with the HIC media. In the first strategy, the exposure of hydrophobic residues of FH8BMP-2 was promoted by the addition of calcium ($CaCl_2$) to the soluble fraction before being applied onto the purification column. This strategy was selected due to the calcium binding properties of FH8 protein and previous work that demonstrate a larger solvent exposed hydrophobic region of FH8 in the presence of calcium [38,39]. In the second strategy, two pH values for the purification buffers were studied (7 and 7.5) using the *Tris* composition (see assays 3 and 4 from Table 10 of materials and methods). Figures 12.b1 and 12.b2 show the SDS-PAGE analyses of purification samples from these assays, where condition i corresponds to pH 7 and condition ii corresponds to pH 7.5 of binding/washing buffer. Regarding to purification results, it is possible to detect important improvements in relation to the previous purification assays: reduction of protein loss in flowthrough and washing samples (Figure 12.b2) and reduction of contaminants level in elution samples (Figure 12.b1). These improvements

demonstrated an increased and specific interaction between protein and hydrophobic resin that can be explained by the calcium-dependent conformation change of FH8 tag.

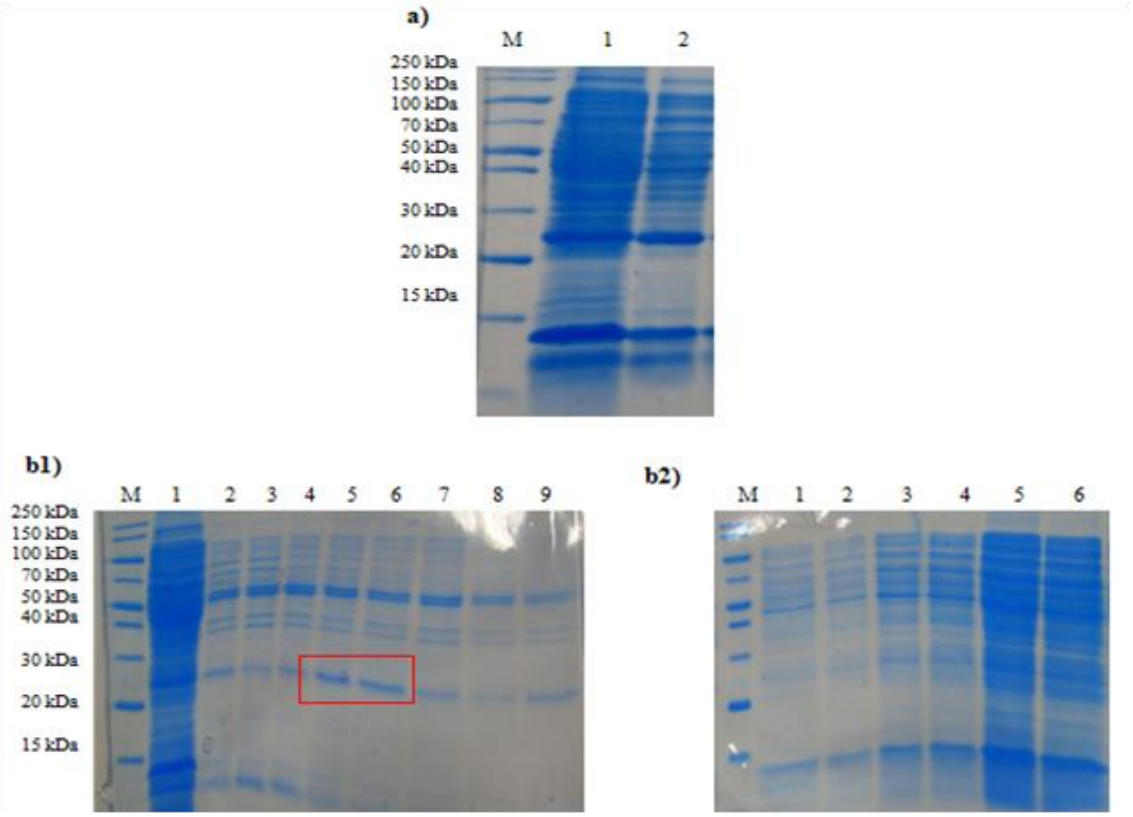

Figure 12: 15%-4% SDS-PAGE analysis of samples obtained from HIC small scale purification assays of FH8BMP-2:
a) Sampling of HIC assays 1 and 2: M - PageRuler™ protein marker (Fermentas); 1 – flowthrough sample; 2 – eluted sample.
b1) Eluted samples of HIC assays 3 and 4: 1 – soluble protein sample charged into the column; 2,5,6,9 – eluted samples from i; 3,4,7,8 - eluted samples from ii.
b2) Flowthrough and washed samples of HIC assay 3 and 4: 1,4 - washed samples from ii; 2,3 - washed samples from i; 5 - flowthrough sample from ii; 6 – flowthrough sample from i.

The purity achieved was not optimal, yet. Regarding the pH strategy, the best results were achieved with the binding buffer at pH 7, where it is verified a stronger protein recovery in eluted samples marked with a red box in Figure 12.b1.

The HIC small screenings outlined the calcium importance for the hydrophobic interaction of FH8BMP-2 fusion protein and a HIC buffer was established to proceed to larger scale purification assays (Table 16).

Table 16: Recipe of buffers of HIC purification step of recombinant FH8BMP-2 protein in *E. coli* M15/pQE-30.

Lysis buffer	Binding buffer	Washing buffer	Elution buffer	Additives
50 mM Tris, 250 mM NaCl, pH 7	50 mM Tris, NaCl 250 mM, pH 7	50 mM Tris, 250 mM NaCl, pH 7 Dilution 1:2	50 mM Tris, pH 10	**5 mM of $CaCl_2$**

In order to explore the fusion protein purification using the 6 histidines presented in the N-terminal of FH8BMP-2, an affinity chromatography using IMAC-Ni technique was performed. The main results of this assay using an imidazole gradient (see screening 1 from Table 12 of materials and methods) are present in Figure 13. Comparing with the previous HIC purification results, notable differences are highlighted, since it can be observed a much lower interaction between FH8BMP-2 and nickel resin. This result is identified in Figure 13 by the reduced amount of protein in eluted samples (marked by a yellow box) and as indicated by a red arrow, the majority of protein amount leaves the column in flowthrough sample. The poor purification of FH8BMP-2 by IMAC can be possibly explained by the fusion protein conformational complexity. The oligomeric tendency of both FH8 tag and BMP2 protein can be hiding the histidines tail and consequently, decreasing the protein interaction with nickel ions.

As the FH8BMP-2 was better purified by HIC than by IMAC, the HIC technique was chosen for the fusion protein purification in larger scale experiments.

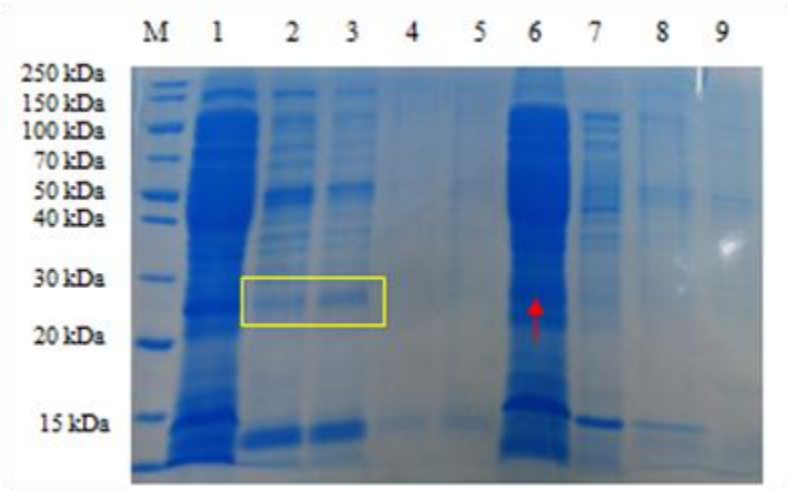

Figure 13: 15%-4% SDS-PAGE analysis of samples obtained from IMAC-Ni small scale purification assay: M - PageRuler™ protein marker (Fermentas); 1 - soluble protein sample charged into the column; 2-5 – eluted samples; 6 – flowthrough sample; 7-9 – washed samples.

3.1.3 Larger scale purification test of soluble recombinant FH8BMP-2 in *E. coli* M15 using the pQE-30 system

Figure 14 shows the SDS-PAGE results of large scale HIC purification assays involving *E. coli* 1 L culture of FH8BMP-2 and using the buffer composition selected in small scale tests (Table 16). As can be seen in Figure 14.a1, the total lysate (lane 1) contains an intense band around 25 kDa, which corresponds to FH8BMP-2 monomer. In contrast, in the soluble fraction applied onto HIC purification column (lane 2), an intense gel band with approximately 50 kDa (indicated by a red arrow) is also detected, in addition to a slight gel band around 25 kDa (indicated by a white arrow). This result is in agreement with previous observations for the soluble expression of FH8BMP-2, in which two gel bands (25 and 50 kDa) were observed. Curiously, in this larger scale expression, the 25 kDa gel band is obtained in lower intensity than the 50 kDa gel band. Regarding the eluted samples presented in Figure 14.a2, it is possible to observe the elution in high amounts of a protein with 50 kDa. The reduced loss of protein in flowthrough and washing samples (Figure 14.a1) contributed to this high protein amount during the elution step. The possible monomeric form of the protein (around 25 kDa) is practically absent in SDS-PAGE, prevailing the possible dimeric state. In order to evaluate this type of conformation obtained by the fusion protein FH8BMP-2, a dynamic light scattering (DLS) analysis was performed. The hydrodynamic radius FH8BMP-2 after purification was measured and it was estimated a molecular weight of 137.80 kDa that may correspond to a complex in pentamer form. This result is in good agreement with the SDS-PAGE analysis, in which were used denaturing conditions. Here, the possible pentamer form was denaturated, resulting in the observation of a gel band with a molecular weight corresponding to the dimeric form (50 kDa).

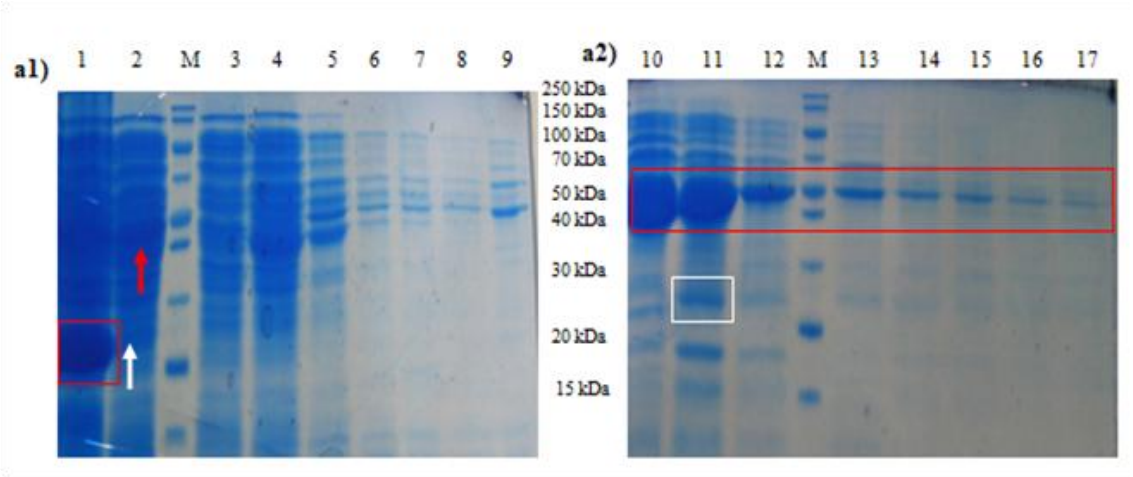

Figure 14: HIC purification assay of 1L E. coli M15/pQE-30 culture of FH8BMP-2:
a1) 1 – total lysate; 2 - soluble protein sample charged into the column; M – PageRuler™ protein marker (Fermentas); 3 – flowthrough sample; 4-8 – washed samples; 9 – eluted sample.
a2) 10-17 - eluted samples**.**

Previous structural studies of the FH8 tag shows a high tendency of this protein for the formation of oligomers, in particular dimers and pentamers, which are also enhanced by the interaction of this molecule in solution with calcium [39,47]. The formation of oligomers of the fusion protein FH8BMP-2 may, therefore, be explained by the oligomerisation tendency promoted by the FH8 tag. However, since it was already observed the presence of a 50 kDa band in solubility tests, even before the addition of calcium, it can be concluded that the transition to a pentamer complex occurs at an early stage. But calcium is in fact an enhancer of this conformational change, as aggregation of the protein in this state is very stable and more prevalent after the addition of calcium. The dynamic structure of the FH8 tag can be influencing the global conformation of the fusion protein, and different molecular forms of FH8BMP-2 can be formed. Additionally, the monomer-to-dimer transition of BMP-2 itself can also be occurring at the same time, as there are proteins that in response to a change in calcium levels, undergo significant conformational changes and dimerize to form an active complex. These include the class of cell matrix proteins, which belongs the BMPs [48]. Naturally, with these conformational changes, the establishment of hydrophobic paths can be favored by the exposure of hydrophobic areas of FH8 tag and, on the other hand, by the solvent-accessible surface of the BMP-2 dimer, which exposes large hydrophobic areas [11]. With all of these possibilities it becomes clear the ability for interaction of the fusion protein with the hydrophobic resin in HIC purification column.

At the end of the purification assay, all eluted samples were dialyzed in PBS 1X, pH 7.4 and then grouped and quantified by Bradford method (described in section 2.9 of materials and methods). A final protein purification yield of 16 mg of purified FH8BMP-2 per litre of *E coli* culture was obtained using the FH8/HIC methodology.

In order to improve purity of protein samples, a second HIC step was performed with eluted fractions 10 and 11 from Figure 14.a2, using the same protocol as previously. The results of this assay are presented in Figure 15. As observed, the second HIC purification improved the purity level of FH8BMP-2. The high stability of FH8BMP-2 aggregation form and the specific interaction with hydrophobic resin, increased by the effect of calcium, was also evident from this second HIC purification. A final yield of 4.85 mg of purified protein per liter of *E. coli* was obtained. This lower yield in comparison to the first HIC purification can be explained by the loss of protein during dialysis process and during the second purification step itself.

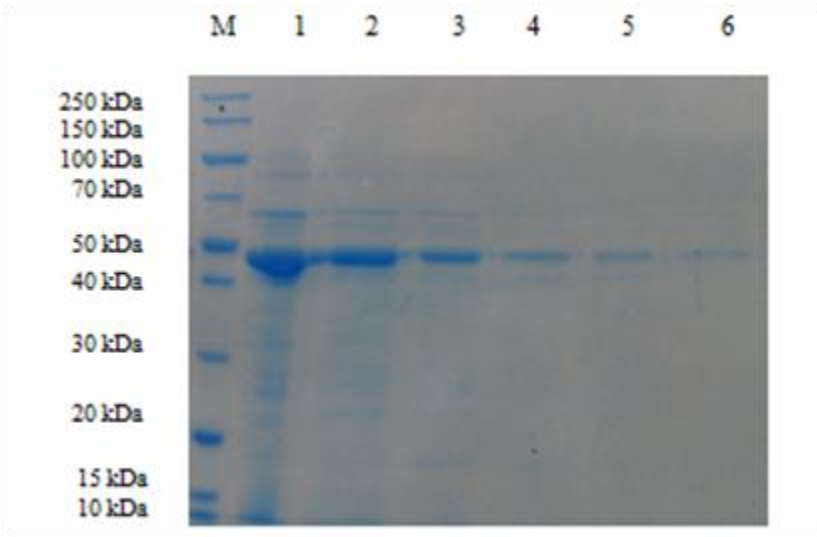

Figure 15:**Second HIC purification of eluted samples of first HIC assay containing recombinant FH8BMP-2**: M – PageRuler™ protein marker (Fermentas); 1-6 – eluted samples.

From this first stage of the project, important conclusions can be taken:

- The FH8 allows the solubilisation of BMP-2 protein, and here, a new strategy was developed to overcome a limitation in obtaining bone morphogenetic protein in soluble form.
- With the purification strategy studied, it was possible to find new directions to purify the fusion protein, namely by the FH8 tag in combination of

calcium and HIC technique. Therefore, it was possible to use the FH8 tag as a purification tag in addition to its solubility enhancer effect.

- A good purity level of protein samples was achieved, but it is still necessary to increase the amount of soluble expression and consequently the amount of purified protein for future biomedical applications.
- The FH8BMP-2 purification by affinity chromatography with nickel was not successful possibly due to an inaccessibility of histidines tail caused by the global structure of the fusion protein.

3.2 Cloning of *bmp-2* gene into pETMFH8 and pStaby1.2FH8 plasmids

In order to increase the production of soluble protein FH8BMP-2 and to obtain larger quantities of purified protein for biomedical applications, two different expression systems were also studied in this work: the pETMFH8 and the pStaby1.2FH8

Figure 16 presents the steps followed to obtain the desired constructs. As it can be seen from Figure 16.a, the template sequence used to produce BMP-2 fragment by PCR was the coding region of the mature BMP-2 from the construction pQE-30/BMP-2. The PCRs performed allowed to amplify the interest fragment and to insert in its ends the cleavage sites of restriction enzymes *NcoI* and *XhoI* (for cloning into pETMFH8) and *SacI* and *XhoI* (for cloning into pStaby1.2FH8). Before performing this step it was important to verify if the selected restriction enzymes (they were chosen according to the MCS of vectors pETM10FH8 and pStaby1.2FH8) do not cut the bmp-2 fragment. According to *NebCutter* analysis (shown in appendix 3), none of these enzymes cut the fragment of interest. The sequence of primers used is presented in section 2.4.2 of materials and methods. In relation to the constructions, it is important to notice the difference of the location of FH8 tag and histidines tail between them. Due this difference, for the cloning process of bmp-2 gene into vector pStaby1.2FH8 it was necessary to introduce a mutation in the natural codon stop of the sequence of BMP-2 to allow expression of the histidines tail located at the C-terminal.

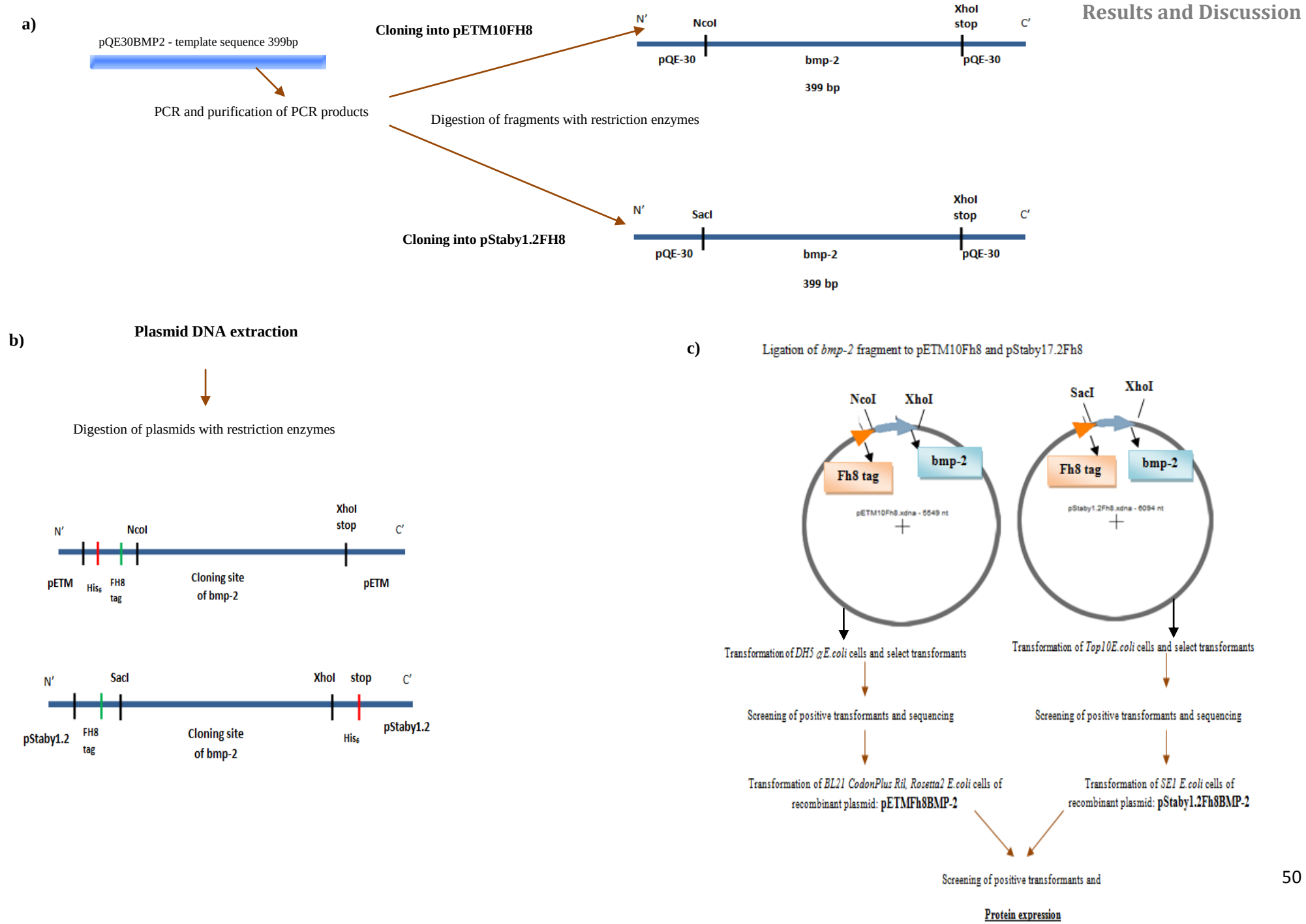

Figure 16: Cloning strategy to pETM/FH8BMP-2 and pStaby1.2/FH8BMP-2 constructs.

The obtained PCR products were analysed in electrophoresis agarose gel and the results are present in Figure 17. Bands of PCR products are slightly above the band of 400 bp, which is concordant with the predicted size, 428 bp (for cloning into pETMFH8) and 444 bp (for cloning into pStaby1.2FH8). It is possible to verify the inexistence of unspecific amplifications and that the intensity of DNA bands is higher for the fragment cloning into pETMFH8 (wells 5 and 6). This result is consistent with the DNA quantification performed which indicates the following DNA concentration: PCR product (pETMFH8) – 167 ng/µl and PCR product (pStaby1.2FH8) – 120 ng/µl.

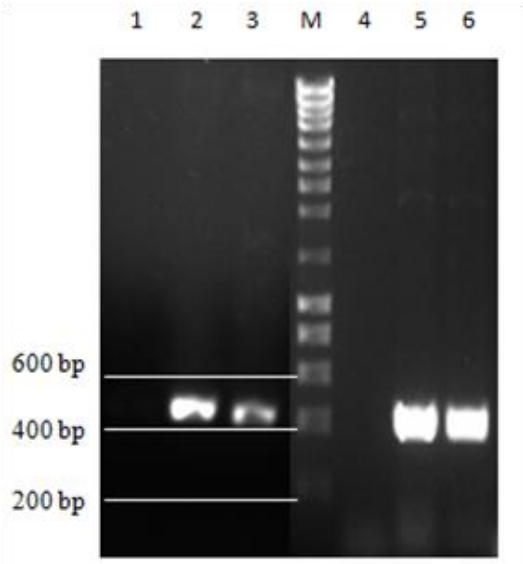

Figure 17: 1% Agarose electrophoresis analysis of *bmp-2* PCR products: 1 – negative control of PCR; 2, 3 - amplified *bmp-2* gene by PCR (for cloning into pStaby1.2FH8); M –NZYDNA Ladder III DNA marker; 4 - negative control of PCR; 5, 6 - amplified *bmp-2* gene by PCR (for cloning into pETMFH8).

PCR products were then purified and digested with the corresponding restriction enzymes. The plasmid DNAs were also digested and analyzed and purified in 1% agarose gel, as it can be seen in Figure 18.

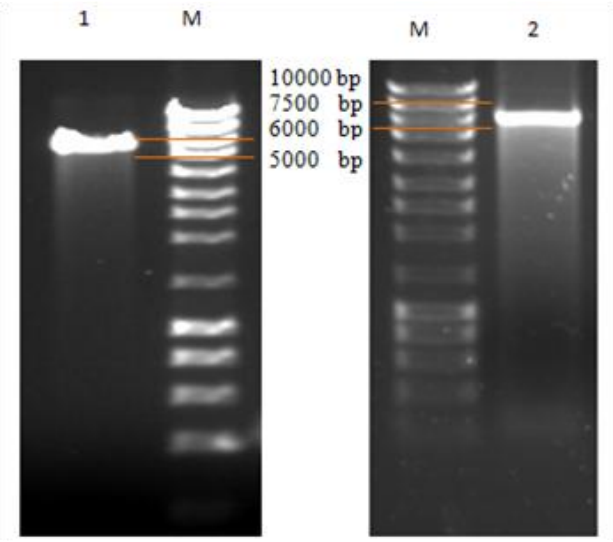

Figure 18: 1% Agarose electrophoresis analysis of DNA plasmids: 1 - *XhoI* and *NcoI* double restriction enzyme digestion of pETMFH8 plasmid; M - NZYDNA Ladder III DNA marker; 2 - *XhoI* and *SacI* double restriction enzyme digestion of pStaby1.2FH8 plasmid.

As expected it can be visualized a unique band for pETMFH8 vector, corresponding to the linearized vector with 5.5 Kbp, approximately. For the pStaby1.2FH8 plasmid digestion, it is possible observe a faint bad around 200 bp, which corresponds to CP12 fragment, as the plasmid DNA of pStaby1.2FH8 was isolated from a culture plate of pStaby1.2/FH8CP12. The band corresponding to linearized vector has a size slightly above 6 Kbp, closer to the predicted size of plasmid pStaby1.2FH8 that is 6.11 Kbp.

Figure 19 shows the obtained colony PCR products of transformants for the two recombinant vectors. All clones of pETM/FH8BMP-2 are positive, as bands are located just above 800 bp and this result is consistent with the predicted size of PCR product of 848 bp. Unspecific amplifications that can be observed in agarose gel may be related to the use of universal primers used in PCR colony. For the case of pStaby1.2FH8BMP-2 only the clone 3 is positive with an estimated size of 816 bp (in agarose gel the clone band is very close to 800 bp band). Similar unspecific amplifications can be also detected in this transformation result. Clone 8 of pETM/FH8BMP-2 was chosen for sequencing to be the one that has a higher level of purity, and for obvious reasons clone 3 of pStaby1.2/FH8BMP-2 was selected to be sequenced.

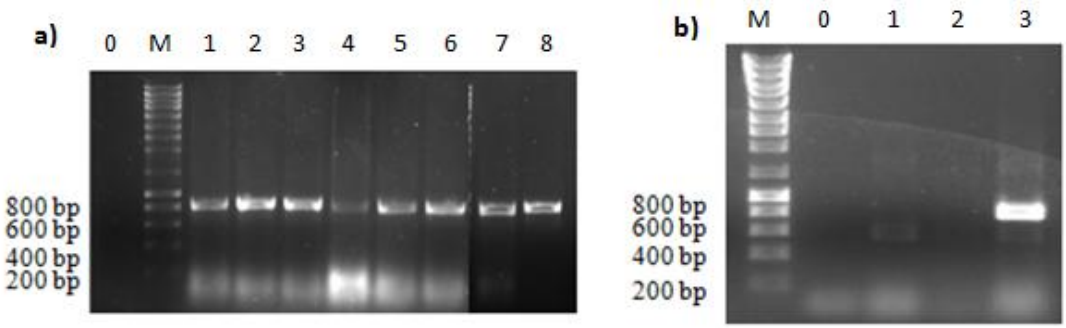

Figure 19: **1.2% Agarose electrophoresis analysis of transformants of recombinant vectors: a) Transformants of pETMFH8BMP-2**: 0 – transformation negative control; M - NZYDNA Ladder III DNA marker; 1-8 – pETMFH8BMP-2 clones. **b) Transformants of pStaby1.2FH8BMP-2:** M - NZYDNA Ladder III DNA marker; 0 – transformation negative control; 1-3 – pStaby1.2FH8BMP-2 clones.

The sequencing results were analysed by the BLASTN tool (NCBI). This analysis showed that the sequence of both clones presents 100% of identity with the bone morphogenetic protein 2 from *Homo sapiens* (accession number EAX10386.1) and 100% of identity with putative calcium binding protein from *Fasciola hepatica* (accession number AAF31420.1). A summary of this analysis is presented in appendix 4.

In the next step, *E. coli* BL21 Codon Plus Ril, Rosetta2 and SE1 expression strains were transformed with the sequenced positive clones of recombinant vectors. It is important to refer that obtained clones of this transformation were also analyzed by colony PCR and then a clone of each transformation was selected for the FH8BMP-2 expression study.

3.3 Soluble expression and purification of recombinant FH8BMP-2 in *E. coli* using pETMFH8 and pStaby1.2FH8 systems

3.3.1 Small scale screening

The expression of recombinant FH8BMP-2 protein using pETM and pStaby1.2 vectors was first evaluated in a small scale screening (20 ml cultures), testing two induction conditions (Table 17) and two different *E. coli* stains (Codon Plus Ril and Rosetta2) for pETM or the *E. coli* SE1 strain for pStaby.

Table 17: Induction conditions of screening tests for soluble expression of recombinante FH8BMP-2.

Type of condition	Description of induction conditions
i	30ºC; 3-4h; [IPTG] - 0.5 mM
ii	20ºC; o/n; [IPTG] - 0.2 mM

Figure 20 presents the results of soluble expression of pETM/FH8BMP-2. It is possible to verify in lanes 4 and 5 of both gels the expression of a protein with a molecular weight around 25 kDa, which corresponds to the estimated weight of FH8BMP-2 in this expression system (25.02 kDa). A band of molecular weight close to 15 kDa, which appears with significant intensity, corresponds to lysozyme, which was used in the lysis buffer. As can be observed from Figure 20, solubility of fusion protein is achieved with both induction conditions, using the two strains. However, the expression of soluble fusion protein is higher for the condition of induction at 20 °C, marked by a red arrow. This result is in agreement with literature, which states that lowering the expression temperature routinely improves the solubility of recombinant expressed proteins in *E. coli*, especially with proteins that are difficult to express in this bacteria, which is the case of BMP-2 protein [21]. Comparing the two strains tested, it is possible to note that the soluble expression results are very similar, as both strains promote the efficient expression of rare codons [21]. Nevertheless, it is possible to determine a slight increase of solubility when using the BL21 Codon Plus Ril strain.

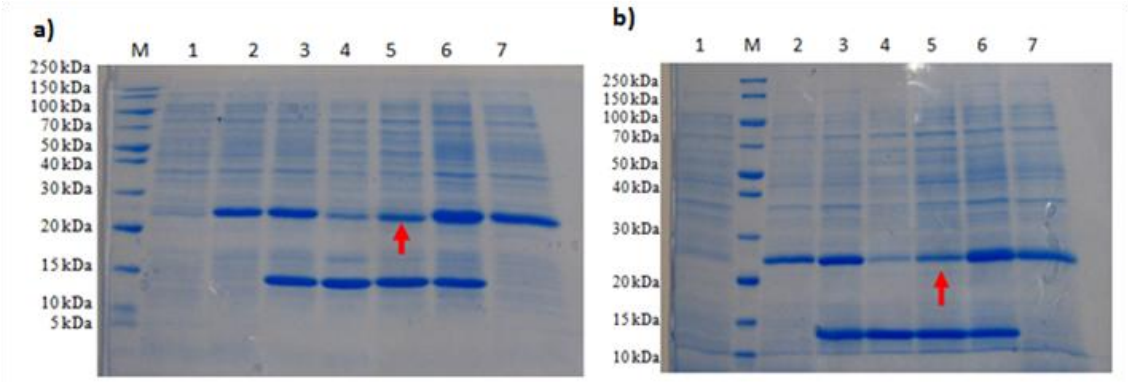

Figure 20: 15%-4% SDS-PAGE solubility analysis of FH8BMP-2 in pETM system in 20 ml cultures: a) *E. coli* BL21 Codon plus Ril culture: M – PageRuler™ protein marker (Fermentas); 1 – uninduced total bacterial protein; 2 – induced total bacterial protein of i); 3 – total lysate of i); 4 – soluble fraction of i); 5 – soluble fraction of ii); 6 – total lysate of ii); 7 - induced total bacterial protein of ii)**.**
b) *E. coli* Rosetta 2 culture: 1 –uninduced total bacterial protein; M – PageRuler™ protein marker (Fermentas); 2 – induced total bacterial protein of i); 3 – total lysate of i); 4 – soluble fraction of i); 5 – soluble fraction of ii); 6 – total lysate of ii); 7 - induced total bacterial protein of ii).

The BL21 Codon Plus Ril strain and condition ii were selected for the large scale production and subsequent purification of FH8BMP-2.

Prior to the large scale experiments, it is important to analyze the results shown in Figure 21, which demonstrate a comparison between the total lysate and soluble fraction samples of the following proteins: FH8BMP-2 expressed using the pETMFH8; HisBMP-2 expressed using the pETM11 and proteins expressed using the corresponding empty vectors without BMP-2 protein (pETM/FH8 and pETM/His), which function as a negative control for the BMP-2 expression.

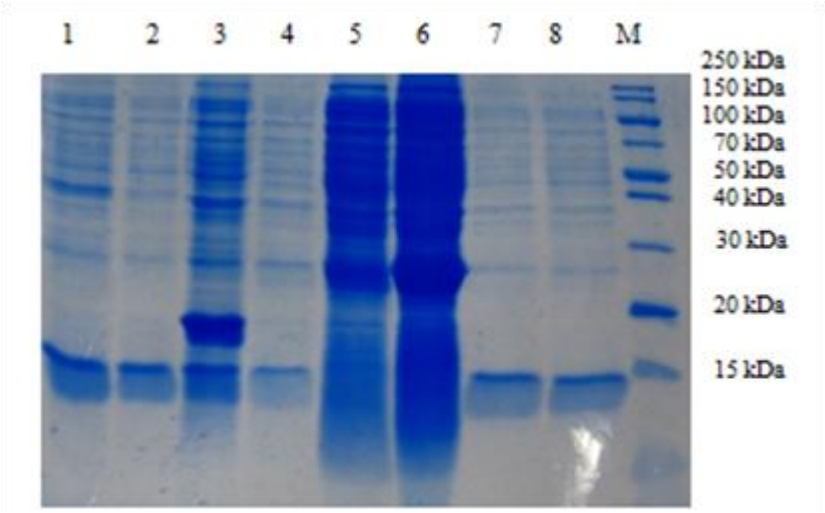

Figure 21: 15%-4% SDS-PAGE solubility analysis of pETM/FH8BMP-2; pETM/HisBMP-2; pETM/FH8 and pETM/His samples: 1 - total lysate of pETM/His; 2 – soluble fraction of pETM/His; 3 – total lysate of pETM/HisBMP-2; 4 – soluble fraction of pETM/HisBMP-2; 5 – soluble fraction of pETM/FH8BMP-2; 6 – total lysate of pETM/FH8BMP-2; 7 – soluble fraction of pETM/FH8; 8 – total lysate of pETM/FH8; M – PageRuler™ protein marker (Fermentas).

Analyzing the gel of Figure 21, it is possible to observe in all lanes of the gel a band with the molecular weight similar to FH8BMP-2 (between 20 and 30 kDa). As this band is also present in the empty vectors pETM/FH8 and pETM/His, it corresponds to a protein from the crude extract of *E. coli*. The expression of the protein in lane 5 of the gel corresponds in fact to the soluble fusion protein FH8BMP-2. FH8 solubilises the BMP-2 as shown by the comparison between soluble fractions of HisBMP-2 and FH8BMP-2 (lanes 4 and 5). These observations are in good agreement with the results obtained with FH8BMP-2 and BMP-2 expressed proteins using pQE-30 vector described in section 3.1.1 of present chapter.

Figure 22 shows the SDS-PAGE analysis of the main results of FH8BMP-2 soluble expression test using the pStaby1.2 system. Lanes 1, 10, 15 and 18 correspond to proteins expressed using the empty pStaby1.2/FH8 vector. None of these lanes present relevant protein expression around 25 kDa, which is the estimated molecular weight for FH8BMP-2 using this system. The total lysate samples (lanes 8 and 9) of FH8BMP-2 expression using the two conditions tested contain two proteins between 20-30 kDa. Looking at soluble fractions, it is possible identify the expression of a protein with a molecular weight higher than 25 kDa, at 20 ºC. On the other hand, at the same temperature, a protein around 25 kDa is expressed in insoluble form. The both bands referred are indicated by a red arrow and are correspondent to bands identified in total lysate samples. The samples of soluble and insoluble fractions were analysed by Western blot (data not shown) and the results are in agreement with what it is observed in Figure 22. Both bands visualized correspond to FH8BMP-2 but present indeed a shift in the molecular weight. The condition ii was selected for further larger scale productions.

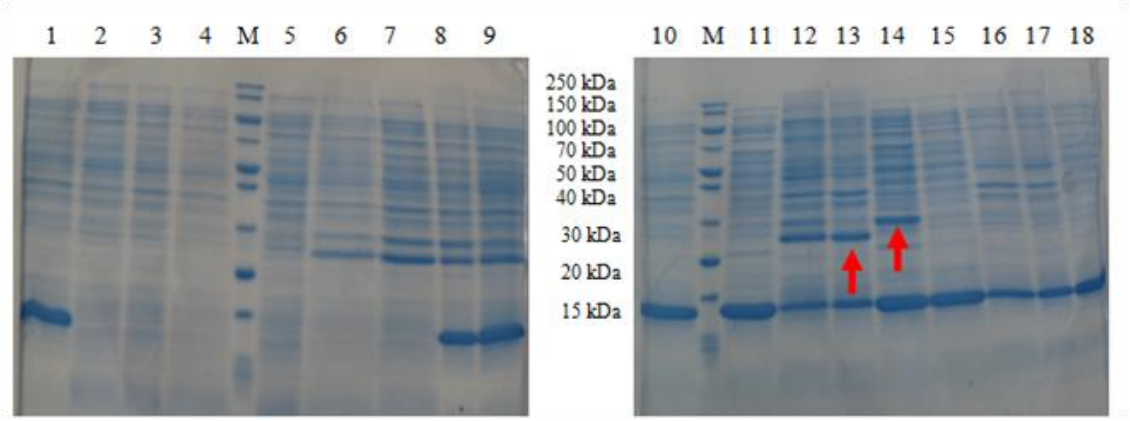

Figure 22: 15%-4% SDS-PAGE Solubility analysis of FH8BMP-2 in pStaby1.2 system in *E. coli* SE1 20 ml cultures in comparison with pStaby1.2/FH8 (negative control –without protein):
1-4,10,15-18: culture samples of pStaby1.2/FH8: 1 –total lysate of ii; 2 - uninduced total extract; 3 – induced total extract of ii; 4 - induced total extract of i; M – PageRuler™ protein marker (Fermentas); 10 – total lysate of i; 15 – soluble fraction of ii; 16 - insoluble fraction of ii; 17 – insoluble fraction of i; 18 – soluble fraction of i.
5-9,11-14: culture samples of pStaby1.2/FH8BMP-2; 5 –uninduced total bacterial protein; 6 – induced total bacterial protein of i); 7 - induced total bacterial protein of ii); 8 – total lysate of ii; 9 – total lysate of i; 11 – soluble fraction of i); 12 – insoluble fraction of i); 13 – insoluble fraction of ii); 14 - soluble fraction of ii.

3.4 Larger scale production and purification tests of soluble recombinant FH8BMP-2 in *E. coli* using pETM system

This section is divided in three parts according to the different chromatographic techniques applied to purify the FH8BMP-2 fusion protein: immobilized nickel ion affinity chromatography (IMAC-Ni); hydrophobic interaction (HIC) and ion exchange (IEX) chromatography.

3.4.1 Immobilized nickel ion affinity chromatography (IMAC-Ni)

In the first part of purification strategy with recombinant protein FH8BMP-2 from pETM system, it was performed small scale purification tests, using buffers recipe described in Table 18 and a more detailed description of incubation conditions can be consulted in section 2.7.2 of materials and methods. As it can be seen, similarly to assays performed with pQE-30/FH8BMP-2 it was used an imidazole gradient in the purification process. Samples obtained with this assay were analysed by SDS-PAGE and the gel is presented in Figure 23. By the observation of the gel, it is possible to conclude that the interaction between fusion protein and nickel resin was weak, as most of protein leaves the column in the flowthrough sample. As occurred with the FH8BMP-2 in pQE-30 system, the fusion protein structure may be hiding the histidines tail present in the N-terminal sequence of BMP-2.

In this way, the purification strategy was targeted to the HIC technique in order to improve the results by the properties of the FH8 tag.

Table 18: Recipe of buffers used in IMAC-Ni purification step of recombinant FH8BMP-2 protein in *E. coli* BL21 Ril/pETM.

Lysis buffer	Binding buffer	Washing buffer	Elution buffer
50 mM Tris, 250 mM NaCl, pH 7.4	50 mM Tris, NaCl 250 mM, 20 mM imidazole, pH 7.4	50 mM Tris, 250 mM NaCl, 40 mM imidazole, pH 7.4	50 mM Tris, 250 mM NaCl; 300 mM imidazole, pH 7.4

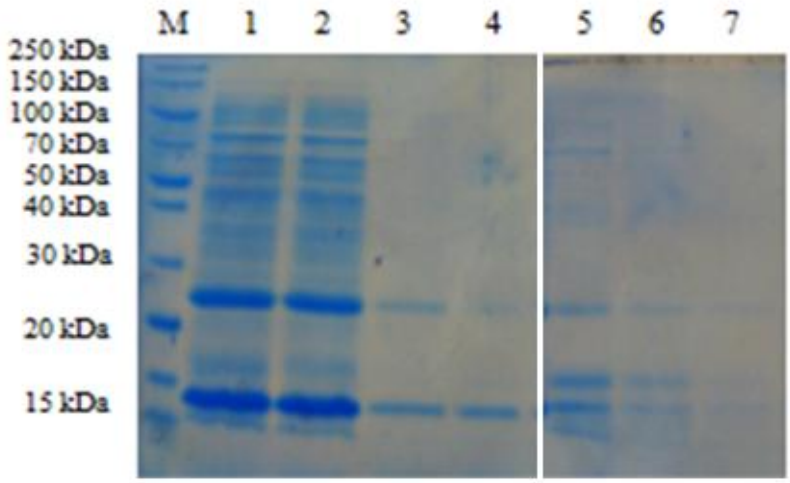

Figure 23: 15%-4% SDS-PAGE analysis of samples obtained from IMAC-Ni small scale purification assay: M - PageRuler™ protein marker (Fermentas); 1 - soluble protein sample charged into the column; 2 - flowthrough sample; 3, 4 – washed samples; 5-7 – eluted samples.

3.4.2 Hydrophobic interaction chromatography

To perform the purification of FH8BMP-2 expressed in *E. coli* BL21 Codon Plus Ril, the previous results of purification of the fusion protein into the vector pQE-30 were taken into consideration, in which there was a good interaction of the FH8 fused protein with the hydrophobic resin after the addition of calcium. Table 19 shows the buffers used in this step and as it can be seen the concentration of calcium used in soluble fraction was the same as that used in previous tests. SDS-PAGE gels of Figure 24 shows samples from 1L of *E. coli* Codon plus BL21 Ril culture of pETM/FH8BMP-2 and the results of the first purification step performed with the soluble fraction recovered from this production.

Table 19: Recipe of buffers used in first HIC purification step of recombinant FH8BMP-2 protein in E. coli BL21 Ril /pETM.

Lysis buffer	Binding buffer	Washing buffer	Elution buffer	Additives
50 mM Tris, 250 mM NaCl, pH 7.4	50 mM Tris, NaCl 250mM, pH 7.4	50 mM Tris, 250 mM NaCl, pH 7.4 Dilution 1:2	50 mM Tris, pH 10	**5 mM of $CaCl_2$**

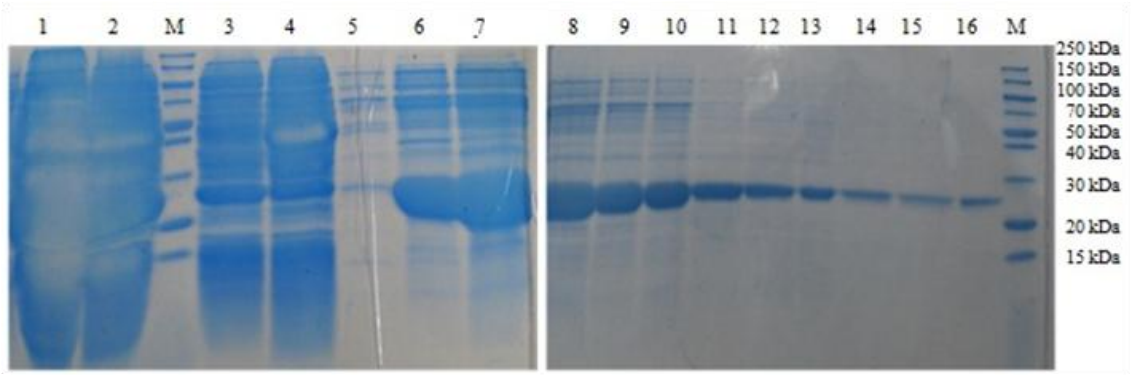

Figure 24: **15%-4% SDS-PAGE gel with samples of 1L of *E. coli* Codon plus BL21 Ril culture of FH8BMP-2 and HIC purification samples**: 1 – total lysate; 2 – soluble fraction charged into purification column; M – PageRuler™ protein marker (Fermentas); 3 – flowthrough sample; 4,5 – washed samples; 6-16 – eluted samples.

Comparing the samples of total lysate and soluble fraction in Figure 24, it is possible to observe that a high expression of the FH8BMP-2 protein in soluble form with a molecular weight of approximately 25 kDa. This result reflects, but on a larger scale, the results from the small-scale analysis. Now, regarding the results of HIC purification performed, it is possible to see that there is loss of fusion protein in the flowthrough and washed fractions (lanes 3 and 4). However despite this loss, a high amount of protein is eluted in the first fractions (lanes 6 and 7) with high amount of contaminants. However, from the eluted fraction 10 it can be observed a good profile of purity of the recombinant protein. In relation to the presence of contaminants, they are not completely eliminated and their presence is more substantial in a molecular weight above the FH8BMP-2 band.

At the end of the purification assay, all eluted samples were dialyzed in PBS 1X, pH 7.4 and then they were grouped and quantified by Bradford method (described 2.9 of materials and methods). A final protein purification yield of 31 mg of purified FH8BMP-2 per litre of *E. coli* culture was obtained using the FH8/HIC methodology.

In order to understand the effect of calcium in the interaction of the fusion protein with hydrophobic resin, it was made a similar purification assay (using the same conditions described in Table 19) using 1L of *E. coli* Codon plus BL21 Ril culture of FH8BMP-2, but now with a lower calcium concentration (2 mM). The same profile of production, as well the first eluted samples, presented in the left gel of Figure 24, was observed, that is, a high expression and amounts of FH8BMP-2. The remaining eluted

samples of this second assay are shown in Figure 25 and it is equivalent to the right gel shown in Figure 24. Comparing the two gels with eluted samples, it is possible to observe that, with a lower concentration of calcium, the interaction between the FH8BMP-2 and the hydrophobic resin is weaker, due to higher level of contaminants identified in the samples of Figure 25. This result supports the previous findings registered on larger scale purification of fusion protein into the vector pQE-30, involving HIC technique and calcium effect. Since the fusion protein in vector pETM has also strong hydrophilic residues (the graph of the degree of hydrophobicity of the protein is shown in appendix 2), addition of calcium allows an increased exposure of hydrophobic regions of the fusion protein. This modification on hydrophobicity of the protein is certainly correlated with the conformational change of the structure of FH8 tag. The binding of calcium to the EF-hand loops of FH8 tag triggers the opening of the structure, which exposes a large hydrophobic area, allowing in this way a more specific binding to hydrophobic resin [38].

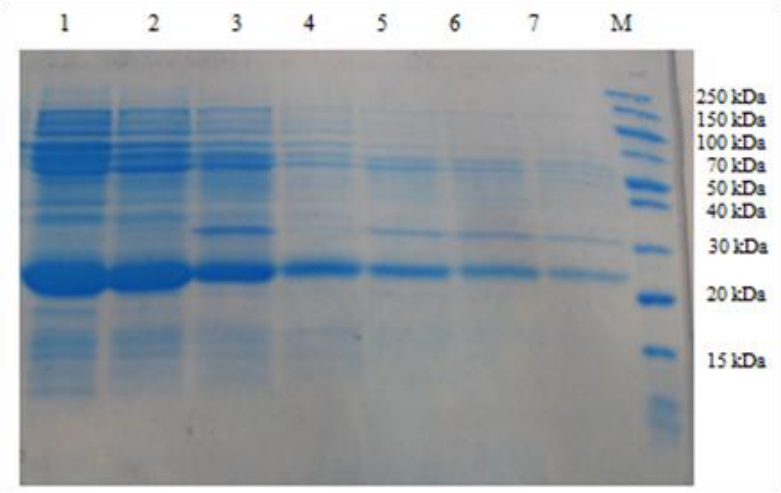

Figure 25: 15%-4% SDS-PAGE gel with HIC purification samples from 1L of *E. coli* Codon plus BL21 Ril culture of FH8BMP-2, with lower calcium concentration: 1-7 – eluted samples; M – PageRuler™ protein marker (Fermentas).

Another aspect of the interaction of the fusion protein with calcium may be related to the exposure of the BMP2 to this reagent. According to some studies, the addition of calcium influences the adsorption of the protein to certain surfaces as hydroxyapatite [49]. At the same time, a DLS analysis was performed using an eluted sample from Figure 24 to explore the previously described calcium effect as an enhancer of fusion protein aggregation for higher molecular weight forms. It was measured the hydrodynamic radius of FH8BMP-2 and it was concluded that the global

arrangement of fusion protein resulted in a molecular weight estimation of 64 kDa, which corresponds to an oligomer of about 2.58 units and this may correspond to a dimeric form of the protein. This is approximately half of the value obtained for the fusion protein expressed in *E. coli* M15 using the vector pQE-30 (about 5.5 units). Once again this result is in agreement with the SDS-PAGE analysis, in which it is visualized a band of 25 kDa as a result of denaturation of the dimeric complex. As referred before, the aggregation of the fusion protein can be a result of combined individual factors related to FH8 tag and BMP-2 protein and calcium can be an enhancer of this aggregation. Additionally, according to features of recombinant bone morphogenetic protein, its dimer formation involves three monomer variants and their combination can lead to at least six dimeric isoforms and all of them have proven to be active *in vitro* cell culture assay [50]. So this possible dimeric form of the fusion protein is extremely relevant for its biological activity in cell culture applications [16,50].

Given the previous results and for improving the elimination of contaminants in the first eluted fractions (indicated in left gel of Figure 24) which have a high soluble amount of FH8BMP-2, a second purification of these fractions in the HIC column was performed, with initial calcium concentration of 5 mM. The results of this second stage are shown in Figure 26. The results of this second purification show the removal of most impurities and unspecific proteins that bound to the resin, but some contaminants are still observed above the FH8BMP-2 band (with 25 kDa).

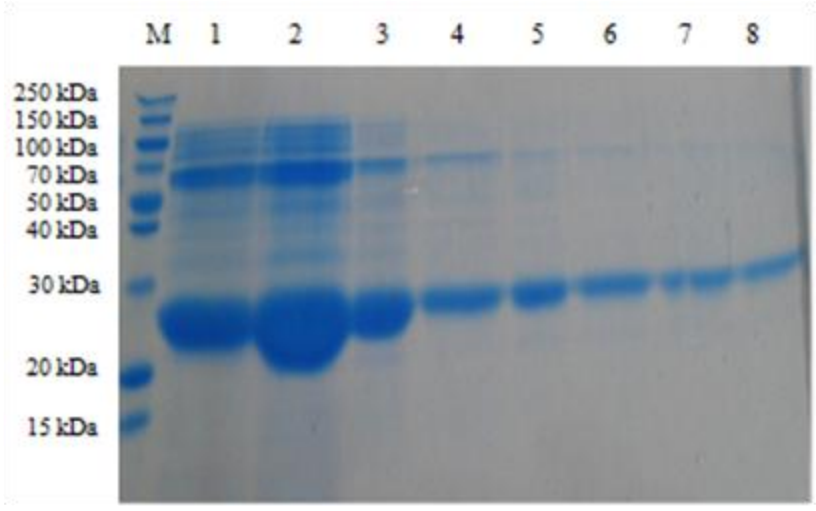

Figure 26: 15%-4% SDS-PAGE gel with samples of second HIC purification performed with eluted samples obtained from first HIC purification assay: M – PageRuler™ protein marker (Fermentas); 1-8 – eluted samples.

At the end of the purification assay, all eluted samples were dialyzed in PBS 1X, pH 7.4 and then they were grouped and quantified by Bradford method. A total yield of purified protein of 15.15 mg per liter of *E. coli* was obtained which corresponds to a production yield of 16.14 mg of protein per g of cell dry weight.
An important goal was successfully achieved with FH8BMP-2 protein in pETM system, that is, a significant increase in purification yield of the fusion protein.

3.4.2.1 Optimization of HIC wash step

In order to reduce the affinity of unspecific proteins during elution in HIC, various agents were tested to optimize the washing step of this purification technique. Table 20 presents the washing buffers tested. The choice of these agents stemmed from a previous work on the optimization of the washing step in the HIC technique [51].

Table 20: Washing buffers tested in HIC purification.

Washing buffers
W1 – distilled H_2O
W2 – 50 mM Tris, 250 mM NaCl, pH 7.4 + 2.5 M $MgCl_2$
W3 – 50 mM Tris, 250 mM NaCl, pH 7.4 + 1.5 M Urea + 5% glycerol

As a negative control, it was used the initial washing buffer (denominated W0, corresponding to lane 1 in gel of Figure 27), which is described in Table 19. It is possible to verify in gel of Figure 27 that significant lost of target protein FH8BMP-2 occurred (around 25 kDa) during the washing process of the column with W0 and W2 buffers. The difference between these two buffers is the presence of magnesium chloride ($MgCl_2$) which is a chaotropic salt that is employed to decrease protein retention on HIC stationary phases. However, the effect of this salt was not favorable for the purification of recombinant FH8BMP-2 protein, since its elution in washed sample with high amounts of contaminants was observed. This result is in agreement to what was tested in previous studies, which showed for other proteins fused to the FH8 tag that action of $MgCl_2$ in a concentration range of 1-3 M is liable to decrease the stability of the interaction of calcium binding protein with the HIC resin [39].

Regarding theW3 buffer, it is possible to verify that the removal of contaminants is minimal and comparing to W2, elution of the target protein is lower. The explanation of this effect is related with two factors: first, the urea concentration was not high enough to allow a decrease in the affinity of unspecific proteins with hydrophobic resin. According to previous work conducted for the optimization of HIC wash step, the most dramatic increase in elimination of non-specific proteins was observed with urea concentrations above 2.0 M [51]. Since the aim of this study is to work with FH8BMP-2 protein in the native state, high concentrations of urea were not used. Secondly, with the synergistic effect of glycerol, the desorbing effect of urea was mitigated and in consequence the washing of contaminants was not successful. Contrary to what it was obtained, previous studies showed that the combination of 1.5 M urea and 5% glycerol is a possible strategy to form a washing buffer to have an increase in the protein purity level by HIC [51].

Finally, analyzing and comparing the effect between control buffer (W0) and distilled water (W1), is evident that although the removal of contaminants is not optimal, the effect of washing water is positive because it allows to obtain a removal of some contaminants and a reduced elution of the protein in the washed samples as indicated by a red arrow in the Figure 27. Thus, face to this result, it was chosen a combination of the two buffers (W0 and W1) to try to purify by HIC technique the washed samples previously obtained from the application of W0,W2 and W3 buffers – samples of lanes 2, 4 and 5. Eluted fractions resulting from this test are shown in the SDS-PAGE gel shown in Figure 28.

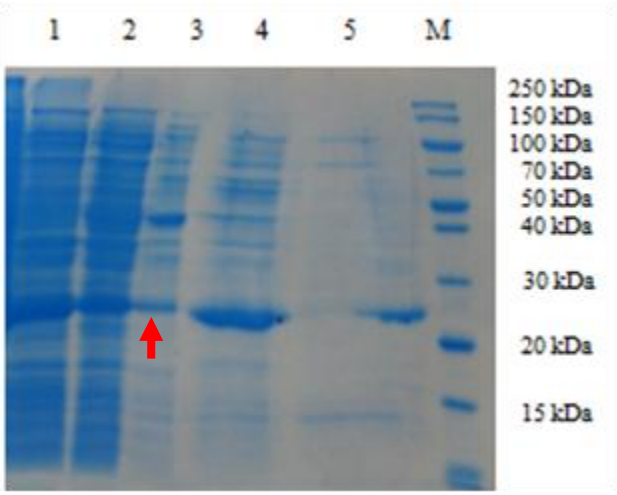

Figure 27: 15%-4% SDS-PAGE gel with samples of HIC purification performed with different wash buffers: 1 – protein sample charged into the column; 2 - washed sample of WO; 3 - washed sample of W1; 4 - washed sample of W2; 5 - washed sample of W3; M - PageRuler™ protein marker (Fermentas).

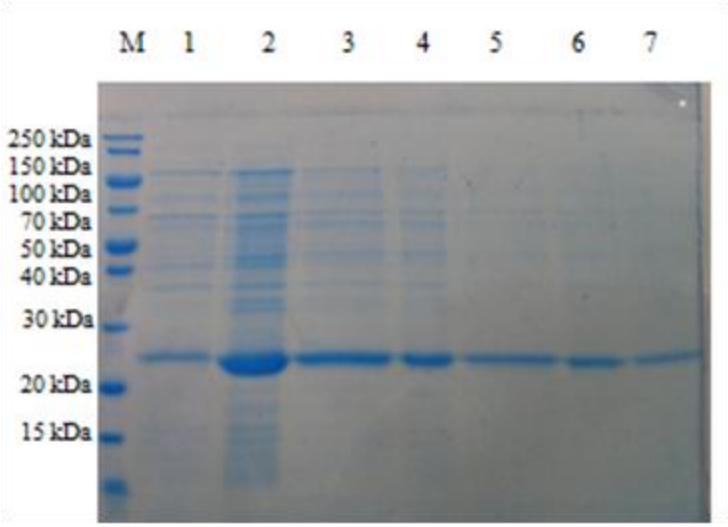

Figure 28: 15%-4% SDS-PAGE gel with samples of HIC purification performed with wash buffers W0 and W1: M – PageRuler™ protein marker (Fermentas); 1-7- eluted samples.

This step of combining W0 and W1 buffers shows encouraging results in relation to purity level of final eluted fractions. With the exception of the eluted protein fraction present in lane 2, it is evident the reduced level of contaminants and it represents an important result in the purification of the fusion protein by HIC. This optimization of HIC washing was incorporated as part of the downstream purification process for this recombinant protein and offered tremendous advantages in terms of minimizing the number of column steps.

With the performance of these purification strategies using the FH8 tag/HIC as purification technique, important guidelines were found for the purification of

FH8BMP-2 protein under native conditions. Thus, the addition of calcium has a predominant effect to increase specificity in the interaction with the hydrophobic resin, allowing for a good purity level and high final yield of recombinant protein. Additionally, a combination of washing buffers, such as distilled water, can have an important role in the process of removing contaminants which have some affinity with the resin.

3.4.3 Ion exchange chromatography

The use of anion exchange chromatography in the purification of protein FH8BMP-2 has two main purposes: firstly, to understand if this technique can be used as a polishing technique after completion of a first purification step by HIC; secondly, with the use of an anionic exchanger, to find some explanations about net surface charge and conformation of fusion protein.

Diagram of Figure 29 illustrates the steps that were executed in this stage of work.

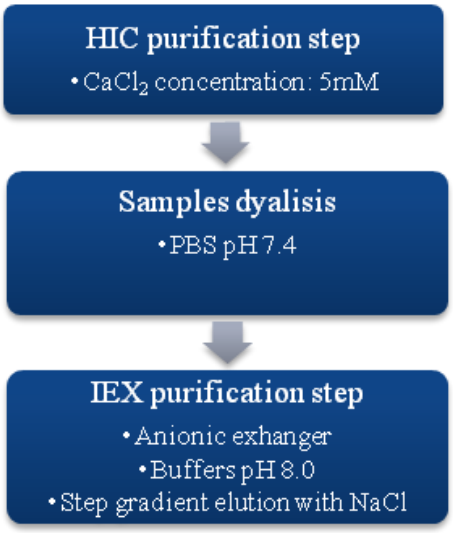

Figure 29: Illustrative diagram of steps performed in IEX procedure.

The isoelectric point (pI) of FH8BMP-2 protein expressed using the vector pETM is 8.35 and the pH of the buffers used was 8.0. Thus, since the pH is slightly lower than the pI, the protein has a neutral or an overall minor positive charge. Therefore, an interaction between the fusion protein and in IEX column is not expected,

and so, it is anticipated to collect the protein in the flowthrough sample, free of contaminants. The aim is to elute contaminants through the NaCl gradient, according to their interaction with the resin, and thereby obtain a protein clean of impurities.

However, analyzing the results obtained after passing the sample through the column of anion exchange purification, a different outcome was obtained from what it was expected. The protein has interacted with the anionic resin and therefore was recovered in eluted samples (data not shown). Taking the obtained result, it was made an extensive study of individual characteristics of surface charge of FH8 tag and BMP-2 protein. So, starting with BMP-2 protein, a study of crystal structure of human BMP-2 shows that pronounced charged regions are located at the fingertips regions of this bone morphogenetic protein where strong negative potential is visible and in the centre of the molecule exists two patches of mixed charge. On the other hand, this analysis shows that a high concentration of positive charges is localized at the N-terminal region [11]. The cloning process followed in this work leads to fusion of FH8 tag to the BMP-2 N-terminal sequence. Analyzing the main properties of the FH8 tag, it is possible to identify a pI of 5.60 and an overall negative charge. Thus, taking into account the properties of this calcium binding protein and its location in the fusion protein, it is possible to note that FH8 tag is in some way preventing the exposure of positively charged regions presented in BMP-2 protein and, in turn, it is favoring the exposure of its negatively charged residues. According to an analysis of FH8 tag and contrarily to the same aligned regions of other characterized calcium binding proteins, it was observed a region clearly negatively charged and a less hydrophilic area [38]. This accessibility of negatively charged residues of FH8 tag allows a correspondence with the results obtained with the HIC purification technique and addition of calcium. That is, it was observed a strong interaction of the fusion protein with the hydrophobic resin, mediated by the binding of calcium to FH8 tag; this binding to calcium ions occurs in turn by the interaction with negatively charged residues of the tag FH8. These two results converge to the same findings in relation to the exposed regions of FH8 tag, in global fusion protein, to the purification resin.

Taking into account the results presented from production and purification strategy studied in relation to fusion protein in pETM vector, it is possible to conclude that:

- The FH8 solubilises BMP-2 protein and it was proved to be a solubility tag with this target protein.
- The HIC technique via the FH8 tag, similarly to what happens to fusion protein in pQE-30 vector shows good results in purification profile of the target protein, when combined with calcium addition to soluble fraction.
- Calcium ions have an important role in reducing retention of unspecific proteins from hydrophobic resin, through conformational changes in structure of FH8 tag and by influencing adsorption mechanisms of BMP-2 to the hydrophobic resin. Additionally, this reagent potentiates aggregation of fusion protein leading to a dimeric form. So, in presence of calcium, the possible dimerization of BMP-2 together with the exposure of hydrophobic regions of the FH8 tag can explain the strong interaction between fusion protein and HIC resin.
- Finally, the yield of purified protein was much higher than the one obtained with the pQE-30/FH8BMP-2 recombinant protein.
- The ion exchange and nickel affinity chromatography purification steps allows to support and to clarify the results obtained with HIC, by given indications of surface net charge and accessibility of histidines tail in the global conformation of fusion protein.

3.5 Larger scale production and purification tests of soluble recombinant FH8BMP-2 in pStaby1.2 system

In the larger scale production of the fusion protein in the vector pStaby1.2 it was not possible to obtain an objective analysis on the soluble expression. Production tests were performed under the same conditions that were made with the fusion protein cloned into the vector pETM. However, the amount of soluble protein obtained was very low, and purification tests were inconclusive. To conduct further study on the expression of the fusion protein in pStaby1.2 system, new strategies should be studied to obtain higher protein solubilisation and thereafter move to the purification strategies followed in the earlier systems studied (pQE-30 and pETM vectors).

Chapter 4: Main Conclusions and suggestions for forthcoming work

The main objective of the present work was the optimization of the soluble production and purification of recombinant BMP-2 in *E. coli*.

One major disadvantage of using *E. coli* as a host is that heterologous proteins are often expressed as insoluble aggregates of folding intermediates known as inclusion bodies. Insoluble recombinant proteins, generally, do not have activity because they lose their native conformation and therefore they can't be used for applications in which such requirements are essential (biomedical applications). In previous reports, production of recombinant BMP-2 was achieved in bacterial inclusion bodies [5,16,17,19,20].

To date, important tools have been developed to overtake this strong limitation, such as the use of fusion tags that enhance the solubility of expressed proteins.

In this work, it was performed the soluble expression of recombinant BMP-2 protein, a protein that is expressed in insoluble form in *E. coli*, using the fusion partners technology with FH8 tag. In this way, three different vectors (pQE-30; pETM and pStaby1.2) were used in order to test the soluble expression of the recombinant fusion protein, FH8BMP-2.

The main results of production and purification of recombinant fusion protein allowed to conclude that:

- The FH8 allows the solubilisation of BMP-2 protein in all the three vectors, so a new approach was developed to obtain bone morphogenetic protein in soluble form;
- The cloning process of *bmp-2* gene in pETM system resulted in a significant increase in soluble expression levels;
- The soluble expression of the recombinant fusion protein in pStaby1.2 system translates into an important advantage since this is a system without the use of antibiotics, but it is still necessary improve soluble expression levels;

- It was not possible to purify recombinant fusion protein by IMAC-Ni, revealing that conformation of FH8BMP-2 might be promoting the exposure of FH8 tag instead of the histidines tail.
- On the other hand, a new purification strategy by FH8/HIC technique was developed, which resulted in a high yield of purified protein in the pETM system.
- The effect of calcium, in the purification process of the recombinant fusion protein by HIC, was determinant, since it promoted the reduction of binding of non-specific proteins to the hydrophobic resin through conformational changes in the structure of the FH8 tag. Additionally, the dynamic structure and behaviour characteristics of FH8 tag in the presence of calcium reflects the results obtained in relation to the aggregation of the fusion protein into a dimeric form. All these factors explain the strong interaction between fusion protein and HIC resin;

After completion of this work and given the results obtained, interesting proposals can be made for future work. It would be interesting to assess the biological response induced by the protein BMP-2 with the FH8 tag and after tag removal, in order to verify if the tag has influence in the biological activity of the target protein.

Another perspective would be the study of biomaterials that can be used as carriers for the delivery of BMP-2. The main role of a delivery system for BMP-2 is to retain this osteoindutive component at the site of injury for a prolonged time, providing an initial support to which cells attach and form regenerated tissue [52]. This concept is an important step to the development of biomedical applications of bone morphogenetic proteins.

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Chapter 6: Appendixes

Appendix 1: Detailed composition of chemical products, solutions and reagents

Table A1: Composition of solutions used in the project.

LB-medium	10 g/L NaCl 5 g/L Yeast extract 10 g/L Tryptone Adjust pH to 7.5 with NaOH 10N
LB-antibiotic plate	LB-medium 2% (w/v) Agar Final concentrations of antibiotics Ampicillin: 50 µg/ml Kanamycin: 50 µg/ml Chloroamphenicol: 33µg/ml
Super Optimal Broth (SOB Medium)	Tryptone 2 % Yeast Extract 0.5 % NaCl 10 mM KCl 2.5 mM $MgSO_4$ 10 mM $MgCl_2$ 10 mM
Super Optimal broth with Catabolite repression (SOC Medium)	SOB medium with 20 mM Glucose. Prepare 1 M Glucose solution (180 g/L) and filter sterilize. Store at 4 ºC. Add 200 µL of this solution to 9.8 mL SOB
TB solution	10 mM PIPES or HEPES 15 mM CaCl2 250 mM KCl Dissolve in milliQ water and adjust pH to 6.7 with KOH or HCl and then add 55 mM MnCl2 and adjust to final volume Sterilize by filtration with 0.2 µm filter and store @ 4°C
TAE buffer	40 mM Tris-acetate 2 mM EDTA
DNA 5x sample loading dye	20 mM EDTA 25% Glicerol
10x running buffer SDS-PAGE	17.7 mM Tris-HCl 0.25(w/v) SDS 0.2 M Glycine
5x loading sample buffer SDS-PAGE	0.15 mM TrisHCl pH6.8 10% (w/v) SDS 50% (v/v) Glycerol 25% (w/v) β-mercapitalethanol 0.01% (w/v) bromophenol blue
10x PBS solution	80 g/L NaCl 2 g/L KCl 2.4 g/L KH_2PO_4 17.80 g/L $Na_2HPO_4.2H_2O$ adjust pH to 7.4 with HCl
***Coomassie Blue* solution**	Dissolve in 1L of distilled water 60-80 mg de Coomassie Brilliant Blue G250; Stir the solution with a magnet for 2 to 4 hours at RT; Add 3 ml of concentrated 37% HCl solution; Store the reagent at RT environment, protected from the light

Appendix 2: Hidrophobicity analysis of FH8BMP-2 using Kyte and Doolittle scale

The hidrophobicity analysis was made by the tool ProtScale available at the platform Expasy, using Kyte and Doolittle scale. In this scale, regions with a negative score correspond to hydrophilic regions of the protein sequence. Those presenting a positive score are hydrophobic regions. The analysis of Figures A1 and A2 demonstrates that the protein FH8BMP-2 expressed using both pQE and pETM vector systems is predominantly hydrophilic, with only one hydrophobic region in the case of pQE-30/FH8BMP-2 and two hydrophobic regions in the case of pETM/FH8BMP-2. In the hydrophilic domain stands an extremely hydrophilic region (close to position 100 identified in Figures A1 and A2).

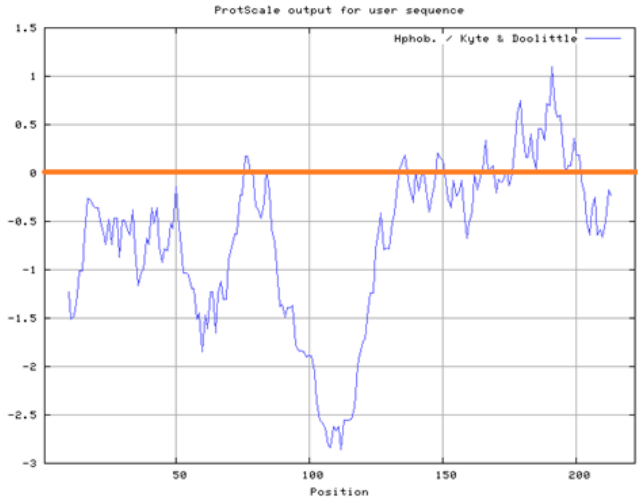

Figure A1: Analysis of the hydrophobicity of pQE-30/FH8BMP-2, according to the Kyte and Doolittle scale.

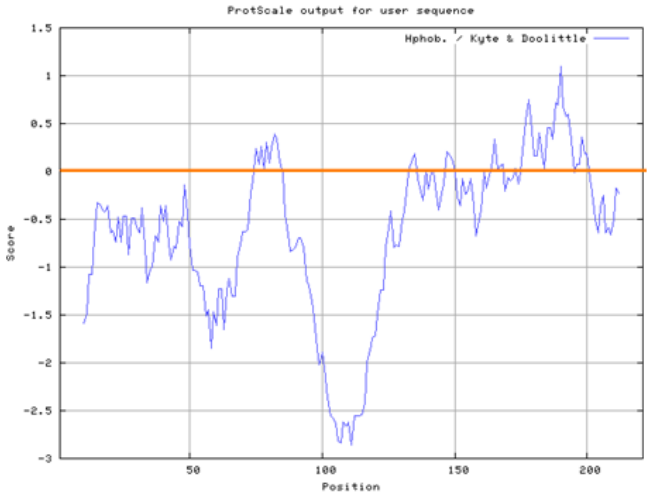

Figure A2: Analysis of the hydrophobicity of pETM/FH8BMP-2, according to the Kyte and Doolittle scale.

Appendix 3: Neb-Cutter analysis

In Table A2 is part of the list of enzymes that do not cut the fragment *bmp-2* to be amplified by PCR. As can be seen, the enzymes *NcoI*, *SacI* and *XhoI* are included in this list.

Table A21: List of enzymes that do not cut the bmp-2 fragment.

Enzymes that don´t cut
NcoI
NdeI
NgoMIV
NhaXI
NheI
SacI
SalI
SapI
Sau96I
SbfI
ScaI
XcmI
XhoI
XmaI
XmnI
ZraI

Appendix 4: BLASTN analysis of the sequencing results of pETM/FH8BMP-2 and pStaby1.2/FH8BMP-2 clones

After performing a manual analysis of the sequencing results, sequences corresponding to BMP-2 and FH8 proteins were analyzed using the software tool BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) and the results are presented in Figure A3. As can be seen, there is a corresponding match of 100% with the sequence of BMP-2 protein from human origin and with FH8 sequence from the organism *Fasciola hepatica*.

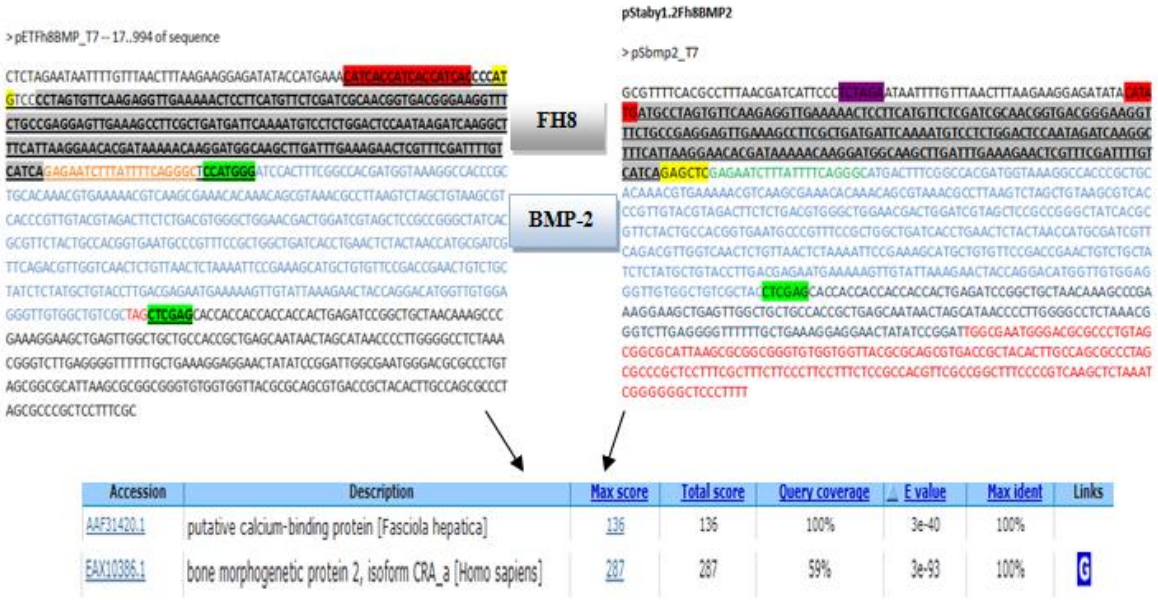

Figure A3: BLASTN analysis of pETM/FH8BMP-2 and pStaby1.2/FH8BMP-2 clones.

To confirm the existence of EF-hands in the FH8 fusion protein, there was made a computer analysis of the domains of the fusion protein tag. This was performed using the software of the NCBI conserved domain search, which proved the existence of domains expected for the fusion protein FH8BMP-2.

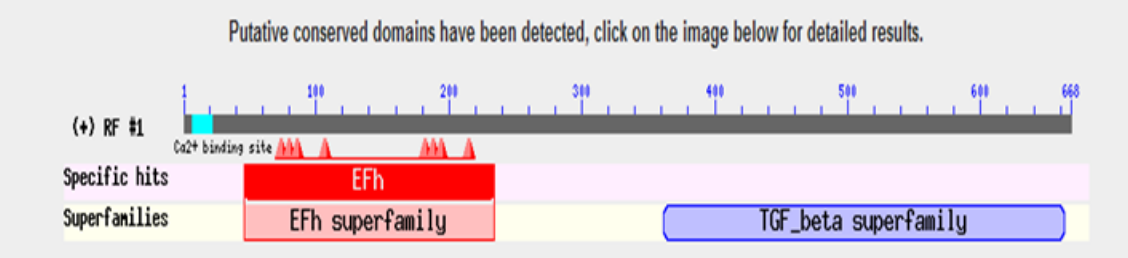

Figure A4: Results of the analysis of protein domains in fusion conserved domain by NCBI software.