# EXPRESSION OF HUMAN DENTIN MATRIX PROTEIN 1 IN *ESCHERICHIA COLI* AND *NICOTIANA BENTHAMIANA* AND THEIR EFFECT ON PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF HUMAN PERIODONTAL LIGAMENT STEM CELLS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Pharmacognosy
Department of Pharmacognosy and Pharmaceutical Botany
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# การแสดงออกของโปรตีนเดนทินเมทริกซ์โปรตีนวันใน *ESCHERICHIA COLI* และ *NICOTIANA BENTHAMIANA* และผลการเพิ่มจำนวนและการเปลี่ยนสภาพเป็นเซลล์สร้างกระดูกในเซลล์เอ็นยึดปริทันต์มนุษย์

นางอัคส์ตาร์ รอสเกียน่า อาหมัด



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ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อผลิตรีคอมบิแนนท์โปรตีน Dentin matrix protein 1 ของมนุษย์ (hDMP1) ใน *Escherichia coli* และ *Nicotiana benthamiana* และศึกษาผลกระทบของโปรตีน hDMP1 ด้านการพัฒนาไปเป็นเซลล์สร้างกระดูกในเซลล์ต้นกำเนิดเอ็นยึดปริทันต์มนุษย์ (hPDLSCs) โดยยีนที่สามารถถอดรหัสเป็นโปรตีน hDMP1 ถูกรวมเข้าไปใน pET22b และ pBY2R2e ซึ่งเป็น expression vectors รีคอมบิแนนท์โปรตีน hDMP1 ผลิตได้เป็นอย่างดีทั้งใน *E. coli* และ *N. benthamiana* ซึ่งโปรตีน hDMP1 ที่ผลิตจาก *E. coli* มีขนาดโปรตีนที่ 60 และ 100 kDa และสภาวะที่เหมาะสมในการผลิตรีคอมบิแนนท์โปรตีน hDMP1 ใน *E. coli* คือที่ความเข้มข้นของ IPTG 0.5 มิลลิโมลาร์ และบ่มที่อุณหภูมิ 37 องศาเซลเซียสเป็นเวลา 6 ชั่วโมง ในขณะที่โปรตีน hDMP1 ที่ผลิตจาก *N. benthamiana* แสดงขนาดโปรตีนที่ 100 kDa นั่นเป็นข้อบ่งชี้ได้ว่ามีกระบวนการปรับแต่งหลังแปรรหัสทางพันธุกรรมเกิดขึ้น และการผลิตโปรตีน hDMP1 จาก *N. benthamiana* ในระดับที่มากที่สุดคือ การเก็บผลผลิตวันที่ 2 หลังจากกระบวนการแทรกซึมโดยอะโกรแบคทีเรียและ ค่าดูดกลืนแสงที่ 600 นาโนมิเตอร์ ($OD_{600}$) เท่ากับ 0.4 รีคอมบิแนนท์โปรตีน hDMP1 สามารถชักนำให้เกิด ALP, BMP2, CBFA1, OSX, OPN และ WNT3a ซึ่งเป็นเครื่องหมายทางพันธุกรรมของเซลล์กระดูกและการสะสมแคลเซียมในเซลล์ hPDLSCs โดยสรุปแล้วผลการทดลองแสดงให้เห็นว่ารีคอมบิแนนท์โปรตีน hDMP1 สามารถกระตุ้นการพัฒนาไปเป็นเซลล์สร้างกระดูกของเซลล์ hPDLSCs อย่างไรก็ตามโปรตีน hDMP1 ที่ผลิตจาก *N. benthamiana* กระตุ้นการพัฒนาไปเป็นเซลล์สร้างกระดูกและมีการสะสมแคลเซียมได้ดีกว่าโปรตีน hDMP1 ที่ผลิตจาก *E. coli* ดังนั้นโปรตีน hDMP1 ที่ผลิตจาก *N. benthamiana* จึงมีศักยภาพที่จะใช้เป็นโมเลกุลเหนี่ยวนำในงานวิศวกรรมเนื้อเยื่อกระดูก

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This study aimed to express recombinant protein human Dentin Matrix Protein 1 (hDMP1) in *Escherichia coli* and *Nicotiana benthamiana* and investigated the osteogenic differentiation effects in human Periodontal Ligament Stem Cells (hPDLSCs). The gene encoding hDMP1 was ligated into the pET22b and pBY2R2e expression vectors. Recombinant hDMP1 protein has been productively expressed in *E. coli* and *N. benthamiana*. *E. coli*-produced hDMP1 resulted in 60 kDa and 100, and the optimal condition to produce highly expression was demonstrated at 0.5 mM IPTG and incubation at 37°C for 6 hr. *N. benthamiana*-produced hDMP1 protein showed in a 100 kDa that indicated the presence of post-translational modification, and the highest-level expression was obtained at 2 dpi and $OD_{600}$ 0.4. Recombinant hDMP1 could induce ALP, BMP2, CBFA1, OSX, OPN, and WNT3a osteogenic marker genes and calcium deposition in hPDLSCc. In conclusion, the results indicated that recombinant hDMP1 could induce osteogenic differentiation in hPDLSCs. However, *N. benthamiana*-produced hDMP1 could induce osteogenic differentiation and calcium deposition better than *E. coli*-produced hDMP1. Therefore, *N. benthamiana*-produced hDMP1 has potential to use as an inductive molecule in bone tissue engineering.



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# CHAPTER I

# INTRODUCTION

Tissue engineering is developed to repair or regenerate the new organ or tissue in the body. Tissue engineering utilizes the living cells in several pathway to restore, maintain or enhance tissues and organs (Akter, 2016; Rodriguez et al, 2015). It has been applied in clinical treatment such as skin, vascular, cartilaginous, bone, and tooth grafts (Cooper et al, 2015a; Murphy et al, 2014). Bone tissue engineering focuses on recovering the form and function of bone tissue insufficiencies. Traumatic injuries and pathological bone diseases such as osteoporosis, osteoarthritis, and incomplete osteogenesis cause damaged bone function and ultimately lead to bone fractures, immobility, severe pain, and deformity (Fernandez et al., 2015; Martini et al., 2018). Therefore, the development of tissue engineering is presently essential for clinical treatment.

The components required for tissue engineering include scaffold, cells, and biological materials. The scaffold provides the environment for the cells to produce new tissue. Biological material functions in inducing and promoting cells to regenerate new organ or tissue (Akter, 2016). The scaffold, which is similar to extracellular matrices, serve to promote cell proliferation and differentiation. Several types of scaffolds have been developed such as collagen (Hendow et al., 2016), and chitosan (Levengood et al., 2014b). Previously, cells used in tissue engineering can be divided into three categories: patient's cells (autologous), human cells obtained from a donor (allogeneic), and cells of animal origin (xenogeneic) (Sangeeta et al., 2014). Another important factor is the biological materials. These molecules function in inducing signalling pathway or cellular component necessary for cell healing or tissue regeneration (Fernandez et al., 2015). The biomolecules should have characteristics such as osteogenicity, osteoconductivity, and osteoinductivity to osteoblasts, osteoclasts, and osteocytes. There are several biomolecules which have been applied in bone tissue engineering such as growth factors and extracellular matrix

(ECM) proteins that could promote bone regeneration (Shrivats et al., 2014; Sun et al., 2013).

The ECM provides several functions such as support systems, regulation of communication intracellular throughout with broad of sequestering growth factors and serves as a local depot. Accordingly, ECM has also been reported to induce the signalling pathway MAPK for bone formation. ECM consists primarily of collagen type I and small amount of non-collagenous proteins (NCPs) (Hyne, 2009; Lin et al., 2015; Staines et al., 2012). Although the NCPs constitute about less than 10% in the matrix, it could encourage and regulate the collagen fibres-forming and crystal growing inside pre-dentin and osteoid which can be converted to bone and dentin (Qin et al., 2007). The major group of NCPs is small integrin-binding ligand N-linked glycoproteins or SIBLING. This group is categorized by the similarity of the biochemical characteristics and the specific peptide motifs such as phosphorylation and integrin-binding arginine-glycine aspartic acid (RGD) (Kulkarni et al., 2000; Lin et al., 2015; Staines et al., 2012). SIBLING group contains large numbers of glutamic acid, aspartic acid, and serine residues which become sites for phosphorylation. Consequently, SIBLING members show a higher function in the post-translational form. The SIBLING increases the protein flexibility and bridges the H factor to either RGD-dependent integrin or CD44 (He et al., 2003; Kulkarni et al., 2000; Lin et al., 2015; Staines et al., 2012). Subsequently, SIBLING has an alternative pathway to form the membrane-bound complex (Akter, 2016). The members of SIBLINGs are osteopontin (OPN), bone sialoprotein (BSP), dentin sialophospoprotein (DSPP), extracellular matrix phospoglycoprotein (MEPE), and dentin matrix protein (DMP) (Fisher et al., 2001; George et al., 2008; Levengood et al., 2014a).



Dentin Matrix Protein (DMP) is expressed in the extracellular matrix of dentin and bone. The DMP is classified into four groups Dentin Matrix Protein 1 (DMP1), dentin phosphoprotein (DPP) or dentin Matrix Protein 2 (DMP2), Dentin Sialoprotein

(DSP) and dentin matrix protein 4 (DMP4). The DMP1 showed the first multifunction of the dentin matrix protein (Hao et al., 2007; Hao et al., 2009).

DMP1 is localized in the bone and dentin. According to the immunofluorescence, the fragments are primarily detected through the lacunar and canaliculi walls of mineralized bone but not exist in the osteoid (Qin et al., 2007; . Feng et al., 2006; Gulseren et al., 2015; He et al., 2005). The DMP1 was firstly cloned from rat without post-translational modification about 53 kDa (George et al., 1993). The DMP1 isolated from dentin rats and mice showed approximately 160–210 kDa (Butler et al., 2009) and 130–150 kDa, in size respectively (Terasawa et al., 2004). Srinivasan also reported the molecular weight without signal peptide of the recombinant rat DMP1-GST in *E. coli* about 90–95 kDa (Srinivasan et al., 2009).

DMP1 modulates an important regulation in the osteogenic differentiation, inorganic phosphate (pi) homeostasis, and nucleator of hydroxyapatite (Feng et al., 2006; Gibson et al., 2013; Narayanan et al., 2003). DMP1 is higher expression in osteocytes than osteoblast cells. In addition, DMP1 can express in the pulp and odontoblast cells. Consequently, DMP1 promotes transcription of osteoblast and odontoblasts markers such as alkaline phosphatase (ALP) and runt-related transcription factor 2 (Runx2) (Eapen et al., 2010; Tabatabaei et al., 2013). Thus, the ECM regulates mineralization via a local and systemic mechanism to promote the signalling pathway in bone regeneration (Feng et al., 2006; Gibson et al., 2013; Narayanan et al., 2003). Several mechanisms supporting the function of DMP1 for bone tissue engineering have been reported. DMP1 has an RGD domain to promote cell adhesion (Gajanan et al., 1999) and cell differentiation by binding to collagen, fibronectin, and vitronectin protein that can activate the signalling pathway (Gajanan et al., 1999; Monique et al., 1989; Rodan, 1998). Further, DMP1 can interact with αβv3 and CD44 cell-surface integrin receptors to control cell differentiation (Qin, 2007; Eapen et al., 2010). Also, DMP1 controls Pi haemostasis through FGF23 hormone

(phosphate regulation) for biomineralization (Qin et al., 2007; Lee et al., 2014). In addition, DMP1 as a hydroxyapatite nucleator bind to a GRP78 receptor in preosteoblasts (Eapen et al., 2010). Furthermore, phosphorylated-DMP1 was delivered into the extracellular matrix to regulate the hydroxyapatite nucleator (Narayanan et al., 2003). Therefore, the recombinant hDMP1 is promising candidate that could be exploited in tissue engineering.

The recombinant proteins are produced from gene manipulation that is generated in several ways to express and manufacture useful commercial products. The recombinant DNA expression technique has been used for the production of antibody, vaccine, therapeutic and dietary proteins such as rabies single-chain antibody-RVG (Phoolcharoen et al., 2017), antibody (mAb) 2C10 and vaccines cholera toxin B subunit (Rattanapisit et al., 2017), osteopontin (OPN) (Rattanapisit et al., 2017), lactoferrin and lysozyme (Cooper et al., 2015b). Different prokaryotic and eukaryotic systems are applied as expression platforms for recombinant proteins production *ie.,* mammalians, insects, yeasts, bacteria, and plants (Bonander et al., 2012; Dalton et al., 2014; Demain et al., 2009; Matthews et al., 2017). In addition, each production host offers advantages and disadvantages. Therefore, the selection of the host is according to the purpose of the research.



Among these platforms, *E. coli* was mostly used in the industry due to fast-growing, generally recognized as safe (GRAS), low-cost, high expression, and accessibility to manipulation and transformation (He et al., 2014; Mattanovich et al., 2012). Nevertheless, *E. coli* lacks post-translational modification. A critical consequence of this system is its inability to produce glycoprotein. However, a number of the recombinant proteins were produced in *E. coli* that showed the potential to treat tissue engineering such as recombinant human bone morphogenetic protein-2 (rhBMP-2) (Kang et al., 2011), and osteocalcin (OCN) (Kim et al., 2015).

The plant is the newest platform developed for recombinant protein production. The advantages of using plants are low cost, high-level accumulation, low contaminant, cheaper to scale-up, and availability for post-translational modifications (Demain et al., 2009). Two methods can be developed to encode the gene-generated into the plant genome, including stable transformation and transient expression. The stable transformation is the first method to transform the gene into the plant genome by stable integration. The process takes about 12-18 months and sometimes results to low level of expression (Gleba et al., 2005). On the other hand, the transient expression method is an improved method of stable transformation that has advantages for rapid and scalability for production of recombinant proteins (Krenek et al., 2015). The full expression process by infiltration with *Agrobacterium* is approximately 3-10 days post infiltration (dpi) (Gleba et al., 2005).

Nowadays, some plant-produced recombinant proteins are available to the market, for example, protein C, interferon α, β, haemoglobin α, β and α-1 antitrypsin inhibitor (Daniell et al., 2001; Thomas et al., 2002). Meanwhile, others are tested for clinical treatment such as gastric lipase for cystic fibrosis and pancreatitis (Ma et al., 2005), vaccine Hepatitis B virus surface antigen (Liz et al., 2000), and rabies vaccine (Yusibov et al, 2002). In addition, plants (tobacco) have produced functional osteopontin (OPN), which induces osteogenic expression (Rattanapisit et al., 2017).



The objectives of this study are to express recombinant protein human DMP1 (hDMP1) in *Escherichia coli* and *Nicotiana benthamiana* and to study the proliferation and osteogenic differentiation effects of this recombinant protein in human Periodontal Ligament Stem Cells (hPDLSCs).

# CHAPTER II

# HISTORICAL REVIEW

## 1. Tissue engineering

Tissue engineering is a multidisciplinary field that applies the principles of engineering, life sciences, cell and molecular biology toward the development of biological substitutes that restore, maintain, and improve tissues function. Tissue engineering aims to develop functional tissues substitutes that can be used for reconstructing damaged tissues or organs. Three general components involve in tissue engineering:

(1) Cells: functional matrix

(2) Scaffolds: transplantation matrix

(3) Bioactive molecules: inductive signalling pathway

These triad constituents can be used as a single component or combine to regenerate the tissues. However, the scaffolds could be developed by collagen or chitosan. Thus, the bioactive molecules will work as inductive molecules. A few growth factor and proteins have been used as an inductive molecule such as extracellular matrix protein (De et al., 2010; Spiller et al., 2017).



## 2. Extracellular matrix proteins

Extracellular matrix proteins are a promising class of biocompatible materials since they comprise the natural environment for cells in the body. They can promote the bone cells as an inducing molecule (Jarvelainen et al., 2009). The constituents of the extracellular matrix protein could bind to the receptors to generate the tissues or organ. Then, they could transduce the signals to the cells, and regulate cellular functions, such as proliferation, migration, and differentiation, which are important for maintaining and repairing the normal homeostasis. The extracellular matrix proteins are served in extracellular matrix (ECM) of bone and

dentin. ECM is a complexly interlinked composite of collagenous molecules, non-collagenous molecules and water-rich mucopolysaccharide ground substance. Cells are integrated into their matrix via integrin and non-integrin receptors or activated the other signalling pathway. ECM mainly contains 90% collagen and minor component of non-collagenous proteins (NCPs). Non-collagenous proteins, an integral component of bone's organic matrix, have been implicated to play a role in bone (Al-Qtaitat, 2014; Rosenthal, 2007; Katherine et al., 2012).

**3. Non-collagenous proteins**

Non-collagenous proteins (NCPs) serve in the extracellular matrix approximately 10%. However, NCPs have a crucial role to provide bone restoring. NCPs actively induce and control collagen fibril mineralization and crystal growth during osteoid, when these tissues are converted into bone and dentin. Non-collagenous constituents involve proteoglycans (PGs) and glycoproteins. Proteoglycans include small leucine-rich proteoglycan (SLRP) family: decorin, biglycan, fibromodulin, lumican, and osteoadherin, large aggregating (versican). The members of glycoproteins are vitamin K-dependent glycoproteins (osteocalcin) secretory calcium-binding phosphoprotein (SCPP) family, osteonectin (SPARC), and SIBLING proteins; osteopontin, dentin matrix protein 1, bone sialoprotein, dentin sialophosphoprotein, matrix extracellular phosphoglycoprotein. However, SIBLING is the major group of NCPs (Ogbureke et al., 2007; Katherine et al., 2012).



The SIBLINGs proteins have a particular domain, an Arginine-Glycine-Aspartate (RGD), which shows a few biological activities by binding to receptor integrins (Fisher et al., 2001). The SIBLING groups in the solution are enlarged proteins which build the bridge complementary between factor H and RGD domains or receptor integrin CD44, thus the conformation of a membrane-bound complex has a function to suppress the alternative pathway (Fisher et al., 2001). The SIBLING proteins include osteopontin, BSP, dentin matrix protein-1 (DMP-1), dentin sialophosphoprotein, and matrix extracellular phosphoprotein. They are secreted proteins that share a

common genetic origin on chromosome 4. However, DMP1 one of the SIBLING proteins has been associated with bone disease (autosomal hypophosphatemia rickets). In addition, loss of the DMP1 in mice affected malformation in bone and tooth within a postnatal process (Ye et al., 2004). Therefore, DMP1 plays a crucial role in bone formation (Ogbureke et al., 2007; Katherine et al., 2012).

## 4. Dentin matrix protein 1

### 4.1 Characteristics of DMP1

The Dentin Matrix Protein 1 (DMP1) gene was isolated from humans, mice, and rats (Fisher et al., 2001; Karen et al., 1997; Macdougall et al., 2009; Seetha et al., 2000). The sequencing of DMP1 cDNA has indicated that the human gene involves an open reading frame of 1539 bp (Karen et al., 1997). The cDNA is categorized into 6 exons that divided into 5 small exons with the size 33 to 104 bp and 6 large exons that consist of 80% of genetic information. The exon 2 relates to the amino acids for the signal peptide and exon 5 with the size 45 bp will be spliced in some species. The intron 1 is the largest part with a size 3791 kb to ~ 6 kb and requires a specific expression of DMP1. Other introns are small, approximately 162-189 bp (Ling et al., 2005; Lu et al., 2007).



The rDMP1 was firstly isolated from rat dentin. It was detected by immunoblotting and showed the full length of 53 kDa without modifications sequence (George et al., 1993). In contrast, modification of rDMP1 was indicated about 160–210 kDa after immunoblotting analysis with an anti-DMP1 antibody (Butler et al., 2009). In addition, mouse DMP1 (mDMP1) was analyzed by using an anti-mouse DMP1 antibody and showed the size 130–150 kDa (Terasawa et al., 2004). Gglycosylation of mouse DMP1 has been shown the size of 89-180 kDa (Sun et al., 2015). The distinct molecular weight of DMP1 is caused by post-translational modification, which might increase the molecular weight of proteins (Terasawa et al., 2004). The recombinant fusion rDMP1-GST in *E. coli* was produced and performed

around 90–95 kDa in SDS-PAGE analysis (Srinivasan et al., 2009).. Subsequently, recombinant mouse DMP1 expressed in wheat germ or *E. coli* that was detected by using immunoblotting and probed with anti-mouse DMP1 (Terasawa et al., 2004).

The DMP1 full-length protein is expected as an initiator to initiate the fragmentation of functional forms (Qin et al., 2004). The DMP1 fragments were short fragments that initially isolated from bone and dentin. The 37 kDa (amino acid residues 1-334) and 57 kDa are from the $NH_2$-terminal and COOH-terminal region, respectively (Gajjeraman et al, 2007). In bone, DMP1 showed in 4 forms, namely 57 kDa C-terminal, 37 kDa N-terminal core protein, glycosylated N-terminal core protein (DMP1-PG), and full-length DMP1 (Qin et al., 2007; Maciejewska et al., 2009). The proteolytic of DMP1 cleavage products are generally the N-terminal and C-terminal fragments. However, rat DMP1 is divided into three potential domains for cleavage. The first region is called GA-domain at $Ser^{74}$, which connects to glycosaminoglycan (GAG) chain. The second region is $Val^{157}$-$Gly^{184}$ of rat that contains a primary cleavage site $NH_2$-terminal peptide bond of $Asp^{181}$. The third region corresponding to $Arg^{449}$-$Tye^{473}$, of rat DMP1 (Qin et al., 2007; Feng et al., 2006; Qin et al., 2003). In addition, the phosphate analysis of DMP1 indicated that there are 12 and 41 phosphates in the 37 and 57 kDa fragments which consensus phosphorylation of the protein. Consequently, DMP1 becomes an acidic protein with an isoelectric point 4.15 (Deshpande et al., 2011; Qin et al., 2003). The DMP1 contains 473 amino acids which involve dominantly of acidic amino acid residues such as serine, glutamic acid, and aspartic acid (George A et al., 1993).



The acidic residues of DMP1 lead to function as a mineralization in bone. Previous study reported that DMP1 contains four acidic domains such as ESNES (376–380), ESQES (386–390), DSQDS (431–435), QESQSEQDS (414–422) which can control crystallization (G. He et al., 2003). Furthermore, DMP1 also comprises of ASARM motif (acidic serine and aspirate rich motif). ASARM motif has an essential effect on regulating the activity of the proteins (Rowe et al., 2004; Staines et al., 2012). In the

DMP1 sequence, ASARM motif localizes in the COOH-terminal fragments (Martin et al. 2008). On the other hand, DMP1 contains the important RGD (arginine-glycine-aspartic acid) domain, which urges the cell binding to a cell and tissue-specific target and then activates the signalling pathway (Butler et al., 2009; Eapen et al, 2011; Kulkarni et al., 2000., George A et al., 1993). The RGD domain is in the middle of the 57 kDa fragment (Qin et al., 2004; Qin et al., 2003). The residues and domains of DMP1 protein conduct to multifunctional as mineralization in bones and teeth.

### 4.2 Function of DMP1

The DMP1 can regulate the mineralization through hydroxyapatite nucleator, osteogenic differentiation and phosphate homeostasis mechanisms (Qin et al., 2007; Feng et al., 2006; Gibson et al., 2013; Nishino et al., 2017).

#### 4.2.1 Hydroxyapatite formation

DMP1 performs the regulation for formation hydroxyapatite (HA) and crystal growth (Tartaix et al., 2004). DMP1 can initiate the hydroxyapatite formation by multi-process, which are started by binding calcium ion, generating the mineral deposition, precipitating the nucleate amorphous calcium phosphate, and forming the nanocrystal. Afterward, these extend and unite into the microscale crystals elongated in the C-axis direction (He, 2003).

The crystals formations of calcium phosphate are under regulate of acid domains DMP1. Whereby self-assembly acidic sites to the beta-sheet template is desired for inducing of a biomineral. The supramolecular of DMP1 stimulates the crystal nucleation. Then, it arranges the step transition of calcium phosphate to carbonate apatite depositions (He et al., 2005; He et al., 2003). The full length and C-terminal of native rat DMP1 could elevate nucleation of hydroxyapatite in the existence of collagen I (Gajjeraman et al., 2007; Tartaix et al., 2004). In addition, DMP1 has two domains of the acid cluster that can interact with type I collagen in the N-

telopeptide region, which indicates collagen fibrogenesis forming (George et al., 2008; He et al., 2003). The domains are DSESSEEDR (249-357) in the C terminal and SEENRDSDSQDSSR (424-437) at the end of DMP1 (He et al., 2008). Based on the site-directed mutagenesis studies, it has been confirmed of collagen-attachment domains. *In vitro* study, DMP1 enhanced the collagen fibril formation and elevated the diameter of the collagen fibrils. Also, DMP1 attach to collagen sites was indicated the calcium and hydroxyapatite depositions (He et al., 2003; He et al., 2004). Other peptides of DMP1 DSESSEEDR-Ahx-ESQES (1.726 kDa) and DSESSEEDR-Ahx-QESQSEQDS (2.185) had reported. These domains bind specifically to collagen-binding and calcium-binding sites. They have been shown the regulation of mineral deposition and hydroxyapatite formation when the physiological exposure to calcium and phosphate (Padovano et al., 2015).

On the other hand, 57 kDa COOH-terminal shows the hydroxyapatite nucleator activity and controlling differentiation cells by targeting and binding between nucleus or cell-surface integrin such as CD44 receptors (Gajjeraman et al., 2007). The binding between DMP1 and cell surface receptor GRP78 are very interesting due to the action protect of stress, depletion of ER Ca2+ and aggregation of un-glycosylated proteins (Eapen et al., 2010; Lien, Kung, Lu, Jeng, & Chau, 2008). The existence of DMP1 can increase the self-assembly of calcium to filaments forming (He et al., 2004).



### 4.2.2 Osteogenesis Differentiation

DMP1 stimulates the migration, regulation, differentiation of the dentin, and bone cells (Widbiller et al., 2018). DMP1 induces the osteogenic-markers genes in the nucleus, such as alkaline phosphate (ALP) and runt-related transcription factor 2 (Runx2), and osteocalcin (Gulseren et al., 2015; He et al., 2005; Eapen et al., 2010). They regulate the mineralization via a local and systemic mechanism to promote the signaling pathway in bone regeneration (Feng et al., 2006; Qin et al., 2004). In addition, DMP1 has an RGD domain to promote

cell adhesion (George et al., 1993; Terasawa et al., 2004) and differentiate the bone cells by binding to collagen IV, fibronectin, and vitronectin protein, which activate the signaling pathway (Butler et al., 2009; George et al., 1993; Terasawa et al., 2004). Subsequently, DMP1 could interact with **αβ**v3 and CD44 receptors to control cell differentiation (Gulseren et al., 2015; Srinivasan et al., 2009). Furthermore, DMP1 could control differentiation of the odontoblast and osteoblast cells (Lu et al., 2007). The DMP1 showed the differentiation of endometrial stem cells. The osteogenic effect also has been shown on dental follicle stem cells (DFSCs) (Rezai et al., 2015). Accordingly, the recombinant DMP1 in *E. coli* could promote differentiation of mouse calvaria preosteoblasts (MC3T3-E1) and C3H10T1/2 cells by inducing run2x and osteocalcin genes. These results also confirmed the osteogenic differentiation effect of DMP1 through binding RGD-integrin to activate the signal-regulated kinase (ERK)-MAPK pathway (Eapen et al., 2010; Eapen et al., 2011).

Furthermore, the osteogenic differentiation of the cells could regulate through calcium signaling. Eapen et al. proposed that DMP1 could increase calcium in systolic which facilitate the downstream to cellular stress. These conditions involve the regulation of calcium and activate the stress-induced p38 MAPK associated osteoblast differentiation. Besides, the binding between GRP78 and DMP1 induce the release and accumulation of the ER2Ca2+, then activate the p38 MAPKs pathway. Subsequently, the activation of the p38 MAPK translocate the nucleus to regulate the modulator transcription of cell differentiation, such as Run2x on embryonic development (Eapen et al., 2010).



### 4.2.3 Phosphate homeostasis

The new finding of DMP1 can control inorganic phosphate (Pi) homeostasis through Fibroblast Growth Factor 23 (FGF23) (Feng et al., 2006; Lorenz-Depiereux et al., 2006). FGF23 is hormone that is mainly secreted by osteocytes and osteoblasts in bone. It is targeted in kidney and extends the function of bone endocrine organ in the regulation of Pi homeostatic (Gattineni et al., 2009). Phosphate haemostatic

indicates the mature of osteoblast and osteocytes. Previous study showed that the deficiency of DMP1 conducts to elevated FGF23 mRNA in the osteocytes (Feng et al., 2006). In related with previous study showed that FGF23 increased in the DMP1 null osteocytes which lead to the Pi homeostasis in the bone (Lin et al., 2015; Quarles, 2012). Apparently, this sharp increase is likely due to defects in the maturation of osteoblasts into osteocytes. The 57 kDa C-terminal plays a key role in maintaining phosphate homeostasis through regulating FGF23 expression in bone (Lu et al., 2011).

In the other hand, studies of DMP1 null mice and DMP1 mutations in patients showed that FGF23 is significantly upregulated in osteocytes, which is released into circulation through the connection between osteocytes and vessels, leading to an increase of FGF23 in serum and in subsequent hypophosphatemia. Specifically, in situ hybridization (measuring mRNA levels) or immunohistochemistry (measuring protein levels), revealed that the FGF23 level in control osteocytes was much lower than that in osteoblasts, indicating differential regulatory mechanisms for FGF23 in bone cells of healthy versus disease conditions. The discovery of elevated level of FGF23 in theDMP1 null osteocytes indicates that bone is an endocrine organ regulating phosphate homeostasis (Lin et al., 2015; Feng et al, 2006).

Based on the functions of DMP1 which is highly potential to induce or promote the cells for bone formation. Therefore, DMP1 can be used to treat the bone diseases caused by damage or accident such as application in bone tissue engineering. Consequently, DMP1 is necessary to produce for developing tissue engineering.



### 4.3 Source of DMP1

Mostly the DMP1 is isolated from animals or humans. However, the limited amount of DMP1 in the tissue makes it difficult to isolate. Also, DMP1 is not easy to purify from other NCPs in the tissues. However, previous study reported the expression of recombinant rDMP1 in *E. coli* (Srinivasan et al., 2009). Therefore, the recombinant protein technique might be a solution to produce hDMP1 protein in properly systems.

**5. Recombinant proteins Production**

Recombinant proteins technology has been used to produce number of proteins in prokaryotic and eukaryotic systems (Greene, 2004; NelsonLehninger & Cox, 2008). Non-glycosylated proteins are widely expressed in prokaryotic (Sahdev Khattar & Saini, 2008). The glycosylated proteins are able to produce in eukaryotic cells such as mammalian cells, insect cells, yeast cells, and plants (Jenkins et al., 1994; Nelson et al., 2008; Palomares et al., 2004).

Among the cells hosts, each host has unique strengths and weaknesses such as cost, scale-up, ability post-translational modification, and protein folding (Clark et al., 2016). Therefore, selecting the system is based on the suitable and purpose of the research. The advantages and disadvantages of the systems are summarized in Table 1. Among the hosts, bacterial platform, which is also known as prokaryotic expression system, is the most common method to produce large amounts of proteins (Bonander et al., 2012; Dalton et al., 2014; Demain et al., 2009; He et al., 2014; Mattanovich et al., 2012).

**Table 1** Overview of the protein expression systems (Dalton et al.,2014)

| Comparison | Plant | Bacteria | Yeast | Insect cell | Mammalian cell |
|---|---|---|---|---|---|
| Cost | Very low | Low | Medium | High | High |
| Scale-up production | Very high | High | High | High | Low |
| Protein yield | High | Medium | High | Low | High |
| Folding accuracy | High | Low | Medium | Low | High |
| Glycosylation | Correct | None | Incorrect | Incorrect | Correct |
| Product quality | High | Low | Medium | Low | High |
| Contaminant risk | Low | High | Low | High | High |

### 5.1 Bacterial system

In bacterial system, *E. coli* host extremely uses to produce recombinant protein because it is easier to manipulate genetics than other microorganisms. This system is appropriate to express a non-glycosylated protein and available for transcription, translation, and protein folding and improved genetic tools. In addition, the *E. coli* genome is rapidly and accurately modified, easy to control the promoter and change the copy number of the plasmid. *E. coli* system shows the metabolic carbon modifications, amino acid analogue preventions, intracellular disulphide bonds forming, and reproducible control by computer. The production of the recombinant protein of this system can be collected more than 80% of its dry weight (Demain et al., 2009). *E. coli* proposes the affordable carbon source necessities for development, rapidly biomass collection, acceptable to high-cell density, and easy to scale-up (Sahdev et al., 2008). *E. coli* was mostly used in the industry due to some benefits involving the rapid growth rate, high expression, recognized as safe (GRAS), low-cost, easy to manipulate and transform (Gaciarz et al., 2016; Rosano et al., 2014). Several parameters have been tried to improve expression in *E. coli* such as modifying the promoter, using appropriate strain, optimizing the conditions, growth medium, and expression of the fragments genes (Demain et al., 2009; Maldonado et al., 2007; Wong, Wu et al.,2008).



Furthermore, bacterial system also has limitations such as containing a toxin and forming a inclusion bodies (Sahdev et al., 2008). Inclusion bodies are frequently insoluble, non-active, and refolding (Demain et al., 2009; Jenkins et al., 1994). The bacteria system is unavailable to synthesize the protein complex, such as monoclonal antibodies and coagulation blood factors. (Clark et al., 2016; Demain et al., 2009). In addition, bacterial system is a prokaryotic cell which is unavailable for post-translational machinery (Sahdev et al., 2008). However, post translational modification protein can be produced in eukaryotic cells such as mammalian cell, insect cell, yeast cell, and plant system (Jenkins et al., 1994; Nelson et al., 2008; Palomares et al., 2004).

### 5.2 Plant system

The use of plant to produce recombinant proteins is an economically significant and promising direction being developed as an alternative for the traditional one. The advantage of plant systems is the lower cost of cell cultivation. These are not subjected to undesirable components, such as bacterial endotoxins, hyperglycosylated proteins produced by yeast, and animal and human pathogens in cell cultures of transgenic animals. Besides, plant is higher eukaryotes, and, therefore, full protein folding and formation of intricate multimeric protein complexes. The significant of posttranslational modifications appears to be analogous of mammalian cells. The plant system being presently developed for recombinant protein expression that is diverse in different technologies of gene transfer, expression strategies and methods of subsequent target protein extraction. The stable transformation (transgenic plant) and transient expression have been developed to express the protein in plant system (Demain et al., 2009; Gerasimova et al., 2016; Zagorskaya et al. 2017).

### 5.3 Stable transformation

Stable transformation or transgenic plant is the first method to transform the gene into the chloroplast genome by stable integration. Transgenic plant can be applied in two methods; transform the interest gene into the plant virus such as the tobacco mosaic virus in the tobacco plant and insert directly into the plant DNA. The limitation of this method probably contains contamination of herbicides, pesticides, and plant metabolites. Also, this method needs time-consuming about 12-18 months (Fitzgerald et al., 2003; Gleba et al., 2005). Forwards, the stable transformation is sometimes low expression in the range 0.01%-2% total soluble protein (Mor et al., 2003).

### 5.4 Transient expression

The transient expression is an improvement of the stable transformation that has advantages, such as rapid and easy to scale up for production. Nowadays, the transient method is developing to serve the rapid systems for producing the recombinant protein. Subsequently, transient has some benefits as simple, effective, exceedingly scalable, safe from contamination, and proper to produce complex proteins. Among the advantages, the most important is the time consumed for the entire expression, only approximately 2-12 days. Several plants have been developed to express the recombinant protein, including alfalfa, soybean, lettuce, potato, spinach, arabidopsis, and tobacco (Cao et al., 2017; Gerasimova et al., 2016). Tobacco has significant benefit, including established protocols of transformation, high yield expression, accessibility to scale up the production, non-consumption food, growth at any-time (Karg et al., 2009; Twyman et al., 2003). Regarding a few species of *Nicotiana*, the *Nicotiana benthamiana* is the most useful species for transient expression for producing recombinant proteins. Moreover, *N. benthamiana* has been performed to produce recombinant protein in this study.

In this study, we have been used *E. coli* and plant systems to express recombinant hDMP1 proteins. *E. coli* system is commonly used to produce recombinant proteins, however, it is unavailable for post-translational modification. Protein expression in plant system involves post-translational modification that can increase the biological activity of the protein. Our study also investigated the effect of post-translational modification of recombinant hDMP1 as an inductive molecule in bone cells. Therefore, human periodontal ligament stem cells (hPDLSCs) were treated with recombinant hDMP1 proteins to study the effect of osteogenic differentiation and calcium deposition.

# CHAPTER III

# EXPERIMENTAL

## 1. Material

### 1.1 Gene

The amino acid sequence of hDMP1 (Gene ID: 1758) with 8xHis tag at the C-terminus was synthesized by Bioneer Corp (Bioneer Corp, South Korea).

### 1.2 Enzymes

Restriction enzyme *EcoR*I (New England BioLabs, USA)

Restriction enzyme *Xba*I (New England BioLabs, USA)

Restriction enzyme *Sac*I (New England BioLabs, USA)

Taq DNA-Polimerase (Promega, USA)

Pfu DNA-Polimerase (Promega, USA)

T4-DNA Ligase (Promega, USA)

## 1.3 Plasmids

### 1.3.1 pET22b-hDMP1

The pET-22b(+) vector was used for expression in bacterial system (*E. coli* strain Rosetta).

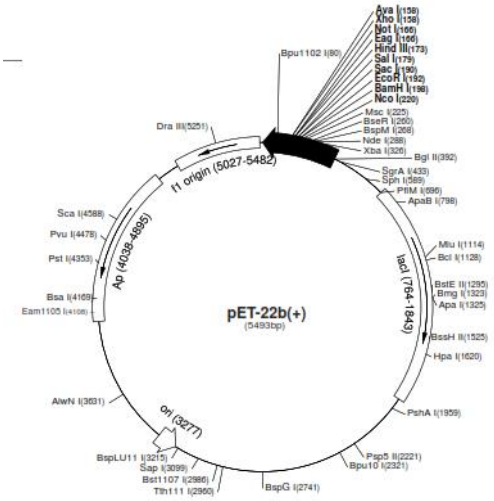


**Figure 1** pET-22b(+) vector

### 1.3.2 pBYR2e Vector

The pBYR2e (bean yellow dwarf virus) vector was used for expression in the *N. benthamiana.*



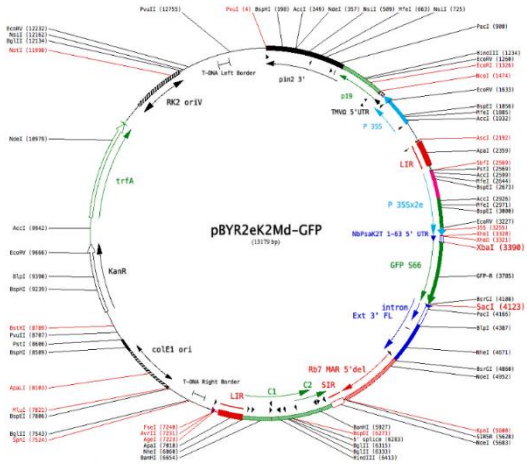


**Figure 2** pBYR2e vector

### 1.3.3 pGEM-T Vector

The pEM-T vector was used for cloning of PCR products. The high copy number pGEM-T vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the αpeptide coding region of the enzyme β-galactosidase.

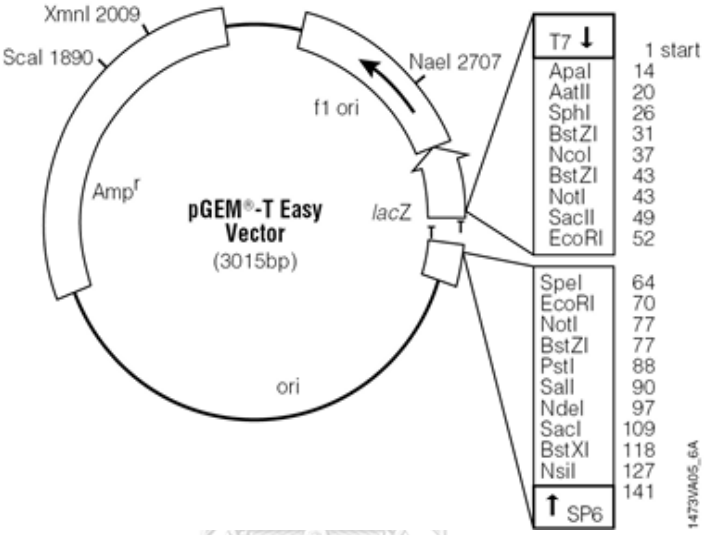


**Figure 3** pGEM-T vector



### 1.4 Molecular biology kits

Plasmid extraction (Bioneer Corp, South Korea)

Gel elution (Bioneer Corp, South Korea)

DMP1 ELISA Kit (ThermoFisher Scientific, USA)

### 1.5 Chemicals

Acrylamide 30% (Himedia, India), Ampicillin (ITW Reagents, Germany), kanamycin (Bio basic, USA), gentamicin (ITW Reagents, Germany), rifampicin (Thermo Fisher Scientific, USA), 2-(N-morpholino), ethanesulfonic acid (MES) (ITW Reagents, Germany), magnesium sulphate (Merck, USA) , tris (Vivantis, Malaysia), natrium chorid, imidazole (Affymetrix, Germany), ammonium sulphate (Merck, USA), syringe filter 0.45

μm (Sigma-Aldrich, Germany), Ni-NTA affinity column (Qiagen Gmbh, Germany), sodium dodecyl sulphate (SDS) (Himedia, India), β-mercaptoethanol, bromophenol blue (Sigma-Aldrich, USA), coomassie Brilliant Blue R250 (ITW Reagents, Germany), methanol (Honeywell, USA), nitric acid (Qrec, New Zeland), ammonium persulphate (Merck, USA), nitrocellulose membrane (ThermoFisher Scientific, USA), Enhanced Chemiluminescence (ECL) (GE Healthcare, UK), MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) (USB Corporation, USA), glycine, Dimethyl sulfoxide (DMSO) (ThermoFisher Scientific, USA), dexamethasone, ascorbic acid, alizarin red S staining solution (Sigma-Aldrich, St. Louis), glycerol, isopropyl-b-D-thiogalactopyranoside (IPTG) (Bio Basic, Canada), DNA-leadder (Vivantis, Malaisya), protein standard (Bio-rad, USA), commercial hDMP1 (R&D systems Inc, USA), 0.45 mm syringe filter (Sigma-Aldrich, Germany), Coomassie Brilliant Blue R250 (ITW Reagents, Germany).

### 1.6 Bacteria

*E. coli* strain DH10B

*E. coli* strain Rosetta (DE3)

*Agrobacterium tumefaciens* strain GV3101



### 1.7 Antibodies

HRP-conjugated goat anti-His antibody (Abcam, UK)

Rabbit anti-human DMP1 antibody (Abcam, UK)

HRP-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research, USA).

**2. Software**

ImageJ

GraphPad Prism 7.0

Expasy

Emboss needle

**3. Tobacco (*Nicotiana benthamiana*)**

The *N. benthamiana* seeds were grown in the soil for about 10 days. After growing, the tobacco plants were transferred into the pot. The tobacco plants were grown at room temperature (28°C) under white light. The tobacco was watered for 2-3 times a week. The tobacco plants will ready to use after 6-8 weeks.

**4. The hPDLSCs (human Periodontal Ligament Stem Cells)**

Periodontal ligament cells were established from periodontal ligament tissue obtained from impacted lower molar extracted for orthodontic reasons at the Department of Surgery, Faculty of Dentistry, Chulalongkorn University. This study was approved by the human subject ethics board of Faculty of Dentistry, Chulalongkorn University, and was conducted to the Helsinki Declaration of 1975, as revised in 2013. Based on the previous report (Osathanon et al., 2013), the hPDLSCs were isolated. Then, the cells were cultured and grew in Dulbecco's modified Eagle's medium that supplemented by 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin and 5 ug/ml amphotericin B. After confluency, cells were detached with 0.25% trypsin-EDTA and sub-cultured at a ratio of 1:3. Cells from the third to the fifth passages were used in the experiments.

**5. Methods**

**6.1 Cloning**

The hDMP1 gene was amplified with the primers (F:5' CTCGAGATCTAGAATGGCTAACAAGCACCTCTCATTGTCTCTCTTCC 3' and R: 5' GAGCTCTTAATGATGGTGATGGTGGTGATGATGATAGCCGTCTTGGCAGTC 3'). Thus, loaded into the agarose gel 1%. The expected size of about 1654 bp was cut and diluted by elution gel kits. Further, the PCR product was ligated with pGEM-T and transformed into the *E. coli* DH10B. The insertion colony was cultured and extracted the plasmid for checking the sequence.

**6.1.1 pET22b-hDMP1**

The hDMP1 gene was ligated into the expression vector pET22b by using *EcoR*I and *Sac*I restriction enzymes. The pET22b-hDMP1 was transformed into the *E. coli* DH10B by using the heat shock method (Froger & Hall, 2007). Then, the insertion colony was determined by PCR and cultured in Luria Bertani (LB) media that supplemented by 100 mg/mL ampicillin at 37°C overnight. Further, the pET22b-hDMP1 was transformed into the *E. coli* Strain Rosetta by using the heat shock method (Froger & Hall, 2007). The insertion colony was determined by PCR.



**Table 2** Thermocycle condition for colony PCR condition colonies

| Step1 | Temperature | Time | Cycles |
|---|---|---|---|
| Denaturation | 94°C | 2 min | 1 |
| Denaturation | 94°C | 2 sec | |
| Annealing | 54°C | 1 min | 30 |
| Extension | 72°C | 1-3 min | |
| Final extension | 72°C | 7 min | 1 |

### 6.1.2 pBRY2e-hDMP1

The encoding hDMP1 was ligated into the expression vector pBYR2e by using *Xho*I and *Sac*I restriction enzymes. The pBYR2e-hDMP1 was transformed into the *E. coli* DH10B by using the heat shock method (Froger & Hall, 2007). The selected colony was determined by PCR and cultured in Luria Bertani (LB) media that supplemented by 50 mg/ml kanamycin at 37°C overnight. The pBYR2e-hDMP1 was transformed into the *Agrobacterium tumefaciens* strain GV3101 by using the electroporation method (Yoshihisa Oda YN & Fukuda, 2018). The bacteria were grown in the agar media (containing kanamycin 50 mg/ml, gentamicin 50 mg/ml, and rifampicin 50 mg/ml) at 28°C for 2 days. Then, the insertion colony was selected by using PCR.

## 6.2 Expression and optimization of recombinant hDMP1

### 6.2.1 *E. coli*-produced hDMP1

The bacteria *E. coli* strain Rosetta were cultured in Luria Bertani (LB) media in the existence of 100 mg/L ampicillin at 37°C overnight. Then, it was subcultured up to optical density 600 nm ($OD_{600}$) approximately 0.4. Afterward, isopropyl-β-D-thiogalactopyranoside (IPTG) was added for inducing the protein expression with a final concentration of 0.2, 0.5, and 1 mM. The bacteria were incubated at either 28°C or 37°C on a rotary shaker at 200 rpm. The cells were harvested every 2, 4, and 6 hours after induction. The expression level was determined by western blot analysis.

### 6.2.2 *Nicotiana benthamiana*-produced hDMP1

The insertion colony of *Agrobacterium* GV3101 was cultured in the LB media that supplemented by kanamycin 50 mg/ml, gentamicin 50 mg/ml, and rifampicin 50 mg/ml at 28°C overnight. The culture of the bacteria was collected by centrifugation

at 4000 g for 15 minutes. Then the pellet was suspended with infiltration buffer. *Agrobacterium* cultures with various OD (0.1, 0.2, 0.3, 0.4, 0.5, and 1) were infiltrated into the *N. benthamiana* using a vacuum with the transient method. The leaves were harvested 1, 2, 3, 4, and 5 days post infiltration (dpi). The expression level was determined by western blot analysis.

### 6.3 Purification of recombinant hDMP1

#### 6.3.1 Purification of *E. coli*-produced hDMP1

The bacterial culture was collected by centrifugation at 4,000 g for 10 minutes. Afterwards, the pellet was suspended with extraction buffer and broke the cell by using ultrasonic lysis. The solution was centrifuged at 6,000 g for 30 min. The supernatant was filtered with a 0.45 µm syringe filter and loaded onto a Ni-NTA affinity column. Subsequently, the column was washed with washing and eluted by eluting buffer.

#### 6.3.2 Purification of *N. benthamiana*-produced hDMP1

The infiltrated leaves of *N. benthamiana* leaves were grounded and extracted with Imac 5 buffer. After that, centrifuged at 15000 rpm for 30 minutes. Then, the rubisco protein was removed by using precipitation with ammonium sulphate (Burgess, 2009). Ammonium sulphate was added into the solution (35%), stirring for 30 min on ice, and then centrifuged at 4000 g for 30 minutes. Afterward, the supernatant was added ammonium sulphate (80%), stirring for 30 minutes and centrifuged at 4000 g for 30 minutes. The pellet was suspended with extraction buffer and filtered by using a 0.45 µm syringe. Subsequently, loaded onto a Ni-NTA affinity column. The column was washed with washing buffer and eluted with elution buffer.

### 6.4 SDS PAGE analysis

The gel acrylamide 10% was used to separate the proteins. The sample was suspended in Z-buffer reducing condition and heated on the 95°C for 5 minutes. As amount 40 µl was loaded into the gel. Afterward, the protein was separated by using electroporation 100 volts. The protein was analyzed by coloring with Coomassie brilliant blue for overnight and followed by distaining with destain buffer. Precision plus protein standard was used as a reference protein size (10-250 kDa).

### 6.5 Western Blot analysis

The proteins on acrylamide gels were transferred to the nitrocellulose membrane by using electroporation at 100 volts for 2 hours. Then, followed by blocking the non-specific proteins with skim milk 5% in 1xTBS buffer. After 1 hour, the membranes were incubated with HRP-conjugated goat anti-his and rabbit anti-DMP1 (HRP-conjugated goat anti-rabbit) antibodies that diluted in the skim milk 3% in 1x TBS at 1:5000. As much three times of TBST buffer was used for washing the membranes. The membranes were probed with ECL (enhanced chemiluminescence) substrate for detecting.



### 6.6 Protein quantification

The crude extracts of the recombinant hDMP1 proteins were determined by the Enzyme-linked immunosorbent assays (ELISA) method. The commercial recombinant hDMP1 was used as a standard by various concentrations. The procedure based on the manual of the hDMP1 ELISA Kit (ThermoFisher Scientific, USA). The absorbance was measured by using a microplate reader at wavelength 450 nm.

### 6.7 DMP1 coating protocol

Before testing the activities of recombinant hDMP1 proteins, the wells were coated by following the protocol. The wells were coated with dopamine for 6 hr. After that, wells were washed by using PBS buffer and coated with recombinant hDMP1 proteins for overnight. Then, as much 50.000 hPDLSCs cells were seeded into each well. Furthermore, the hPDLSCs cells were incubated and treated to follow the assay protocol.

### 6.8 Proliferation assay

The 12-well plates were coated with recombinant hDMP1 proteins (1 and 2 μg/ml) for overnight. The hPDLSCs were seeded at a density of 50,000 cells per wells in 24-well-plate. The cells were cultured on DMP1 for 24 and 72 hr. The cells were assessed for the cell vitality by thiazolyl blue tetrazolium bromide. Then 500 μl MTT (0.5 mg/ml) solution was added into each well, followed by incubation for 30 minutes at 37°C to allow formazan formation. Then, 500 μl of Glycine buffer: DMSO (1:9) was added to dissolve the formazan crystals. Measuring the absorbance was performed at the 570 nm in a microplate reader.



### 6.9 Osteogenic expression

The wells were incubated with DMP1 (0.5, 1, and 2 μl /ml) overnight. Then, the hPDLSCs at 50,000 density were seeded into the wells and incubated for three days. Total RNA was extracted by using Isol-RNA Lysis reagent, and 1 μg/ml of RNA per sample was converted to cDNA using a reverse transcriptase kit. Quantitative RT-PCR was performed using a Lightcycler Nano real-time polymerase chain reaction machine. The primers ALP, BMP2, CBFA1, COL1, OPN, OSX, WNT3a ( Table ***3***) were used for this protocol, and the conditions of the RT-PCR was set on

denaturation at 94 ℃ for 10 min, at 60℃ for 10 sec for annealing, and extension at 72 ℃ for 10 sec for 45 cycles.

**Table 3** RT-PCR primers for differentiation- specific gene expression

| Gene | Sequence | Reference |
|---|---|---|
| ALP | F: 5’ CGAGATACAAGCACTCCCACTTC 3’<br>R: 5’ CTGTTCAGCTCGTACTGCATGTC 3’ | NM000478.3 |
| CBFA1 | F: 5’ ATGATGACACTGCCACCTCTGA 3’<br>R: 5’ GGCTGGATAGTGCATTCGTG 3’ | NM001024630.3 |
| COL1 | F: 5’ GTGCTAAAGGTGCCAATGGT 3’<br>R: 5’ ACCAGGTTCACCGCTGTTAC 3’ | NM000088.3 |
| OSX | F: 5’ GCCAGAAGCTGTGAAACCTC 3’<br>R: 5’ GCTGCAAGCTCTCCATAACC 3’ | NM001300837.1 |
| OPN | F: 5’ AGGAGGAGGCAGAGCACA 3’<br>R: 5’ CTGGTATGGCACAGGTGATG 3’ | NM001040060.1 |
| WNT3a | F: 5’ CTGTTGGGCCACAGTATTCC 3’<br>R: 5’ GGGCATGATCTCCACGTAGT 3’ | NM033131.3 |
| BMP2 | F; 5’CTCAGCGAGTTTGAGTTGAGG 3’<br>R; 5’GGTACAGGTCGAGCATATAGGG 3’ | NM-017178.1 |

**6.10 Calcification assay**

The wells were incubated with recombinant hDMP1 (2 µl/ml) for overnight. The hPDLSCs were seeded with 50,000 cells per wells in Serum-Free Media (SFM) and incubated for 4-6 hr. The cells were changed to be in Osteogenic Medium (OM) and General Medium (GM) for control. They were incubated for 14 days, then fixed with methanol for 10 minutes. After washing by deionized water, the cells were

stained with 1 % alizarin red S solution at room temperature. Each specimen was washed by deionized water and dried.

### 6.11 Data Analysis

The experiments were performed in three replicates. The statistical analysis was performed by Graph Pad 7 and one-way ANOVA (*: $p<0.05$, **: $p< 0.01$).

# CHAPTER IV

# RESULTS AND DISCUSSION

## 1. Codon optimization and gene synthesis

The nucleotide sequence of hDMP1 (Gene ID: 1758) with 8xHis tag at the C-terminus was optimized *in silico* for preferred codon usage in *N. benthamiana* and synthesized by Bioneer Corp (South Korea) (Figure 4). The full length of hDMP1 gene contains nucleotide acid 1647 bp and 549 amino acids, including the modifications sequences poly-histidine tag (8x-Histidine). The poly-histidine tag was utilized to enable detection of the recombinant protein using anti-histidine antibody and purification by Immobilized Metal Affinity Chromatography (IMAC). In addition, hDMP1 could be detected using anti human DMP1 antibody. The poly-histidine tag is appropriate to purify by nickel column (Knecht et al., 2009).

```
1     GAATTCATGGCTAACAAGCACCTCTCATTGTCTCTCTTCCTTGTGCTCCTTGGTCTTTCT  60
1           M  A  N  K  H  L  S  L  S  L  F  L  V  L  L  G  L  S   20
61    GCTTCTCTTGCTTCTGGTAAGATCAGCATCCTGCTGATGTTCCTTTGGGGCCTTTCTTGC  120
21     A  S  L  A  S  G  K  I  S  I  L  L  M  F  L  W  G  L  S  C   40
121   GCTTTGCCTGTGACTAGGTATCAGAACAACGAGAGCGAGGACAGCGAAGAGTGGAAGGGT  180
41     A  L  P  V  T  R  Y  Q  N  N  E  S  E  D  S  E  E  W  K  G   60
181   CATCTTGCTCAAGCTCCTACTCCTCCGCTTGAGTCATCTGAATCTAGCGAGGGTAGCAAG  240
61     H  L  A  Q  A  P  T  P  P  L  E  S  S  E  S  S  E  G  S  K   80
241   GTGTCCTCTGAGGAACAGGCTAACGAAGATCCTAGCGACTCTACCCGTCTGAAGAGGGTC  300
81     V  S  S  E  E  Q  A  N  E  D  P  S  D  S  T  Q  S  E  E  G   100
301   TTGGGATCTGATGACCACCAGTACATCTACAGGCTTGCTGGTGGTTTCTCTCGGTCTACT  360
101    L  G  S  D  D  H  Q  Y  I  Y  R  L  A  G  G  F  S  R  S  T   120
361   GGTAAAGGCGGGGATGATAAGGATGATGACGAGGATGATAGCGGCGACGATACCTTCGGT  420
121    G  K  G  G  D  D  K  D  D  D  E  D  D  S  G  D  D  T  F  G   140
421   GATGATGATTCTGGTCCTGGTCCTAAGGATAGGCAAGAAGGTGGTAACTCTAGGCTGGGC  480
141    D  D  D  S  G  P  G  P  K  D  R  Q  E  G  G  N  S  R  L  G   160
481   TCTGATGAGGATTCCGACGATACAATTCAGGCCAGCGAAGAATCTGCTCCTCAAGGTCAA  540
161    S  D  E  D  S  D  D  T  I  Q  A  S  E  E  S  A  P  Q  G  Q   180
541   GATAGCGCTCAGGATACCACTAGCGAGAGCAGAGAACTGGATAACGAGGACAGGGTTGAC  600
181    D  S  A  Q  D  T  T  S  E  S  R  E  L  D  N  E  D  R  V  D   200
601   TCCAAGCCTGAAGGTGGTGATTCTACCCAAGAGAGCGAGTCAGAGGAACATTGGGTTGGA  660
201    S  K  P  E  G  G  D  S  T  Q  E  S  E  S  E  E  H  W  V  G   220
661   GGTGGTAGTGATGGTGAGTCCTCTCATGGTGATGGTAGCGAGCTTGATGATGAGGGTATG  720
221    G  G  S  D  G  E  S  S  H  G  D  G  S  E  L  D  D  E  G  M   240
721   CAGTCTGACGACCCTGAGTCTATTAGGTCTGAGAGGGGCAACTCCAGGATGAACTCTGCT  780
241    E  T  Q  S  D  D  P  E  S  I  R  S  E  R  G  N  S  R  M  E   260
781   GGCATGAAGTCCAAAGAGAGCGGAGAAAATTCCGAGCAGGCTAACACTCAGGATAGCGGT  840
261    T  N  S  A  G  M  E  T  K  S  K  E  S  G  E  N  S  E  Q  A   280
841   GGTTCTCAGCTTCTTGAGCATCCGAGCCGGAAGATTTTCCGGAAGTCTAGGATCAGCGAG  900
281    N  T  Q  D  S  G  G  S  Q  L  L  E  H  P  S  R  K  I  F  R   300
901   GAAGATGACAGGTCCGAGCTGGATGACAACAACACTATGGAAGAGGTGAAGTCCGACAGC  960
301    K  S  R  I  S  E  E  D  D  R  S  E  L  D  D  N  N  T  M  E   320
961   ACCGAGAACTCTAACTCAAGAGATACCGGTCTGTCTCAGCCTCGGAGAGATTCTAAGGGT  1020
321    T  E  E  V  K  S  D  S  T  E  N  S  N  S  R  D  T  G  L  S   340
1021  GATAGCCAAGAGGACTCTAAAGAGAACCTGTCTCAAGAGGAATCCCAGAACGTTGACGGC  1080
341    Q  P  R  R  D  S  K  G  D  S  Q  E  D  S  K  E  N  L  S  Q   360
1081  CCATCTTCTGAGTCATCACAAGAGGCTAACCTGTCCAGCCAAGAGAACAGCTCTGAGTCC  1140
361    E  E  S  Q  N  V  D  G  P  S  S  E  S  S  Q  E  A  N  L  S   380
1141  CAAGAGGAAGTGGTTTCTGAGAGCAGGGGTGATAATCCTGATCCGACTACCAGCTACGTT  1200
381    S  Q  E  N  S  S  E  S  Q  E  E  V  V  S  E  S  R  G  D  N   400
1201  GAGGACCAAGAGGATAGCGACAGCTCCGAAGAGGATTCTTCTCACACCCTGAGCCACAGC  1260
401    P  D  P  T  T  S  Y  V  E  D  Q  E  D  S  D  S  S  E  E  D   420
1261  AAGTCTGAGTCAAGAGAAGAACAGGCCGACAGCGAGTCTAGCGAGTCACTTAATTTCTCC  1320
421    S  S  H  T  L  S  H  S  K  S  E  S  R  E  E  Q  A  D  S  E   440
1321  GAGGAAAGCCCAGAGAGCCCTGAGGACGAAAACTCTTCAAGTCAAGAGGGCCTCCAGAGC  1380
441    S  S  E  S  L  N  F  S  E  E  S  P  E  S  P  E  D  E  N  S   460
1381  CACTCTTCATCTGCTGAATCTCAGTCCGAAGAGTCCCACAGTGAAGAGGATGACAGCGAT  1440
461    S  S  Q  E  G  L  Q  S  H  S  S  S  A  E  S  Q  S  E  E  S   480
1441  AGCCAGGACAGCTCTAGGTCTAAAGAGGACAGCAACTCTACCGAGAGCAAGTCCTCAAGC  1500
481    H  S  E  E  D  D  S  D  S  Q  D  S  S  R  S  K  E  D  S  N   500
1501  GAAGAAGATGGTCAGCTGAAGAACATCGAGATCGAGTCCAGAAAGCTGACCGTGGATGCT  1560
501    S  T  E  S  K  S  S  S  E  E  D  G  Q  L  K  N  I  E  I  E   520
1561  TACCACAACAAGCCTATCGGCGATCAGGATGATAACGACTGCCAGGACGGTTACCATCAT  1620
521    S  R  K  L  T  V  D  A  Y  H  N  K  P  I  G  D  Q  Y  H  H   540
1621  CACCACCATCACCATCATTAAGAGCTC                                    1647
541    H  H  H  H  H  H  -                                            549
```

**Figure 4** Nucleotide acid and deduced amino acid sequence of hDMP1

**2. *Escherichia coli*-produced hDMP1**

**2.1 Cloning pET22b-hDMP1**

Cloning is the process of generating a genetically identical copy of a cell or an organism (Howe et al., 2007). After the codon optimization of hDMP1 gene, it was amplified by PCR (Figure 5). Then, hDMP1 gene was ligated into the pGEM-T easy vector and nucleotide sequence was confirmed by DNA sequencing. The correct plasmid was cloned into *E. coli* strain DH10B (Figure 6). *E. coli* DH10B is the most useful host for cloning. It has been engineered for the construction of large DNA library. *E. coli* DH10B has high efficiency for transforming and maintaining the large plasmid.

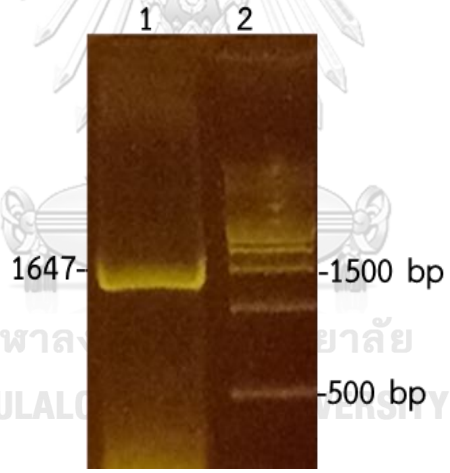


**Figure 5** PCR amplification of hDMP1

The PCR product was separated on 1% agarose gel. Lane 1: marker, lane 2: PCR product of hDMP1.

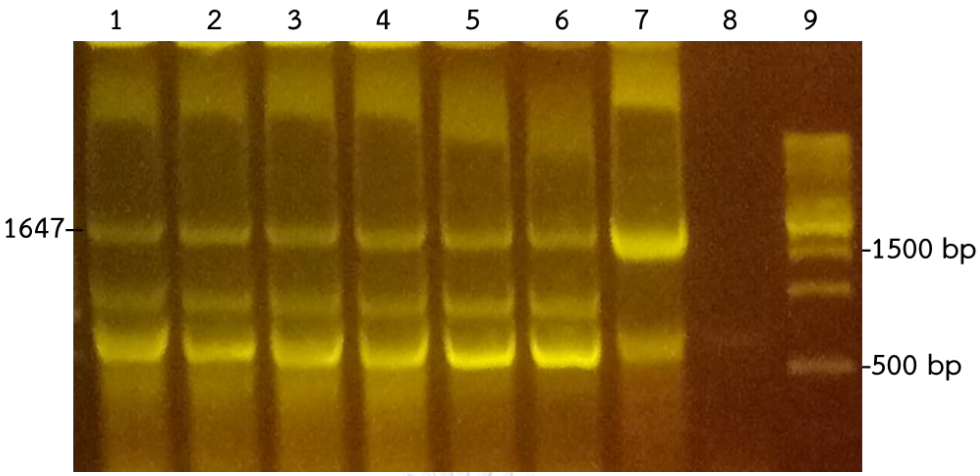


**Figure 6** Agarose gel electrophoresis of colony PCR pGEMT-hDMP1
The pGEMT-hDMP1 was transformed into *E. coli* DH10B. The PCR product was separated on 1% agarose gel. lane 1-6: colonies, lane 7: positive control, lane 8: negative control, lane 9: marker.

Furthermore, the correct sequence of the pGEMT-hDMP1 plasmid was cut by using restriction enzyme *EcoR*I and *Sac*I (Figure 7) and ligated to an expression vector. In this experiment, we used the pET22b vector which is a suitable expression vector in prokaryotic systems (Shafee et al., 2017). The hDMP1 gene was ligated to the pET22b vector (Figure 8). The pET vector is extremely used for producing recombinant protein in *E. coli*. The plasmid has many common restriction sites, not only in front of the T7 promoter but also in other places. Also, the pET vector contains T7 promoter, which is a well-known strong promoter that can produce large amounts of protein (Pan et al., 2018). As a consequence, the T7 promoter can completely induce the cell resources for expression of the target gene (Sadeghi et al., 2007). In addition, pET vector encodes a signal sequence (*pelB*) for inducible expression of proteins in the periplasm. Furthermore, pET22b-hDMP1 was cloned into the *E. coli* DH10B (Figures 9). Continuously, the plasmids pET22b-hDMP1 was transformed into the *E. coli* strain Rosetta for expression (Figure 10).

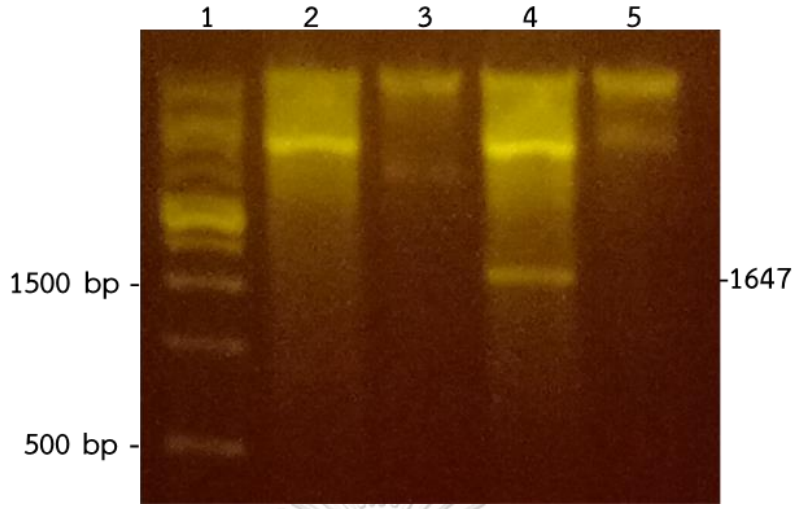


**Figure 7** Agarose gel electrophoresis of restriction pGEMT- hDMP1

The product was separated on 1% agarose gel. Lane 1: marker, lane 2: *EcoR*I and *Sac*I digested plasmid1-pGEMT- hDMP1, lane 3: uncut plasmid 1-pGEMT- hDMP1, lane 4: *EcoR*I and *Sac*I digested plasmid 2-pGEMT- hDMP1, lane 5: uncut plasmid 2-pGEMT-hDMP1.


**Figure 8** Vector map of the pET22b-hDMP1

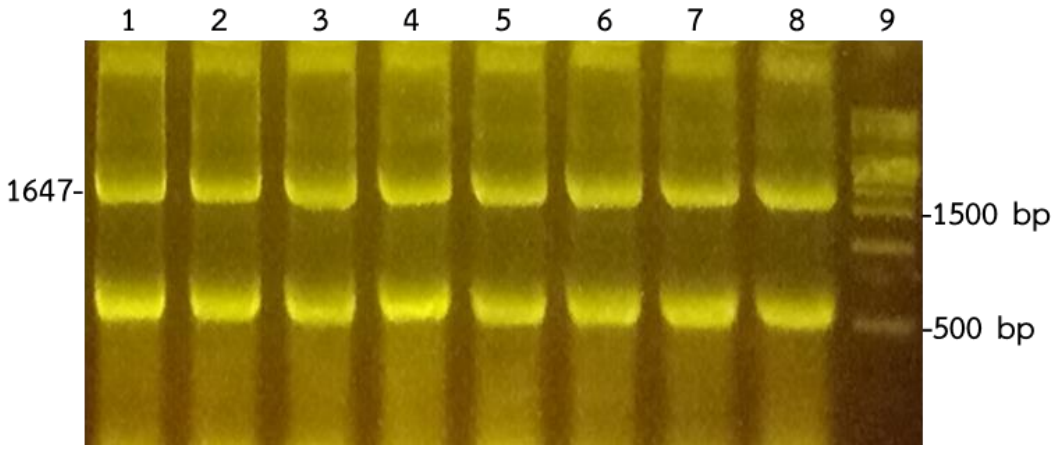


**Figure 9** Agarose gel electrophoresis of colony PCR pET22b-hDMP1.
The pET22b-hDMP1 was transformed into *E. coli* DH10B. The PCR product was separated on 1% agarose gel. Lane 1-7: colonies, lane 8: positive control, lane 9: marker.

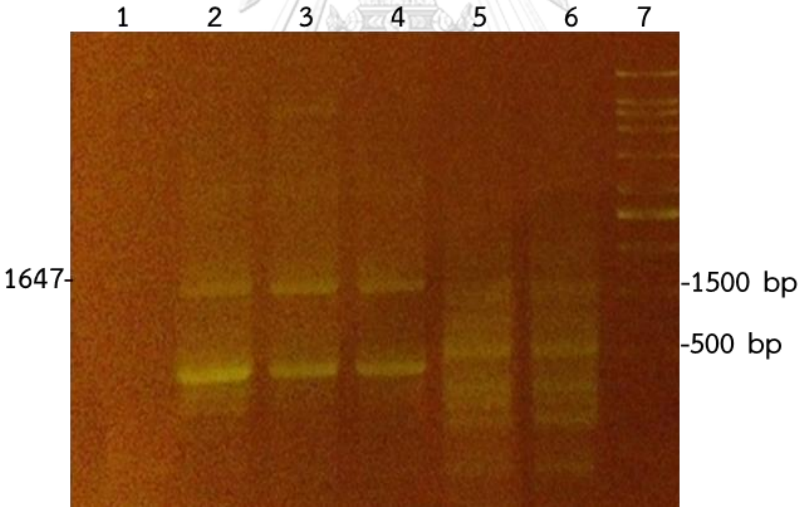


**Figure 10** Agarose gel electrophoresis of colony PCR pET22b-hDMP1
The pET22b-hDMP1was transformed into from *E. coli* strain Rosetta. The PCR product was separated on 1% agarose gel. Lane 1: negative control, lane 2: positive control, lane 3-6: colonies, lane 7: marker.

**2.2 Recombinant hDMP1 expression**

A few strains of *E. coli* have been engineered as an expression system for recombinant protein. Prior studies showed that *E. coli* strain Rosetta is a suitable host for prokaryotic gene expression (Novy, 2001) and is most widely used to produce recombinant proteins at present. *E. coli* strain Rosetta contains T7 RNA polymerase, which is driven by the expression of the gene encoding the target protein and transcribes eight times faster than *E. coli* RNA polymerase. T7 RNA polymerase specifically recognizes the T7 promoter, which drives the expression of the target gene from a plasmid (Baumgarten et al., 2018; Rosano et al., 2014). Therefore, we used *E. coli* strain Rosetta to produce hDMP1.

In this study, the protein expression in *E. coli* was detected by using western blotting under reducing condition and probed with HRP-conjugated anti-histidine (Figure 11) and anti-human DMP1 and HRP-conjugated goat anti-rabbit IgG antibodies (Figure 12). The expression was compared with non-induced and negative control of *E. coli* Rosetta (without gene insertion). The result showed two bands of recombinant hDMP1 with a molecular size of approximately 60 kDa and 100 kDa, respectively. Moreover, non-induced *E. coli* indicated the low expression of hDMP1 protein. The predicted size of hDMP1 protein was at 60 kDa. In previous study, DMP1 in the solution could undergo dimerization to prevent further aggregation or precipitation (He et la, 2005). Accordingly, the recombinant rat DMP1 in *E. coli* has been produced at 61 kDa. Meanwhile, recombinant fusion rDMP1-GST in *E. coli* was detected with a molecular weight of about 90-95 kDa, with GST at 26 kDa (Srinivasan et al., 2009). Thus, both bands might indicate the monomer and dimer forms of hDMP1 protein.

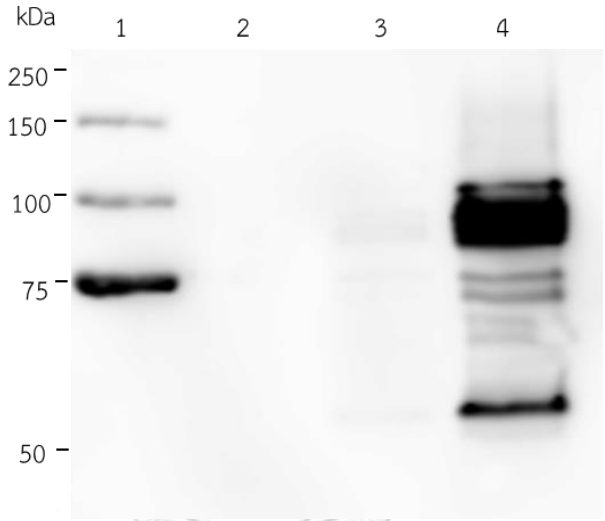


**Figure 11** Western blot analysis of hDMP1 expression in *E. coli*

The crude extracts of hDMP1 protein was treated under reducing condition. The acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: marker, lane 2: *E. coli* without pET22b-hDMP1, lane 3: non-induced, lane 4: induced-IPTG 0.5 mM.

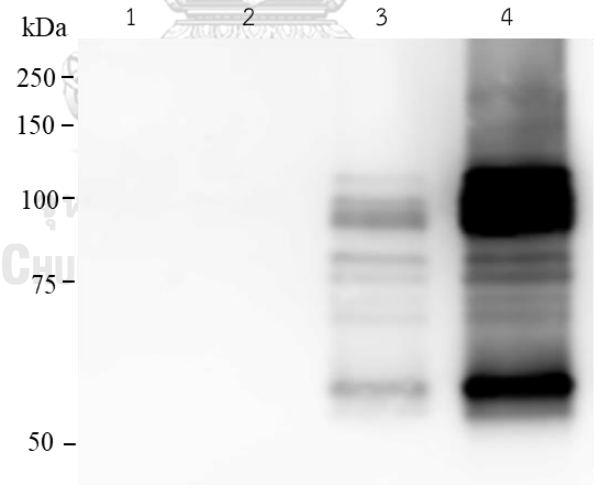


**Figure 12** Western blot analysis of hDMP1 expression in *E. coli*

The crude extracts of hDMP1 protein was treated under reducing condition. The acrylamide gel 10% was used to separate the proteins. Lane 1: marker, lane: *E. coli* without pET22b-hDMP1, lane 3: non-induced, lane 4: induced-IPTG 0.5 mM.

The yield number expression of recombinant hDMP1 proteins was determined by using the hDMP1 Elisa kit. Recombinant hDMP1 from *E. coli* was extracted and performed into the wells. According to the standard curve (Figure 13), protein quantification was calculated (Table 5). The results showed that the yield expression of recombinant hDMP1 in *E. coli* is 1.38 µg/ml.

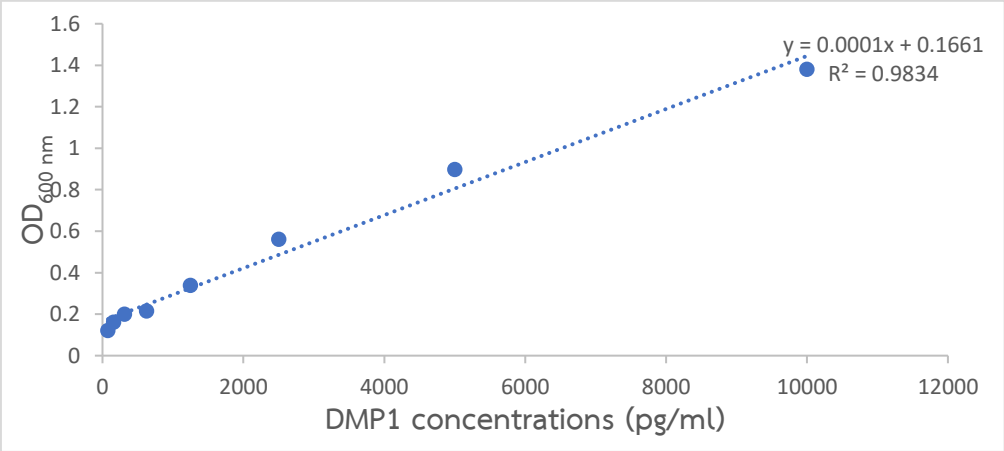


**Figure 13** Standard curve of DMP1 protein quantification

*Table 4* Quantification of yield expression of *E. coli*-produced hDMP1

| Sample | $OD_{600}$ | Media (ml) /Buffer (ml) | Yield expression (µg/ml) |
|---|---|---|---|
| *E. coli*-produced hDMP1 | 0.901<br>0.826<br>0.860<br>0.833 | 20 /20 | 1.38 |

**2.3** Optimization of hDMP1 expression

Each protein has different characteristics, therefore, optimization for the best condition is necessary to produce a high-level expression. In this study the concentration of IPTG, incubation time and temperatures were optimized. IPTG regulates the rate of expression, solubility, and activity of the proteins. It stimulates the induction of lac repressor promoters that could significantly enhance the protein expression. In contrast, the expression of the protein is lower in the absence of IPTG as an inducer. IPTG has a limited concentration to increase the recombinant protein expression (Malik et al., 2016; Zhang et al., 2017). In this experiment, we used various concentrations of IPTG (0.2, 0.5, and 1 mM) and the highest expression of the DMP1 was obtained at the final concentration 0.5 mM IPTG (Figure 14). This finding is consistent with the other studies that had shown the lower concentration of IPTG to induce the recombinant proteins.

After adding the IPTG, the expression of the DMP1 was incubated at different temperatures on 28 and 37°C for 2, 4 and 6 hr incubation time. The temperature is an important parameter that influences the expression level of recombinant proteins (Ran et al., 2015; Sadeghi et al., 2011). As shown in figure 14, western blot analysis probed with HRP-conjugated anti-histidine antibody confirmed highest expression of the DMP1 protein at the optimal temperature of 37°C. Incubation time has a direct correlation not only with bacterial growth but also with protein expression (Sadeghi et al., 2011; Zhang et al., 2017). A short cultivation time results to insufficient growth of bacteria and cell density. In contrast, long induction time influences the accumulation of protein as inclusion bodies (Ran et al., 2015; Zhang et al., 2017). In the present study, the direct relationship of hDMP1 expression and incubation time was observed which yielded to increased hDMP1 expression at 6 hr incubation time. Overall, the result revealed that the optimal condition to produce the recombinant hDMP1 in *E. coli* was with 0.5 mM IPTG induction and incubation at 37°C for 6 hr (Figure 14).

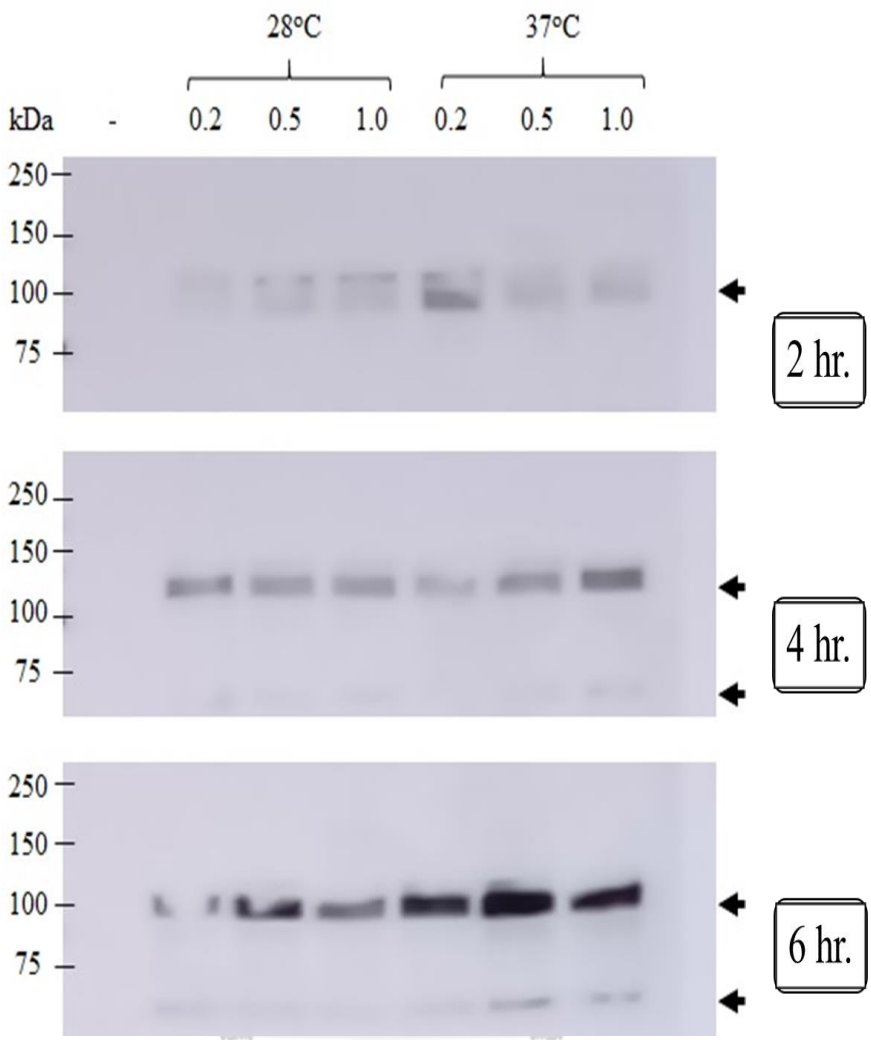


**Figure 14** Western blot analysis of optimization the hDMP1 expression in *E. coli*

The crude extracts of hDMP1 protein was treated under reducing condition. The acrylamide gel 10% was used to separate the proteins. The parameters are culturing temperature (28 and 37°C), incubation time (2, 4 and 6 h), and concentrations of the IPTG (0.2 mM, 0.5 mM and 1 mM) with negative control (*E. coli* without pET22b-hDMP1).

### 2.4 Purification of *E. coli*-produced hDMP1

The sequence of hDMP1 is tagged with poly-histidine that was purified by immobilized-metal affinity chromatography (IMAC) method. *E. coli*-produced hDMP1 was required to break cells before loading into the column. The cells were broken

by using sonicator to disrupt cellular membranes and release the contents of the cells. Subsequently, the cells were loaded into the column and washed with various concentrations of washing buffer (consist of imidazole 20-50 mM) for optimization. The washing buffer removes non-specific and weakly-bound proteins. Consequently, the his-tagged DMP1 protein is eluted and recovered with eluting buffer (consist of imidazole 250 mM). Based from the result, purification with Ni-NTA column was successful. Western blot analysis was performed by using HRP-conjugated anti-histidine antibody (Figure 15). SDS-PAGE gel was stained with Coomassie brilliant blue solution (Figure 16). Despite early elution of some hDMP1 protein in flow through, most was recovered and purified at 80% maximum yield.

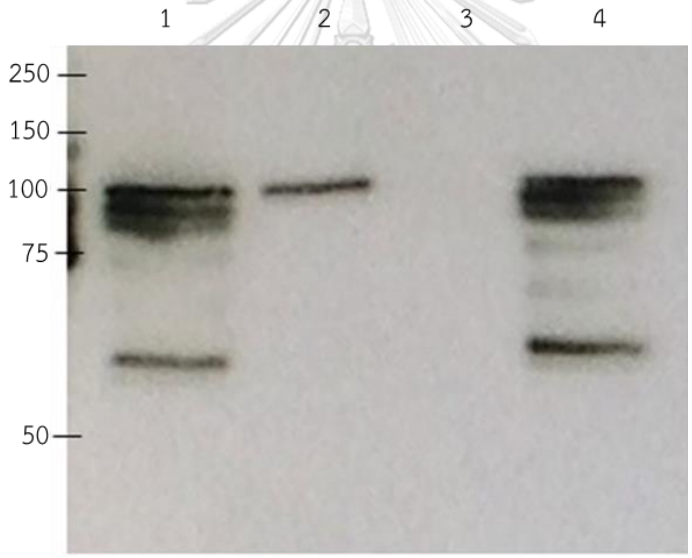


**Figure 15** Western blot analysis of purification *E. coli*-produced hDMP1

The crude extracts of hDMP1 protein was treated under reducing condition. The acrylamide gel 10% was used to separate the proteins. The western blot was probed with anti-histidine antibody. Lane 1: crude extract, lane: flow through, lane 3: wash, lane 4: elute.

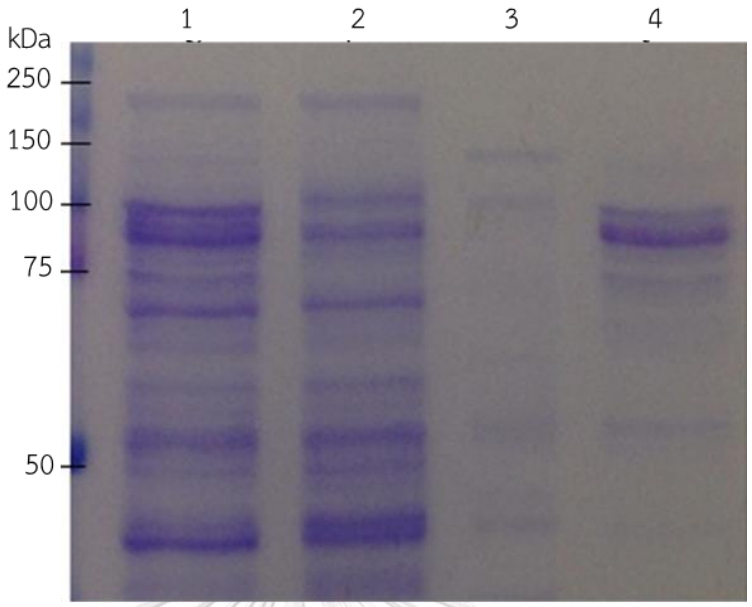


**Figure 16** SDS-PAGE analysis of purification *E. coli*-produced hDMP1

The crude extracts of hDMP1 protein was treated under reducing condition. The acrylamide gel 10% was used to separate the proteins. The SDS page was stained with Coomassie blue dye. Lane 1: crude extract, lane: flow through, lane 3: wash, lane 4: elute.

### 2.5 Proliferation assay of *E. coli*-produced hDMP1

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium reagent that will be reduced to a formazan product by a mitochondrial dehydrogenase. The number of formazan product represents the metabolic activity of viable cells at a particular time point. In this research, the effect of *E. coli*-produced hDMP1 on hPDLSCs viability was investigated. The *E. coli*-produced hDMP1 with concentrations 1 µg/ml and 2 µg/ml and the negative control (*E. coli* without gene insertion) were treated to hPDLSCs. The cells number were determined by the MTT assay after 24 and 72 hr treatment. The results showed that all proteins significantly stimulated the proliferation of hPDLSCs. However, this study also showed that there was no significant difference between the viability of hPDLSCs treated with 1 µg/ml and 2 µg/ml. In accordance with the previous studies, DMP1 could induce

proliferation in the different cell types (Hase et al., 2015; Maglic et al., 2015; Ye et al., 2005). In addition, the RGD domain of DMP1 was postulated to enhance the proliferation of odontoblast like cells (Hase et al, 2005). These data confirmed that *E. coli*-produced hDMP1 was not toxic to hPDLSCs (Figure 17).

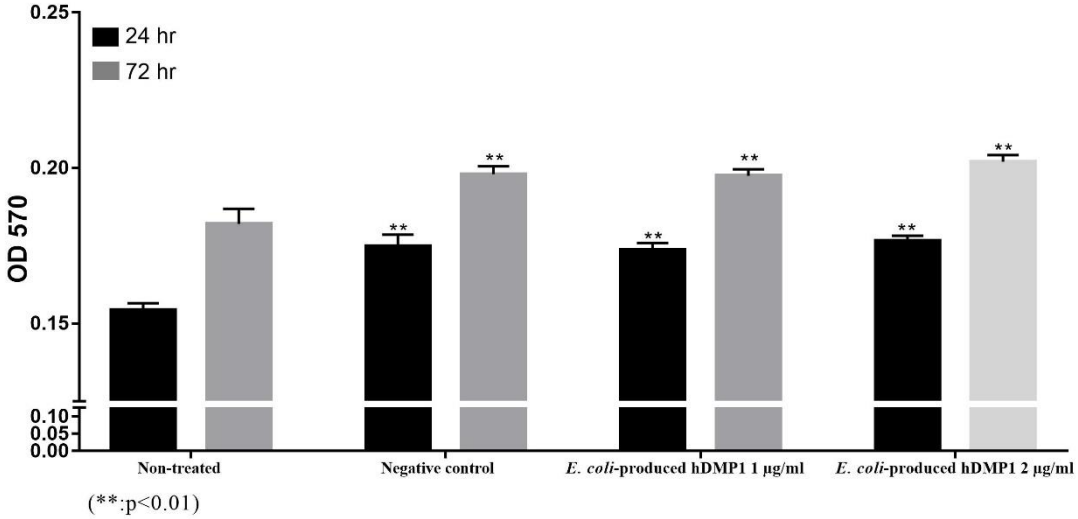


**Figure 17** Proliferation assay of *E. coli*-produced hDMP1 in hPDLSCs.
Three lines of hPDLSCs were collected from three different donors. The hPDLSCs were cultured on plates coated with *E. coli*-produced hDMP1 (1 µg/ml and 2 µg/ml), negative control (*E. coli* without gene insertion), and a non-coated plate for 24 and 72 h before the MTT assay was performed. Data represent the absorbance at 570 nm (indicates a significant difference compared to the cells growing on the non-treated, **: $p < 0.01$).

### 2.6 Osteogenic differentiation of *E. coli*-produced hDMP1

DMP1 was reported that it is expressed in osteoblast and odontoblasts cells (Eapen et al., 2010; Tabatabaei et al., 2013; Feng et al., 2006). A few studies reported that DMP1 played an important regulation for osteogenic differentiation in dentin and bone cells (Rezai et al., 2015). Therefore, the effect of recombinant hDMP1 on the genes-related of osteogenic differentiation in hPDLSCs was tested. The periodontal ligament stem cells (PDLSCs) were appropriate to promote in cementoblast-like cells, adipocytes, or collagen fibres. The PDLSCs periodontal tissues could regenerate reparative dentin through natural processes (Brent et al., 2003). The alkaline phosphatase (ALP), osteopontin (OPN), osterix (OSX), type I collagen (COL1), core binding factor alpha 1 (CBFA), bone morphogenetic protein-2 (BMP2), and Wnt3a osteogenic marker genes were performed to calculate the expression by RT-qPCR.

In this study, the cells were treated with 0.5 μg/m, 1 μg/ml and 2 μg/ml of *E. coli*-produced hDMP1. The mRNA was collected and investigated for expression of ALP, BMP2, CBFA1, COL1, OPN, OSX, WNT3a genes by qRT-PCR. The results showed that 2 μg/ml *E. coli*-produced hDMP1 coated surface could up-regulate the expression of ALP, BMP2, CBFA1, COL1, OPN, OSX, and WNT3a genes. These osteogenic-related genes significantly increased in hPDLSCs. DMP1 was previously expressed in *E. coli* and stimulated the Runx2 gene in mouse calvaria preosteoblasts MC3T3-E1 cells (Eapen et al., 2010 & Eapen et al., 2011). This data suggested that *E. coli*-produced hDMP1 could strongly induce osteogenic differentiation in hPDLSCs (Figure 18).

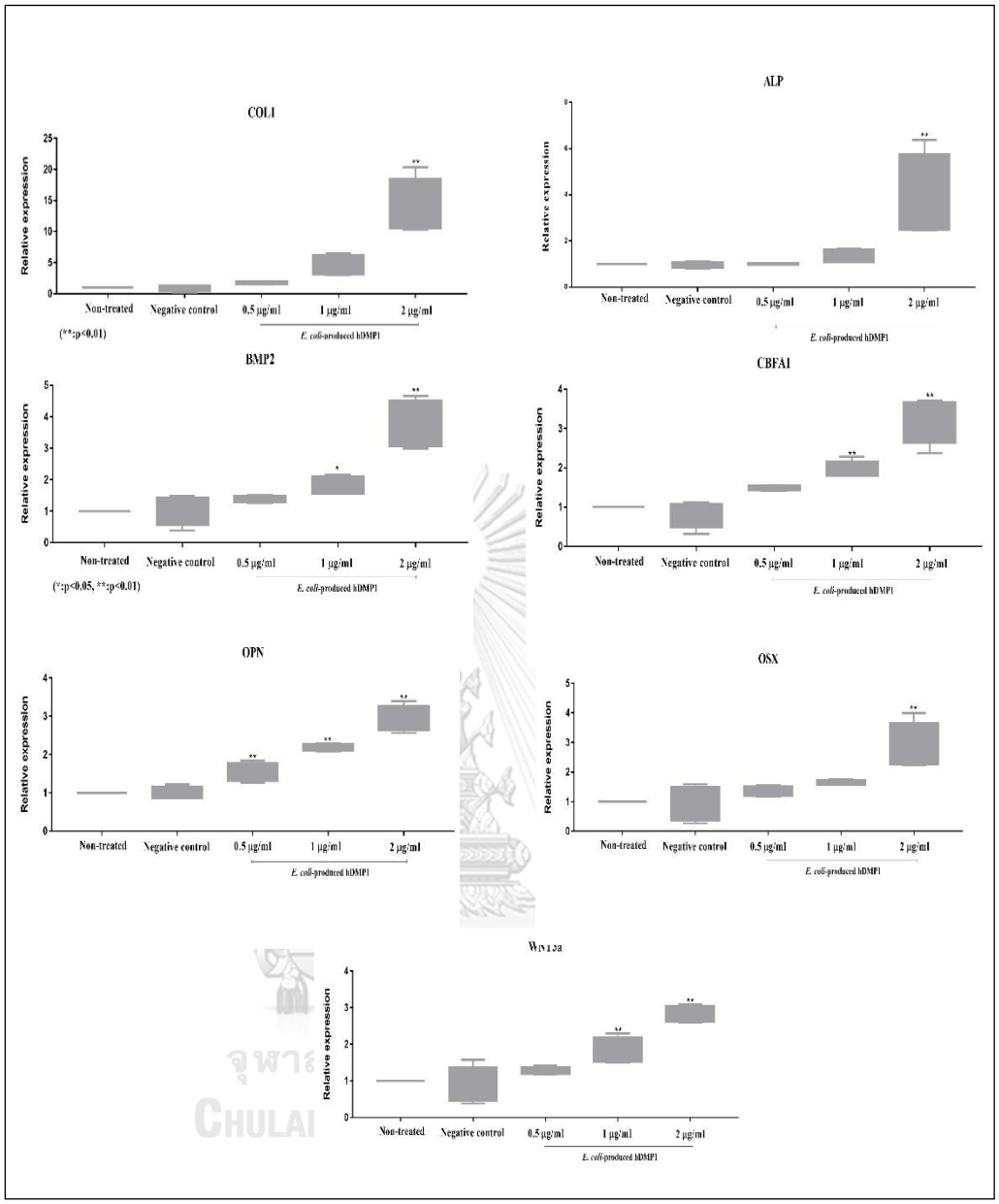


**Figure 18** *E. coli*-produced hDMP1 induced osteogenic differentiation

Four lines of the hPDLSCs were collected from three different donors. The hPDLSCs were cultured on plates coated with *E. coli*-produced hDMP1 (0,5; 1 and 2 µg/ml), negative control (*E. coli* without gene insertion) and non-coated plates for 3 days. The mRNA was extracted, and RT-PCR was performed to analyze the expression of ALP, BMP2, CBFA1, COL1, OSX, WNT3a, and OPN (* indicates a significant difference compared to the cells growing on a non-treated, *: $p < 0.05$, **: $p < 0.01$).

### 2.7 Calcification assay of *E. coli*-produced hDMP1

Supporting the effect of osteogenic differentiation, *E.* coli-produced hDMP1 was tested for calcium deposition in hPDLSCs. To evaluate the impact on the osteogenic differentiation, the hPDLSCs were cultured in general medium (GM) and osteogenic medium (OM). The osteogenic media consists of ascorbic acid and dexamethasone. Dexamethasone elevates the phenotype markers on osteoblast cells; therefore, it could stimulate the osteogenic differentiation *in vitro*. Ascorbic acid is an enzyme cofactor for hydroxylating proline and lysine in pro-collagen (Langenbach et al., 2013).

The hPDLSCs were grown in the general medium and osteogenic medium for 14 days. The hPDLSCs were stained with Alizarin Red S as shown on Figure 19 (A-F). The hPDLSCs cells cultured in general media (GM) were not red, but the cells were light red when they were cultured in osteogenic medium. When the cells treated with proteins purified from wild type *E. coli* (negative control), 0.5 µg/ml, 1 µg/ml and 2 µg/ml of *E. coli*-produced hDMP1 growing in osteogenic medium, the cells also showed light red similar to the nontreated cells cultured in osteogenic medium. However, the highest intensity of the red colour appeared in the *E. coli*-produced hDMP1 at 2 µg/ml that indicated the calcium deposition. Previous study reported that PDLSCs have capability to induce the calcium deposition in mesenchymal stem cells (Seo et al., 2004). This result described that the recombinant hDMP1 in *E. coli* could increase calcium deposition in hPDLSCs.

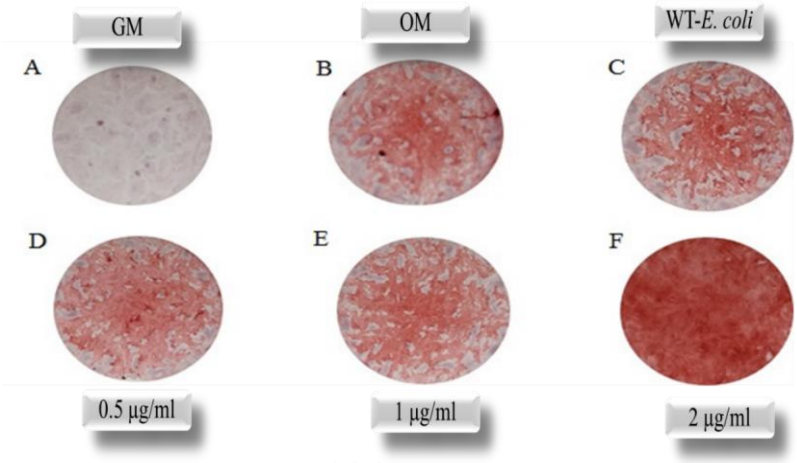


**Figure 19** Calcification assay of *E. coli*-produced hDMP1

The control hPDLSCs growing in general medium (GM) (A), hPDLSCs growing in osteogenic medium (OM) (B, hPDLSCs treated with the proteins purified from negative control (*E. coli* without gene insertion) in osteogenic medium (C), hPDLSCs treated with 0.5 µg/ml, 1 µg/ml and 2 µg/ml of *E. coli-produced* hDMP1 (D-F), respectively in osteogenic medium for 14 days. The wells were stained with Alizarin Red dye.

In this study, hDMP1 protein has been productively expressed in *E. coli* in both monomeric and dimeric forms at 60 kDa and 120 kDa. The effect of *E. coli*-produced hDMP1 osteogenic differentiation in hPDLSCs was investigated. The result revealed that 2 µg/ml of *E. coli*-produced hDMP1 could induce the expression of ALP, BMP2, CBFA1, COL1, OPN, OSX, and WNT3a genes and increase calcium deposition in hPDLSCs. However, *E. coli*-produced hDMP1 did not undergo post-translational modifications which can increase the biological activity of the protein. Hence, to improve the activity of recombinant hDMP1, expression of protein in eukaryotic cells was examined. In the study, further investigation is needed to produce hDMP1 in plant system (*N. benthamiana*) and to confirm the effect of plant-produced hDMP1 on the osteogenic differentiation and calcium deposition in hPDLSCs.

### 3. *Nicotiana benthamiana*-produced hDMP1

#### 3.1 Cloning pBYR2e-hDMP1

After cloning pGEMT-hDMP1 into the *E. coli* DH10B, the plasmid was cut and ligated by *Xba*I and *Sac*I (Figure 20) restriction enzymes into pBYR2e vector. The T-DNA region of the hDMP1 gene (Figure 21) was encoded into the geminiviral vector pBYR2e (bean yellow dwarf virus) vector for expression in the *N. benthamiana* (Huang et al, 2009)

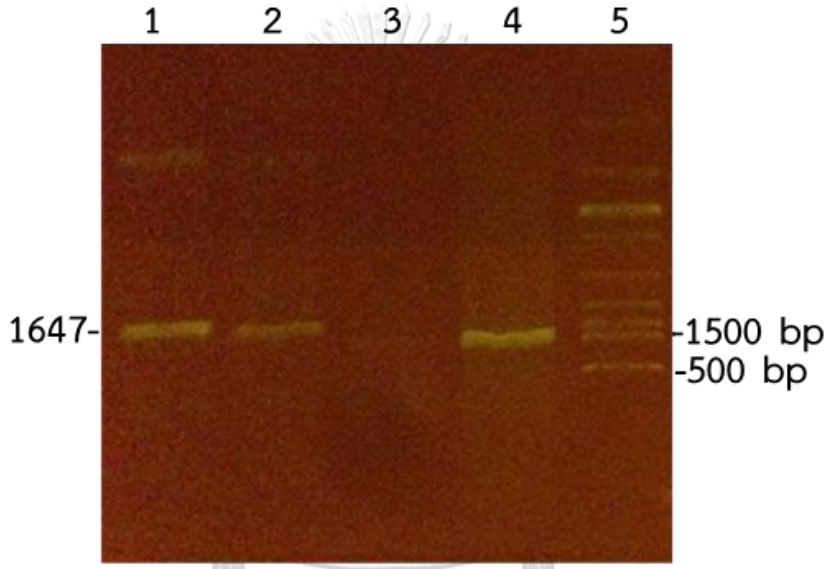


**Figure 20** Agarose gel electrophoresis of restriction pGEMT-hDMP1

The product was separated on 1% agarose gel. Lane 1- 4: *Xba*I-*Sac*I digested plasmids pGEMT-hDMP1, lane 5: marker.



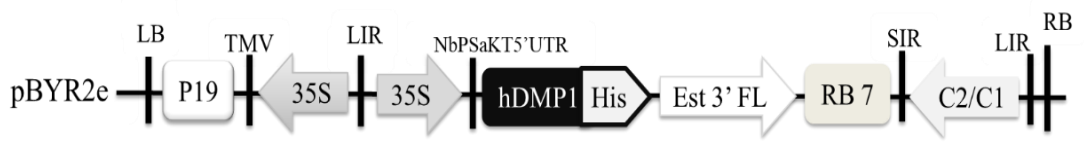


**Figure 21** Schematic representation of the T-DNA in the geminiviral vector

Geminivirus has a replication machinery of the single-strand DNA (ssDNA) virus that can elevate the copy number and expression levels. By rolling circle mechanism, double-stranded DNA facilitates the template for transcription on open reading frames (Mor et al., 2003). The T-DNA is present between the left border (LB) and right border (RB). The T-DNA of pBYR2e contains the bidirectional cauliflower mosaic virus 35S promoter (CaMV 35S) (Regnard et al., 2010), expressed sequence tags-full length (Est 3' FL), tobacco RB7 promoter (Rb 7), replication initiation protein (C2/C1), long intergenic region (LIR), short intergenic region (SIR), P19-Tomato Bushy Stunt Virus (TBSV). Then, it was cloned into the *E. coli* DH10B (Figure 22).

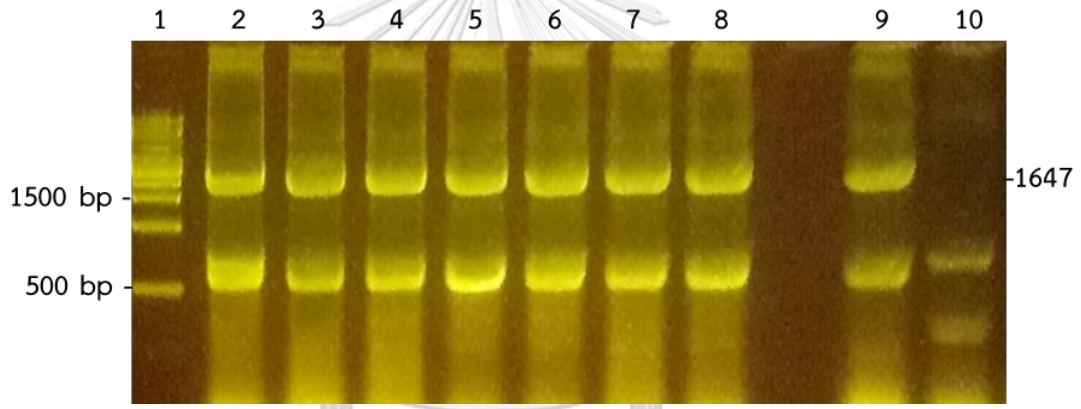




**Figure 22** Agarose gel electrophoresis of colony PCR pBYR2e-hDMP1
The pBYR2e-hDMP1 was transformed into *E. coli* DH10B. The PCR product was separated on 1% agarose gel. Lane 1: marker, lane 2-8: colonies, lane 9: positive control, lane 10: negative control.

*Agrobacterium tumefaciens* is a soil phytopathogen that causes crown gall disease in plants. It is commonly used for the delivery of foreign genes into plant cells and transformed by bacteria plasmid. However, the T-DNA is the small part localized in the Tumour-inducing (Ti) plasmid and inserted into the nuclear genome of the plants. This region generates and mediates T-DNA transport. In addition, *A. tumefaciens* naturally contains the Ti plasmid that becomes a binary vector which

primarily comprises multiple unique restriction endonuclease sites (Gelvin, 2003; Kim et al., 2007). Thus, the hDMP1-pBRY2e was transformed into the *Agrobacterium tumefaciens* strain GV3101 by using the electroporation method (Figure 23).

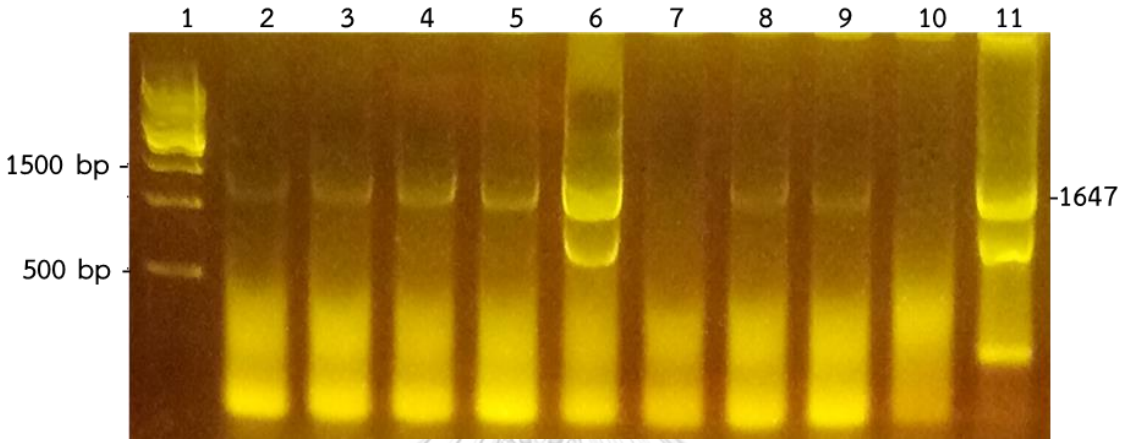


**Figure 23** Agarose gel electrophoresis of colony PCR pBYR2e-hDMP1
The pBYR2e-hDMP1 was transformed into *A. tumefaciens* GV3101 by electroporation method. The PCR product was separated on 1% agarose gel. Lane 1: marker, lane 2-9: colonies, lane 10: negative control, lane 11: positive control.

### 3.2 Recombinant hDMP1 expression

Tobacco has several benefits and the most useful for *Agrobacterium* infiltration. Tobacco has many species that have been used for transient expression. However, *N. benthamiana* has shown the highest-level of protein expression by using 35S promoter among other species of *Nicotiana* (Sheludko et al., 2007). Therefore, we used *N. benthamiana* to express the recombinant hDMP1.

The hDMP1 protein in *N. benthamiana* was treated under reducing and non-reducing condition and was then compared with non-infiltrated *N. benthamiana* extract. The expression of hDMP1 was confirmed by using Western blot by using rabbit anti-human DMP1 (Figure 24) and anti-histidine conjugated-HRP (Figure 25) antibodies. The band of recombinant hDMP1 in *N. benthamiana* was detected at

approximately 100 kDa. The results showed that band patterns were not different between reducing and non-reducing conditions. However, the protein size is higher than the expected size 60 kDa that indicates the presence of post-translational modification. This data were supported by previous studies in which modification of rat DMP1 could increase the size of this molecule to 160–210 kDa, as indicated by immunoblotting analysis with anti-DMP1 antibody (Butler et al., 2009). In addition, mouse DMP1 was investigated via Western blot using an anti-mouse DMP1 antibody and showed a size of 130–150 kDa (Terasawa et al., 2004). Subsequently, glycosylation of mouse DMP1 has been shown the size of 89-180 kDa (Sun et al., 2015). The different molecular weights of DMP1 is affected by post-translational modification, which may vary the molecular weight of the desired protein (Terasawa et al., 2004). DMP1 is one of the SIBLING members which is highly phosphorylated. In addition, DMP1 contains many sites for glycosylation (George et al., 1993; Qin et al., 2004; Qin et al., 2003; Tartaix et al., 2004).

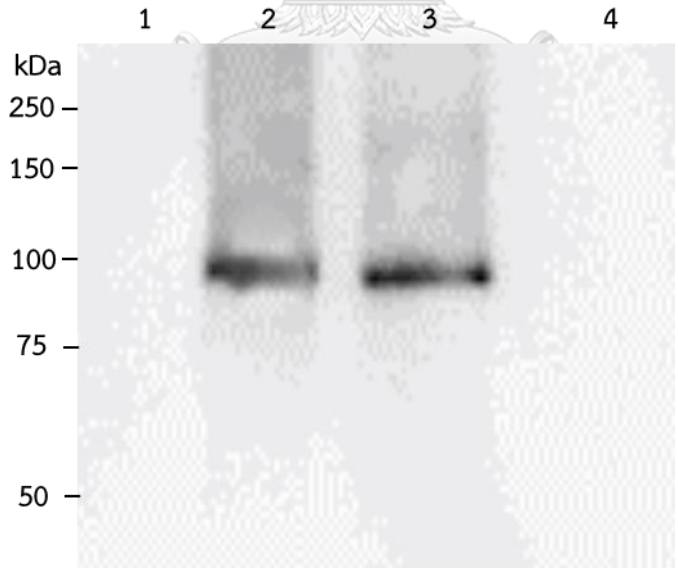


**Figure 24** Western blot analysis of hDMP1 expression in *N. benthamiana*
The Acrylamide gel 10% was used to separate the proteins. The western blot was developed with rabbit anti-DMP1. Lane 1: marker, lane 2: non-reducing condition 3: reducing condition, lane 4: non-infiltrated plant.

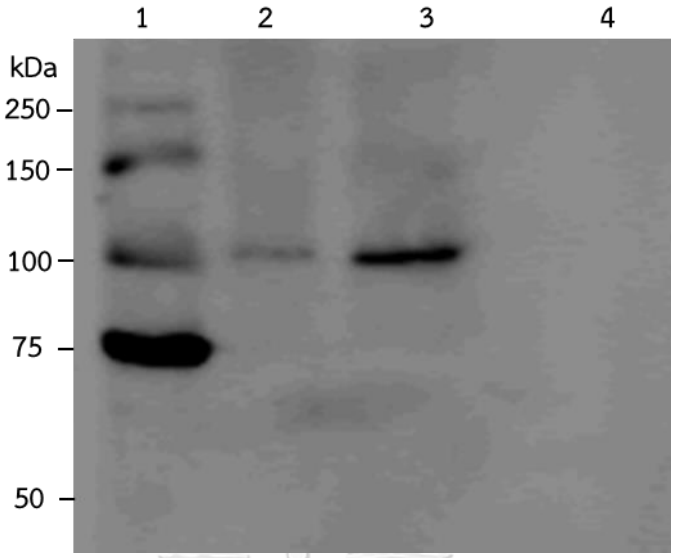


**Figure 25** Western blot analysis of hDMP1 expression in *N. benthamiana*
The Acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: marker, lane 2: non-reducing condition 3: reducing condition, lane 4: non-infiltrated plant.

The concentration of plant-produced hDMP1 protein was quantified by using the hDMP1 Elisa kit. *N. benthamiana*-produced hDMP1 was measured according to the standard curve (Figure 26). The expression level of recombinant hDMP1 was calculated (Table 6). The results showed that the expression yield of the recombinant hDMP1 in *N. benthamiana* was 0.3 µg/g leaves fresh weight.

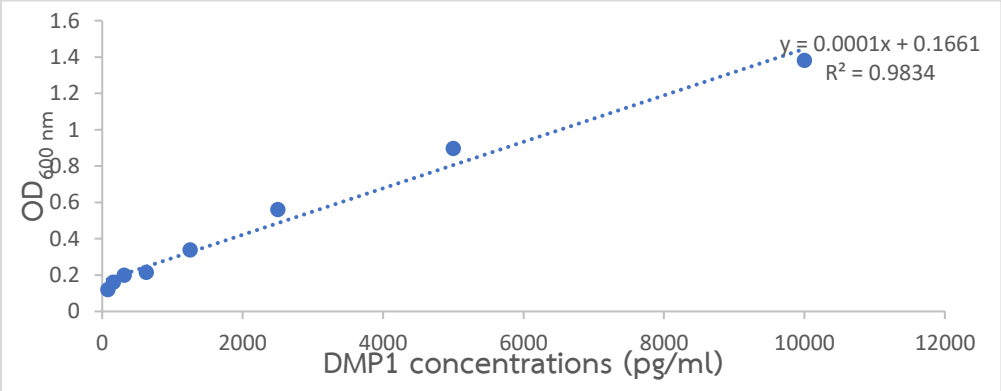


**Figure 26** Standard curve of DMP1 protein quantification

**Table 5** Quantification of yield expression of *N. benthamiana*-produced hDMP1

| Sample | $OD_{600\ nm}$ | Leaves (g)/ Buffer (ml) | Yield expression (μg/g) |
|---|---|---|---|
| Plant-produced hDMP1 | 0.317<br>0.323<br>0.318<br>0.317 | 50/100 | 0.3 |

### 3.3 Optimization of hDMP1 expression

Production of recombinant proteins in plants requires consideration of crucial parameters that can influence the protein expression level. Few studies have sought to apply the day post infiltration and cell culture density of the *A. tumefaciens* to increase the yield number of recombinant protein in the plants (Cao et al., 2017; Norkunas et al., 2018).

In this study, we varied the density of *A. tumefaciens* GV3101 ($OD_{600}$) and time to harvest leaf biomass or days post infiltration (dpi). The concentrations of the *A. tumefaciens* ranging from 0.1, 0.2, 0.3, 0.4, 0.5 were used for infiltration. To determine the level of expression, the Western blot was probed with HRP-conjugated anti-histidine antibody. Findings confirmed that the highest expression of hDMP1 was detected in $OD_{600}$ 0.4 (Figure 27). The bands of SDS-PAGE (Figure 28) was stained with Coomassie brilliant blue and digitalized by scanning of the jpg file with ImageJ. The similarity the peaks was indicated that the amount of the total protein in samples were not significantly different.

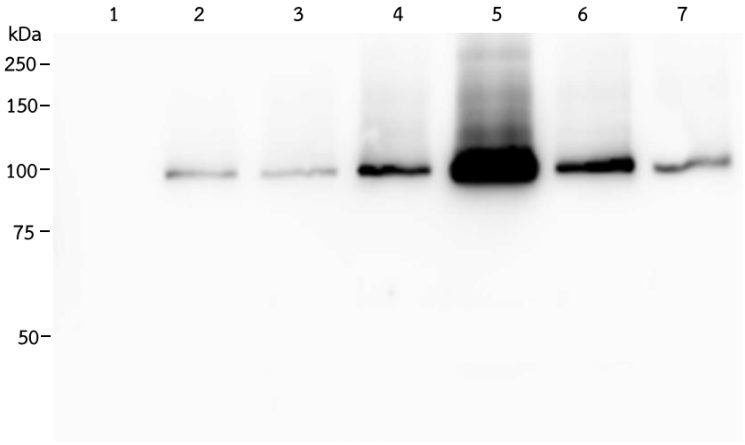


**Figure 27** Western blot analysis of the optimization the $OD_{600}$ of *Agrobacterium*
The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: non-infiltrated plant (WT), lane 2: 0.1, lane 3: 0.2, lane 4: 0.3, lane 5: 0.4, lane 6: 0.5, lane 7: 1.

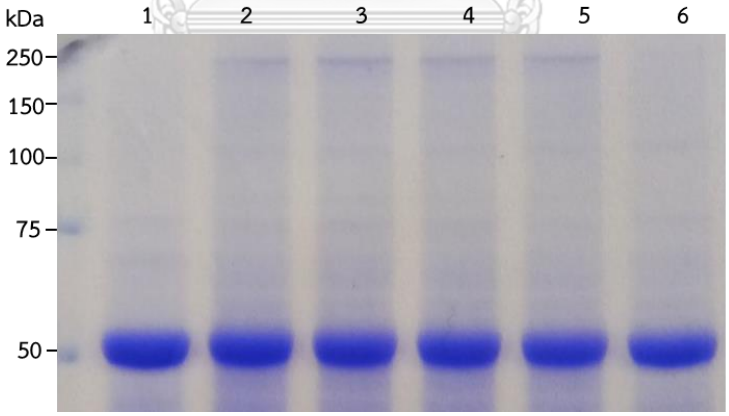


**Figure 28** SDS-PAGE analysis of optimization the $OD6_{00}$ of *A. tumefaciens*
The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The gel was stained with Coomassie blue. Lane 1: non-infiltrated plant (WT), lane 2: 0.1, lane 3: 0.2, lane 4: 0.3, lane 5: 0.4, lane 6: 0.5, lane 7: 0.6.

To optimize the day post infiltration, the *A. tumefaciens* cells $OD_{600}$ 0.4 harbouring hDMP1 was infiltrated into the leaves of *N. benthamiana*. Then, the leaves were collected at day 1-5 post infiltration (dpi). In Figure 29, the symptoms of leaf necrosis rapidly occurred on 2 dpi. This experiment used wild type of *A. tumefaciens* GV3101 (without gene interest) for comparison, which showed no necrosis effect on the leaves. The optimal harvest time or day post infiltration was investigated by Western blot and SDS-PAGE analyses. The SDS-PAGE (Figure 30) gel was stained with Coomassie brilliant blue solution and was visualized by ImageJ to measure the total soluble protein. The data showed no significant difference from the total soluble protein loaded into each sample per well. Then, Western blot analysis was performed with HRP-conjugated anti-histidine antibody. A strong band was observed on 2 dpi. Therefore, we concluded that the highest level of hDMP1 expression was obtained 2 dpi (Figure 31).

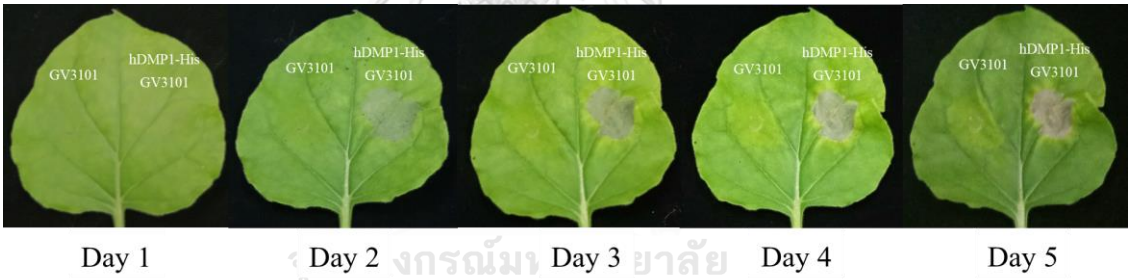


**Figure 29** Necrosis symptoms in *N. benthamiana* leaves

Left side: infiltrated with *A. tumefaciens* without insertion pBYR2e-hDMP1 $OD_{600}$ 0.4. Right side: infiltrated with *A. tumefaciens*-infected pBYR2e-hDMP1 $OD_{600}$ 0.4.

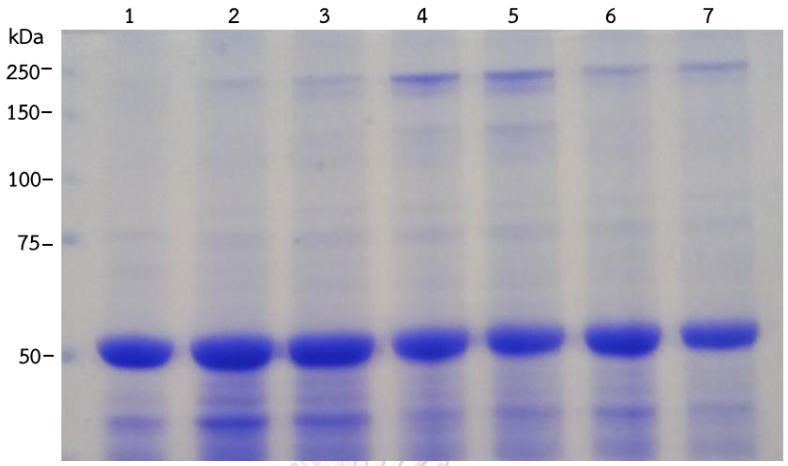


**Figure 30** SDS-PAGE analysis of optimization the day post infiltration (dpi)
The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The gel was stained with Coomassie blue. Lane 1: non-infiltrated plant (WT), lane 2: 1 dpi, lane 3: 2 dpi, lane 4: 3 dpi, lane 5: 4 dpi, lane 6: 5 dpi.

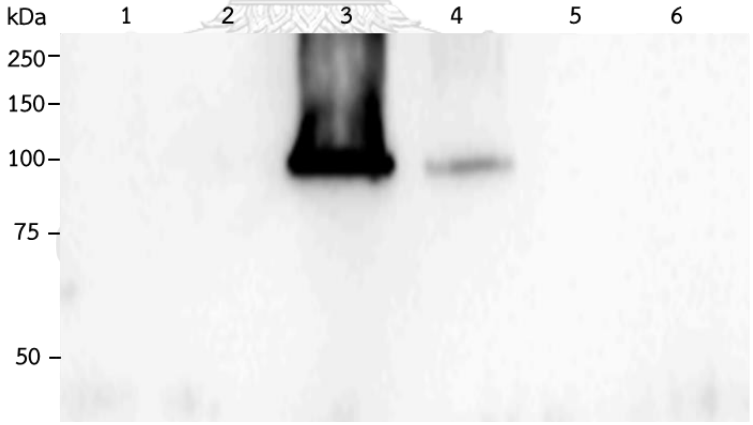


**Figure 31** Western blot analysis of optimization the day post infiltration (dpi)
The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: non-infiltrated plant, lane 2: 1 dpi, lane 3: 2 dpi, lane 4: 3 dpi, lane 5: 4 dpi, lane 6: 5 dpi.

### 3.4 Purification of *N. benthamiana*-produced hDMP1

*Nicotiana benthamiana* leaves naturally produce the Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Therefore, before loading into the Ni-NTA column, removal of the most abundant RuBisCO is necessary. The common method to separate the RuBisCO protein from the plant extract is by ammonium sulphate precipitation. Ammonium sulphate is an inert chemical that will not alter the protein properties.

The RuBisCO was precipitated by ammonium sulphate. The appropriate concentrations of the ammonium sulphate that could precipitate RuBisCO and hDMP1 were optimized and detected by blotting and SDS-PAGE. Western blot analysis was probed with HRP-conjugated anti-histidine antibody (Figure 32). SDS-PAGE gel was stained with Coomassie brilliant blue solution (Figure 33). Accordingly, the result showed that RuBisCO and hDMP1 were precipitated in the concentrations of 35% and 80% ammonium sulphate, respectively (Figure 34 and Figure 35).

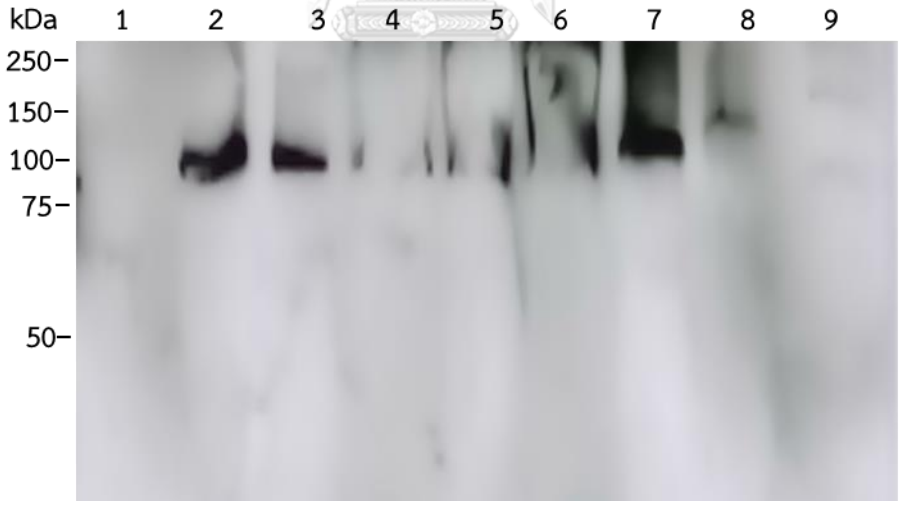


**Figure 32** Western blot analysis of optimization the ammonium sulphate
The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: non-infiltrated plant (WT), lane 2: crude extract, lane 3: 20%, lane 4: 30%, lane 5: 40%, lane 6: 50%, lane 7: 60%, lane: 8: 70%, lane: supernatant.

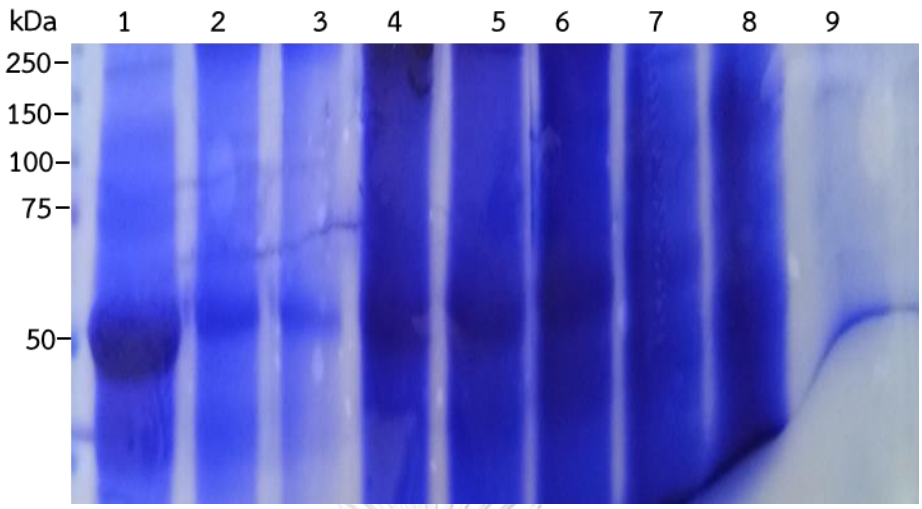


**Figure 33** SDS-PAGE analysis of the optimization the ammonium sulphate

The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The gel was stained with Coomassie blue. Lane 1: non-infiltrated plant (WT), lane 2: crude extract, lane 3: 20%, lane 4: 30%, lane 5: 40%, lane 6: 50%, lane 7: 60%, lane: 8: 70%, lane: supernatant.

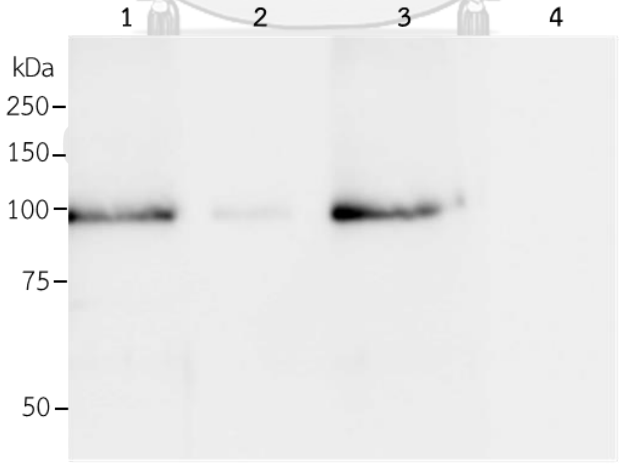


**Figure 34** Western blot analysis of hDMP1-precipitation

The crude extracts of hDMP1 protein was treated under reducing condition. The acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: crude extract, lane 2: 35%, lane 4: 80%, lane 5: supernatant.

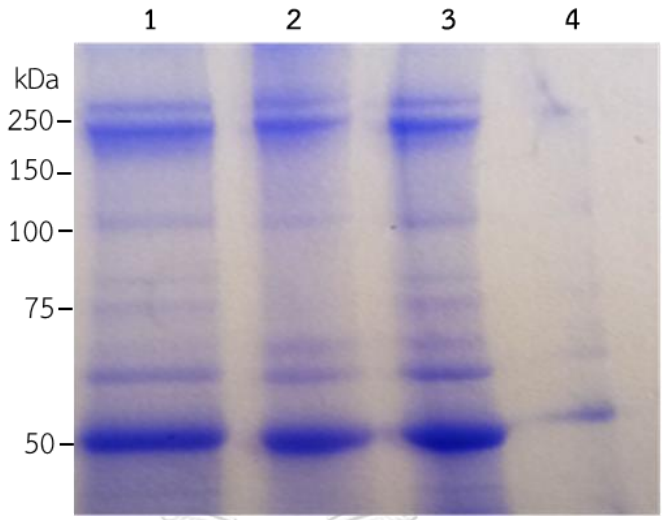


**Figure 35** SDS-PAGE analysis of hDMP1-precipitation

The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The gel was stained with Coomassie blue. Lane 1: crude extract, lane 2: 35%, lane 4: 80%, lane 5: supernatant.

The nickel affinity chromatography column was developed to purify the hDMP1 protein tagged with 8x poly-histidine. The 80% pellet was resuspended in extraction buffer and filtered to remove the particles. Then, it was slowly loaded into the Ni-NTA column. The purification process was washed with washing buffer and eluted with eluting buffer (Figure 36). Based form the result (Figure 37), the gels stained with Coomassie showed the presence of RuBisCO in the elution fraction. However, prior precipitation of RuBisCO by ammonium sulphate indicated reduced amount of RuBisCO host proteins in the plant extract.

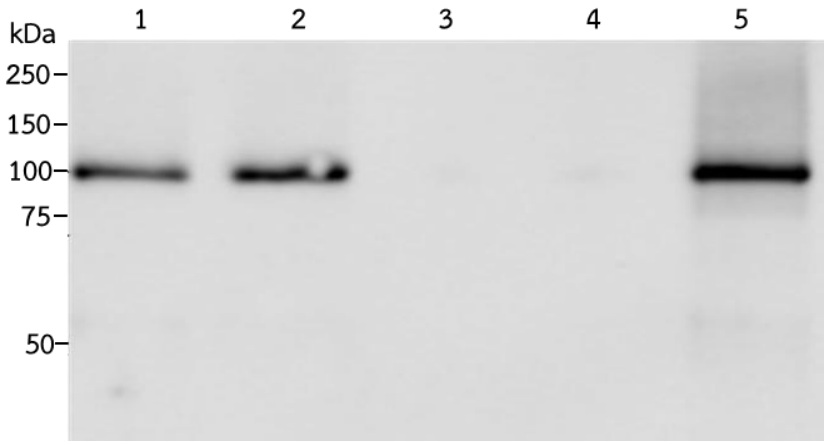


**Figure 36** Western blot analysis of purification *N. benthamiana*-produced hDMP1
The crude extracts of hDMP1 protein was treated under reducing condition. The acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: crude extract, lane 2: pellet 80%, lane 3: flow through, lane 4: wash, lane 5: elute.

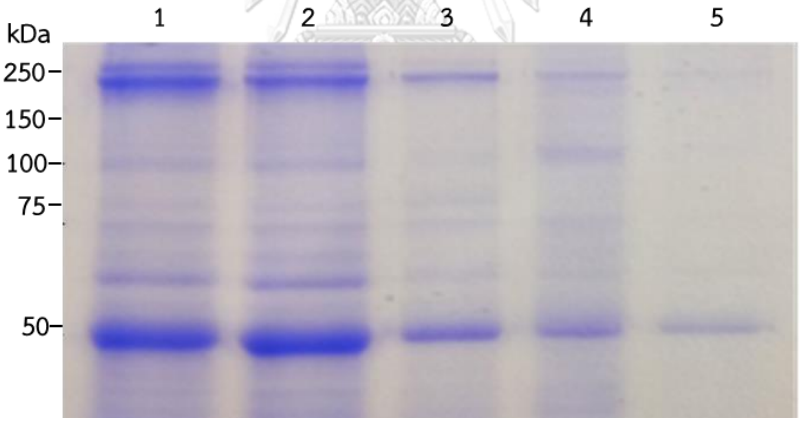


**Figure 37** SDS-PAGE analysis of purification *N. benthamiana*-produced hDMP1
The crude extracts of hDMP1 protein was treated under reducing condition. The acrylamide gel 10% was used to separate the proteins. The gel was stained with Coomassie blue. Lane 1: crude extract, lane 2: pellet 80%, lane 3: flow through, lane 4: wash, lane 5: elute.

To see the effect of the ammonium sulphate precipitation, it was compared with normal purification (without-precipitation). Western blot probed with anti-histidine conjugated-HRP and SDS-PAGE stained with Coomassie brilliant blue were performed for protein analysis. Our data showed that the purification by salt precipitation collected a large amount of the recombinant hDMP1 protein (Figure 38) than normal purification (Figure 39). Accordingly, the SDS-PAGE data showed less amount of RubisCO in the purification by precipitation (Figure 40) than normal purification (Figure 41). The results concluded that two-step purification with ammonium sulphate precipitation and nickel affinity chromatography indicated a more suitable method to improve the purity and yield of recombinant hDMP1 than purification with nickel affinity chromatography alone.

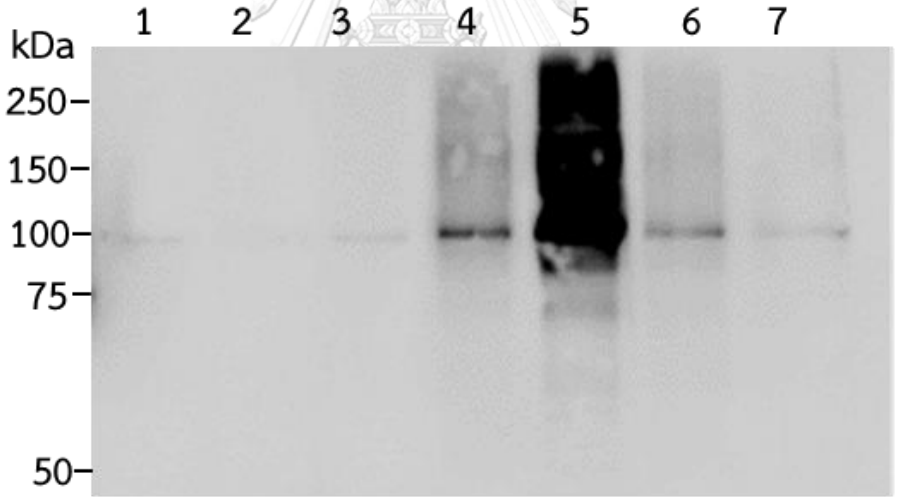


**Figure 38** Western blot analysis of precipitation-purification of hDMP1
The acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: crude extract, lane 2: flow through, lane 3: wash, lane 4: elute 1, lane 5: elute 2, lane 6: elute 3, lane 7: elute 4.

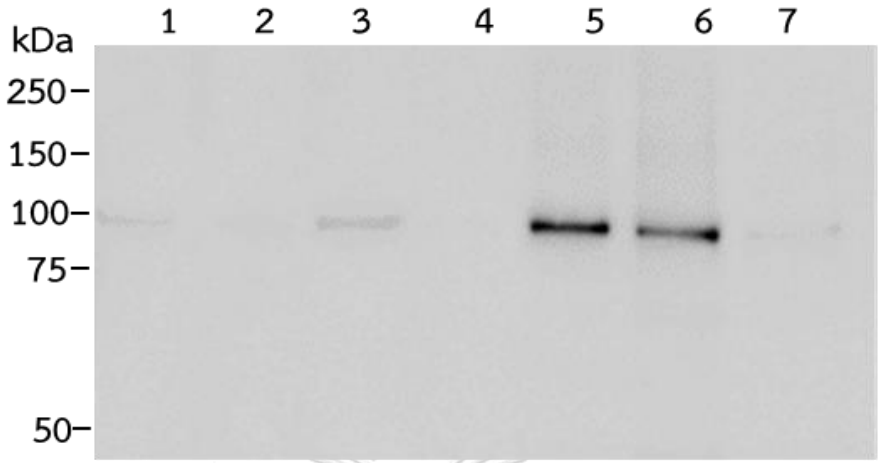


**Figure 39** Western blot analysis of normal purification of hDMP1

The acrylamide gel 10% was used to separate the proteins. The western blot was developed with ant-histidine antibody. Lane 1: crude extract, lane 2: flow through, lane 3: wash, lane 4: elute 1, lane 5: elute 2, lane 6: elute 3, lane 7: elute 4.

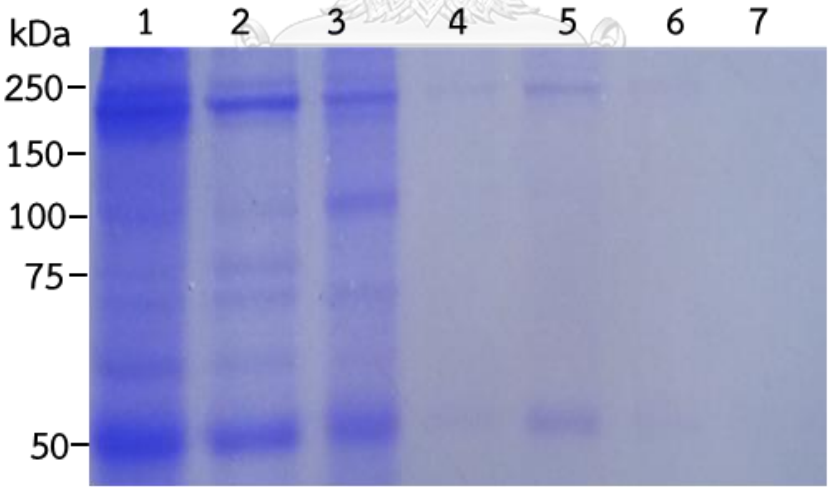


**Figure 40** SDS PAGE analysis of precipitation-purification of hDMP1

The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The gel was stained with Coomassie blue. Lane 1: crude extract, lane 2: flow through, lane 3: wash, lane 4: elute 1, lane 5: elute 2, lane 6: elute 3, lane 7: elute 4.

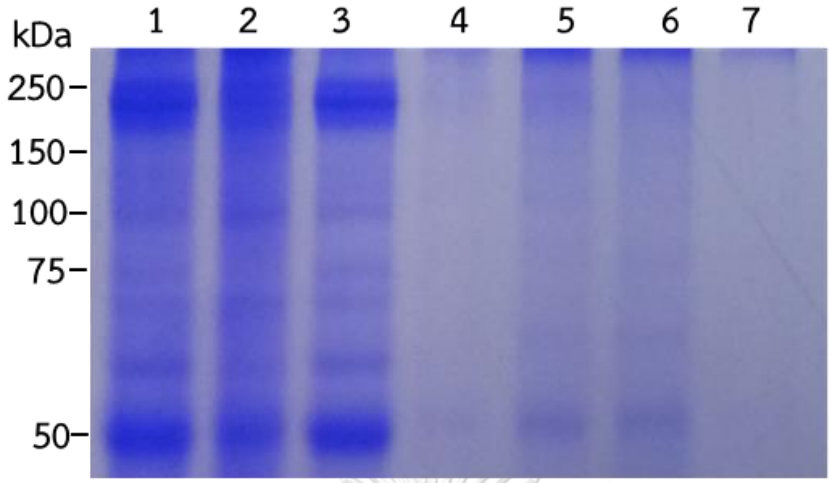


**Figure 41** SDS PAGE analysis of normal purification

The crude extracts of hDMP1 protein was treated under reducing condition. The Acrylamide gel 10% was used to separate the proteins. The gel was stained with Coomassie blue. Lane 1: crude extract, lane 2: flow through, lane 3: wash, lane 4: elute 1, lane 5: elute 2, lane 6: elute 3, lane 7: elute 4.

### 3.5 Proliferation assay of *N. benthamiana*-produced hDMP1

The effect of *N. benthamiana*-produced hDMP1 in hPDLSCs viability was investigated. *N. benthamiana*-produced hDMP1 (1 ug/ml and 2 ug/ml) was compared with *E. coli*-produced (2 ug/ml). Purified non-infiltrated plant was used as a negative control. The hPDLSCs number were determined by the MTT assay after 24 and 72 hr treatment. The results showed that both types of recombinant proteins significantly increased the number of hPDLSCs. There was no significant difference between the viability of hPDLSCs treated with 1 µg/ml and 2 µg/ml. However, hPDLSCs treated with *N. benthamiana*-produced hDMP1 showed the highest survival rate. These data confirmed that *N. benthamiana*-produced hDMP1 was not toxic to hPDLSCs (Figure 42).

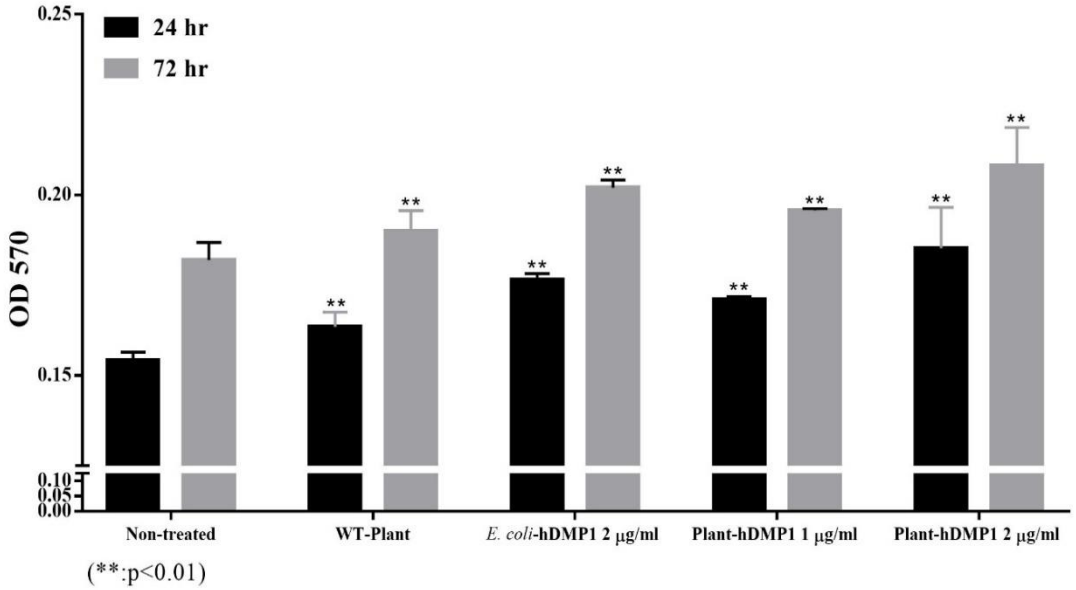


**Figure 42** Proliferation assay of *N. benthamiana*-produced hDMP1 in hPDLSCs

Three lines of hPDLSCs were collected from three different donors. The hPDLSCs were cultured on plates coated with *N. benthamiana*-produced hDMP1 (1 μg/ml and 2 μg/ml), *E. coli*-produced hDMP1 (2 μg/ml), non-infiltrated plant, and a non-coated plate for 24 and 72 h before the MTT assay was performed. Data represent the absorbance at 570 nm. (indicates a significant difference compared to the cells growing on the non-treated, **: $p < 0.01$).

### 3.6 Osteogenic differentiation of *N. benthamiana*-produced hDMP1

The expression levels of osteogenic genes including alkaline phosphatase (ALP), osteopontin (OPN), osterix (OSX), type I collagen (COL1), core binding factor alpha 1 (CBFA), bone morphogenetic protein-2 (BMP2), and Wnt3a were examined using real-time PCR. Human periodontal ligament stem cells (hPDLSCs) were cultured on culture plates coated with 0.5 μg/ml, 1 μg/ml and 2 μg/ml of *N. benthamiana*-produced hDMP1, 2 μg/ml of *E. coli*-produced hDMP1, and wild type plant for three days. The mRNA was extracted and subjected to RT-PCR for gene expression analysis. The expression levels of these genes were compared to non-treated and treated cells. The results indicated that *N. benthamiana*-produced hDMP1 protein could significantly induce the expression of the ALP, BMP2, CBFA1, OSX, WNT3a, and OPN genes (Figure 43). The data revealed that *N. benthamiana*-produced hDMP1 showed higher effect on osteogenic differentiation than *E. coli*-produced hDMP1. This data suggests the impact of post-translational modification, which increases the protein activity or binding to the receptor. Previous studies have reported that glycosylation of DMP1 such as DMP1-PG (DMP1-$S^{89}$) elevated laminin and ZO1. DMP1-PG increased mRNA expression levels of Runx2 and ALP of bone mesenchymal stem cells (BMSCs) when compared with wild type (WT). In addition, DMP1-PG showed higher activity for fracture healing through promoting chondrogenesis of mice models than WT group. In addition, phosporylated-DMP1 showed higher activity as HA nucleator than non-phosporylated-DMP1 and induced the formation of parallel arrays of crystallites with their C-axes co-aligned (Cai et al., 2019; Eapen et al., 2010; Tabatabaei et al., 2013; Xue et al., 2019). This data suggested that post-translational modification of hDMP1 has a potential to increase the osteogenic differentiation of the cells.

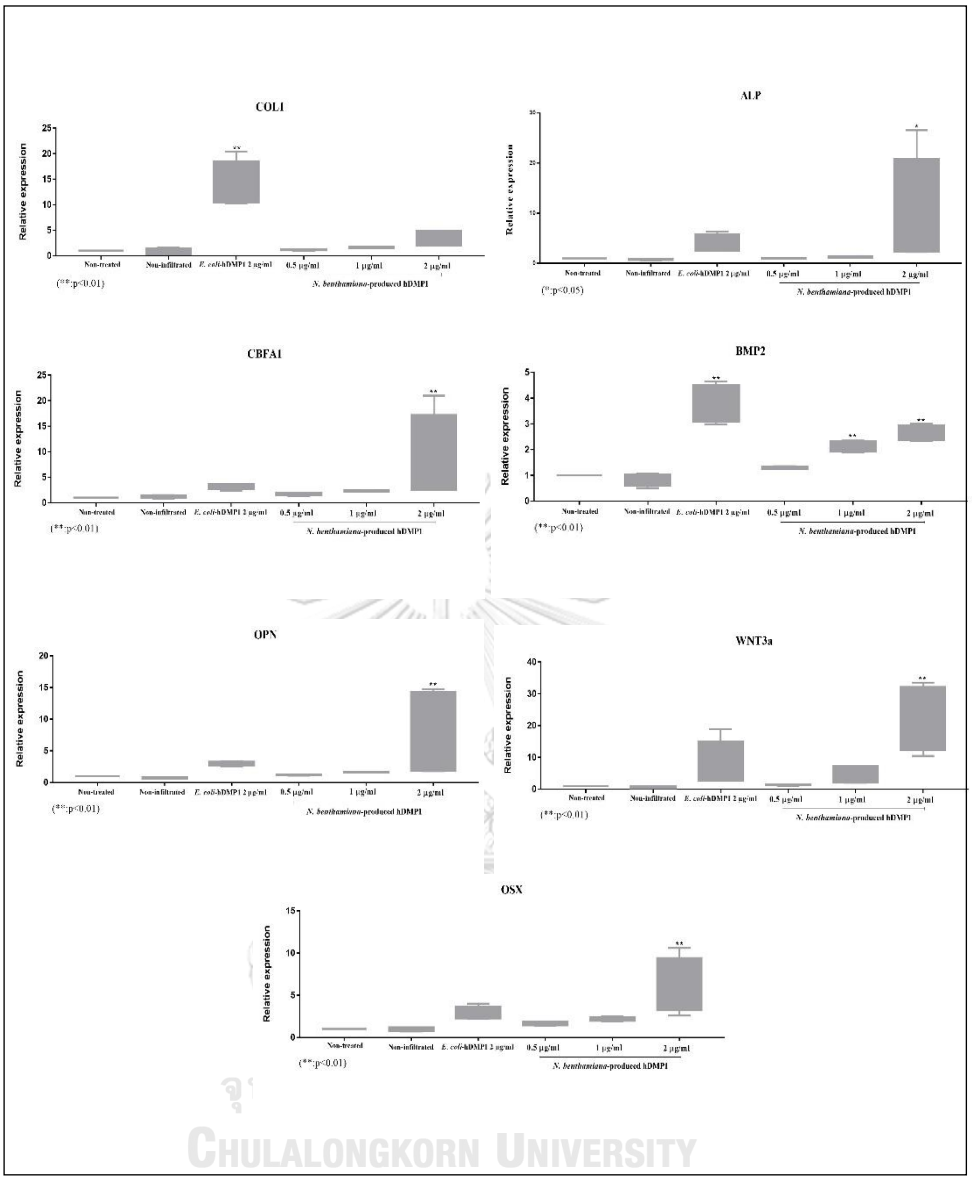




**Figure 43** *N. benthamiana*-produced hDMP1 induced osteogenic differentiation

Four lines of the hPDLSCs were collected from three different donors. The hPDLSCs were cultured on plates coated with *N. benthamiana*-produced hDMP1 (0,5; 1 and 2 μg/ml), *E. coli*-produced hDMP1 (2 μg/ml) negative control (*E. coli* without gene insertion) and non-coated plates for 3 days. The mRNA was extracted, and RT-PCR was performed to analyze the expression of ALP, BMP2, CBFA1, COL1, OSX, WNT3a, and OPN (* indicates a significant difference compared to the cells growing on a non-treated, *: $p < 0.05$, **: $p < 0.01$).

The osteogenic differentiation effect of *N. benthamiana*-produced hDMP1 was compared with commercial hDMP1 (produced in a mouse myeloma cells). Based from the results (Figure 44), the highest expression with *N. benthamiana*-produced hDMP1 on genes ALP, BMP2, CBFA1, OSX, WNT3a, and OPN was obtained among *E. coli*-produced hDMP1 and commercial hDMP1. It demonstrated the proof-of-concept that plant-produced protein can influence cellular behaviour similar to the protein produced in mammalian cells. Human periodontal ligament cells have been shown to possess mesenchymal stem cell characteristics and played important roles in the repair and regeneration of periodontal tissue, including alveolar bone (Carter et al., 2017; Li et al., 2014).

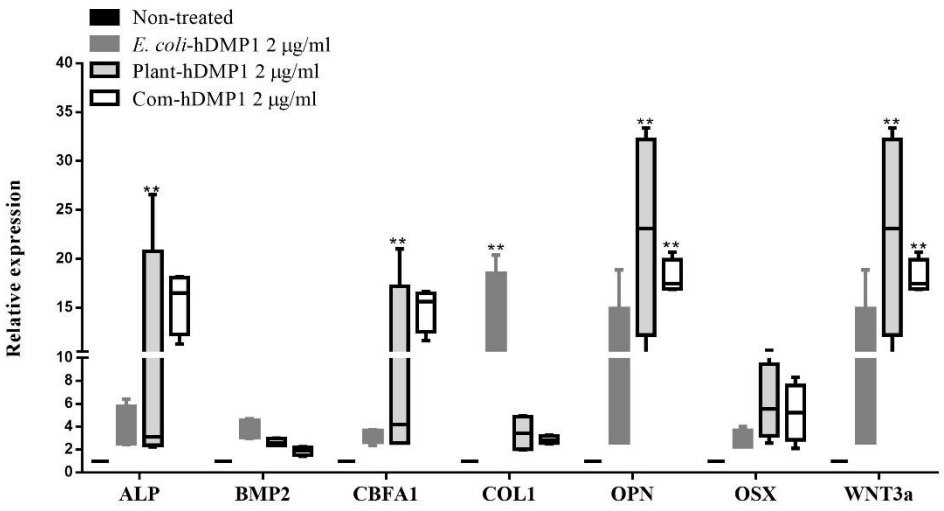


**Figure 44** Recombinant hDMP1 proteins induced osteogenic differentiation. Four lines of the hPDLSCs were collected from three different donors. The hPDLSCs were cultured on plates coated with 2 µg/ml of *N. benthamiana*-produced hDMP1, *E. coli*-produced hDMP1 commecial-hDMP1 and non-coated plates for 3 days. The mRNA was extracted, and RT-PCR was performed to analyze the expression of ALP, BMP2, CBFA1, COL1, OSX, WNT3a, and OPN (* indicates a significant difference compared to the cells growing on a non-coated plate, *: $p < 0.05$, **: $p < 0.01$).

### 3.7 Calcification assay of *N. benthamiana*-produced hDMP1

The effect of *N. benthamiana*-produced hDMP1 on *in vitro* calcification was examined. The hPDLSCs were cultured on the surface coated with *N. benthamiana*-produced hDMP1 2 µg/ml in osteogenic medium (OM) and compared with untreated cells. The hPDLSCs in general medium (GM) were used as a negative control. The alizarin red S solution was used for staining after culturing for 14 days. The result showed that untreated cells in the general medium did not display calcium deposition. Interestingly, hPDLSCs in osteogenic medium showed the development of red colour, which was indicative for the positive alizarin red S stained cells. Moreover, *N. benthamiana*-produced hDMP1 showed higher intensity of the red colour than untreated cells (Figure 45). In accordance with the previous studies, pulp and MCT3-E1 cells showed the calcium deposition after incubation 14 days (Eapen et al., 2010; Widbiller et al., 2018).

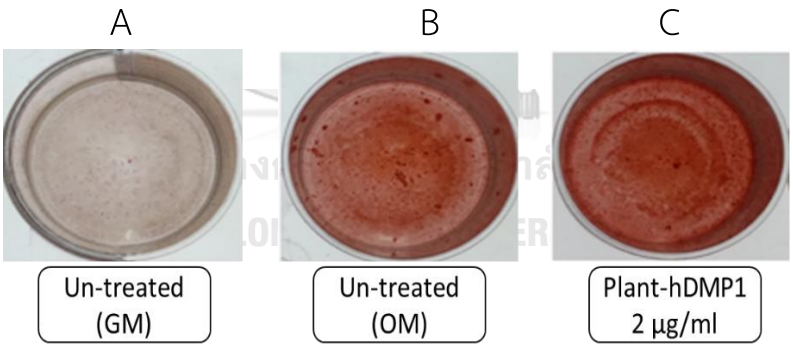


**Figure 45** Calcification assay of *N. benthamiana*-produced hDMP1
The control cells growing in general medium (GM) (A), control cell growing in osteogenic medium (OM) (B), cells treated with 2 µg/ml of *N. benthamiana*-produced hDMP1 in osteogenic medium (OM) (C) for 14 days. The wells were stained with Alizarin Red dye.

The calcification deposition effect in hPDLSC of *N. benthamiana*-produced hDMP1, *E. coli*-produced hDMP1, and commercial hDMP1 were compared. The untreated cells were cultured in osteogenic medium (OM) and general medium (GM) as a control. The hPDLSCs were stained with Alizarin red S solution after culturing for 14 days. The result showed that the red colour appeared in osteogenic medium (OM). However, plant-produced hDMP1 showed higher levels of calcium deposition than *E. coli*-produced hDMP1 and commercial hDMP1 (Figure 46). In relation to previous study, bone mesenchymal stem cells (BMSCs) in the osteogenic medium showed a larger mineralization stained area in DMP1-PG than WT-group (Cai et al., 2019).

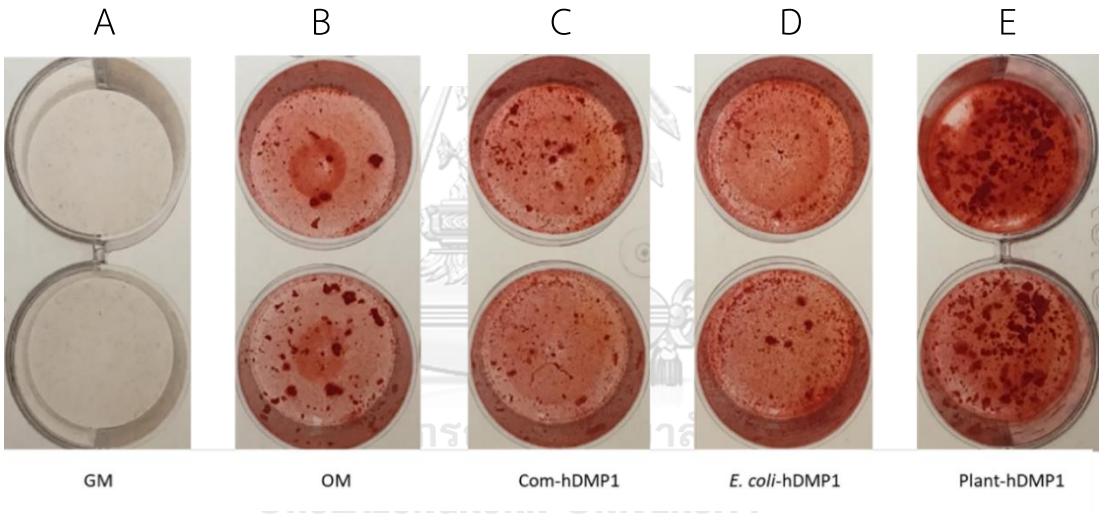


**Figure 46** Summary of calcification assay of recombinant hDMP1 proteins
The control hPDLSCs growing in general medium (GM) (A), control cell growing in osteogenic medium (OM) (B), hPDLSCs treated with 2 µg/ml commercial hDMP1 (C), hPDLSCs treated with 2 µg/ml of *E. coli*-produced hDMP1 (D), cells treated with 2 µg/ml of *N. benthamiana*-produced hDMP1 (E) in osteogenic medium (OM) for 14 days. The wells were stained with Alizarin Red dye.

In summary, hDMP1 has been successfully produced in *N. benthamiana*. The protein was detected at 100 kDa indicating further protein processing and post-translational modifications. Subsequently, *N. benthamiana*-produced hDMP1 could increase the expression of gene marker-related to osteogenic differentiation and calcium deposition in hPDLSCs. However, this result also confirmed that *N. benthamiana*-produced hDMP1 could induce higher osteogenic differentiation and calcium deposition in hPDLSCs than *E. coli*-produced hDMP1. Moreover, *N. benthamiana*-produced hDMP1 has high potential to be considered as a new candidate for tissue engineering due to inductive properties. However, further investigations for the associated post-translational modifications and molecular mechanism of hDMP1 on osteogenic differentiation are required.

# CHAPTER V

# CONCLUSION

Recombinant hDMP1 has been productively expressed in *E. coli*-produced hDMP1 and *N. benthamiana*- produced hDMP1. Recombinant hDMP1 produced in *E. coli* and *N. benthamiana* showed a distinct pattern. *E. coli* could produce recombinant hDMP1 1.38 µg/ml at optimal condition 0.5 mM IPTG and incubation at 37°C for 6 hr. In *N. benthamiana*, the highest-level expression for producing hDMP1 was obtained at 2 dpi and $OD_{600}$ 0.4 with 0,3 µg/g leaf fresh weight.

The proliferation assay assured that recombinant hDMP1 proteins are not toxic to hPDLSCs. *E. coli*-produced hDMP1 could induce ALP, BMP2, CBFA1, OSX, OPN, and WNT3a osteogenic marker genes. However, *N. benthamiana*-produced hDMP1 showed the expression of the gene marker ALP, BMP2, CBFA1, OSX, WNT3a, and OPN higher than *E. coli*-produced hDMP1. In addition, *E. coli*-produced hDMP1 could increase the calcification at 2 ug/ml. *N. benthamiana*-produced hDMP1 showed higher levels of calcium deposition than *E. coli*-produced hDMP1. The results showed that the plant system has the potential to produce recombinant hDMP1 as an inductive molecule in bone tissue engineering.

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# APPENDIX

## Media

The media for bacteria were autoclaved before use. The media and supplements of cells were sterilized and handled under sterile conditions.

1. **Media Luria Bertani (LB) agar**

   Peptone 5 g, yeast extract 2.5 g, NaCl 5 g, agar 7.5 g, added $ddH_2O$ 500 ml

2. **LB broth**

   Peptone 5 g, yeast extract 2.5 g, NaCl 5 g, added $ddH_2O$ 500 ml

3. **General media DMEM**

   DMEM 4.5 g/l glucose, glutaMAX, fetal Bovine Serum 10 %, penicillin 1 %

4. **Osteogenic medium**

   10% DMEM supplemented with:

   100 nM dexamethasone, 50 mg/ml ascorbic acid, 10 mM b-glycerophosphate



## Buffers

1. **DNA loading 6 x DNA loading dye**

   Glycerol 38 % (w/v), bromphenolblue 0.08 % (w/v), xylencyanol 0.08 % (w/v)

2. **Reducing Z-buffer**

   Tris HCl 125 Mm, sodium dodecyl sulphate 12%, glycerol 10%, β-mercaptoethanol 22%, bromophenol blue 0,001% pH 6.8

3. **Non-reducing Z-buffer**

   Tris HCl 125 mM, sodium dodecyl sulphate 12%, glycerol 10%, bromophenol blue 0,001% pH 6.8

4. **Buffer for producing recombinant hDMP1 in *E. coli*:**

   **Extraction buffer**

   Tris HCl 100 mM pH 7.4, sodium chloride 200 mM, imidazole 5 mM

   **Washing buffer**

   Tris HCl 100 mM pH 7.4, sodium chloride 200 mM, imidazole 25 mM

   **Eluting buffer**

   Tris HCl 20 mM pH 7.4, sodium chloride 50 mM, imidazole 250 mM

5. **Buffer for producing recombinant hDMP1 in *N. benthamiana***

   **Extraction buffer**

   Tris HCl 20 mM pH 7.4, sodium chloride 50 mM, Imidazole 5 mM

   **Washing buffer**

   Tris HCl 20 mM pH 7.4, sodium chloride 50 mM, Imidazole 20 mM

   **Eluting buffer**

   Tris HCl 20 mM pH 7.4, sodium chloride 50 mM, imidazole 250 mM.

6. **TBS 10X (Tris-buffered saline)**

   Tris base 24 g, sodium chloride 88 g, dissolve in 900 mL distilled water – pH to 7.6 with 12 N HCl, added $ddH_2O$ to 1 L.



7. **TBST (Tris-buffered saline, 0.1% Tween 20)**

   TBS 10X – 900 mL, tween 20 1 mL, added $ddH_2O$ to 1L

8. **Destaining solution**

   Methanol 50%, acetic acid 10%, added $ddH_2O$ 40%

9. **Coomassie Brilliant Blue**

   Coomassie Brilliant Blue R-250 0.25 g, dissolved in 90 ml of methanol: $H_2O$ (1:1, v/v) and 10 ml of glacial acetic acid, Filtered with a Whatman No. 1

**10. Running buffer 10x**

Tris Base 30. 4 g, glycine 144.4 g, sodium dodecyl sulphate 10.0 g, added $ddH_2O$ to 1 L.

**11. Transfer buffer 1x**

Tris Base 3.03 g, glycine 14.4 gr, methanol 150 ml, added $ddH_2O$ 1 L

**12. Infiltration buffer**

2-(N-morpholino) ethanesulfonic acid 100 mM, MgSO4 100 mM pH 5.5

**13. PBS 1x (Phosphate-buffered saline)**

Potassium chloride 2.7 mM, sodium chloride 137 mM, potassium phosphate 1.76 mM

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