Cloning, Characterization and Expression of Human Dentin Matrix Protein1 (DMP-1)

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Abstract: This study aimed to produce dentin matrix protein 1 (DMP-1) by cloning of DMP-1 cDNA and transfecting FS 293 cells as wellas isolation and purification of the expressed protein which would be used *in vitro* and *in vivo* dental and bone experiments. DH5α competent cells were transformed with plasmid carrying human DMP-1 gene (Trueclone, catalog No TC303479, Origene) and distributed on plates containing ampicillin then incubated at 37 ^{o}C for 24 hrs. Ampicillin resistant colony was grown in sterile glass tubes containing 5 ml LB medium and incubated at 37 ^{o}C for 16-17 hrs with vigorous shaking. DNA was then isolated using an Invitrogen miniprep kit. Recovered miniprep DNA samples were digested with NOT I enzyme (Invitrogen) to release the DMP-1 or control insert and run on 1% agarose gel. One DNA vector with the correct sequence was retransformed into DH5α *E.coli* and then grown up in large quantities for DNA maxipreps. DNA samples were digested with NOT I enzyme to release the DMP-1 or control insert and run on 1% agarose gel. FS293 cells (Freestyle ^{TM} 293 Expression System, Invitrogen) were transfected with DMP-1 cDNA for protein expression. Partial Purification of DMP 1 was done. Conditioned medium (600 ml) was applied to a 20-ml DEAE-Sephacel (Sigma) column. Absorbance of eluted proteins was monitored at 230 nm with 2.4 ml fractions collected. Those containing DMP-1 were identified by Dot Blotting. BCA protein Assay Kit (Pierce) was used to quantify the amount of protein present in the pooled fractions.Then, protein was visualized using SDS PAGE and Western Blotting. Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis was performed using NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen). Two staining techniques were used, Coomassie blue to visualize the protein bands and estimate their molecular weight. The second technique is Stains All (SIGMA) was used to differentiate between acidic glycoproteins and phosphoproteins which stain blue and other classes of proteins which stain red. Samples containing DMP1 were further purified using the Hydroxyapatite column. Dot blotting the fractions using DMP-1 antibody (LF 148 Takara inc.) revealed the presence of DMP-1 in fractions which could be used in dental and bone injuries and experiments.
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Key words: DMP1, Dentine, Bone, cDNA, cloning, transfection, DH5α competent cells.

1. Introduction:

Dentin matrix protein 1 (DMP1) is a non-collagenous protein expressed in bone and dentin (George *et al.,* 1993). It is an acidic protein rich in aspartic acid, glutamic acid, and serine residues. Most of the serine residues are phosphorylated by casein kinase II (George *et al.,* 1994; . D'Souza *et al.,* 1997; MacDougall *et al.,* 1998).

DMP1 was originally thought to be found only in dentin, but later it was also detected in bone, cartilage, and non-mineralized tissues such as the brain, pancreas, kidney, and salivary glands (George *et al.,* 1995; Begue-Kirn *et al.,* 1998; Feng *et al.,* 2003; Ogbureke and Fisher 2004, 2005, 2007).

DMP1 is a multifunctional protein that has been found to regulate cell attachment to the extracellular matrix (Kulkarni *et al.,* 2000)) and cell differentiation (Narayanan *et al.,* 2001; Kalajzic *et al.,* 2004) to activate matrix metalloproteinase-9,(Fedarko *et al.,* 2004) and has been postulated to play a significant role in biomineralization.(Ye *et al.,* 2004) as well as having a role in expressing osteocalcin, alkaline phosphatase and DSPP determined by over expression studies in mesenchymal stem cells (Narayanan *et al.,* 2001). DMP1 is also involved in calcium and phosphate metabolism through the kidney.(Terasawa *et al.,* 2004).

DMP1 is highly acidic, a property that is necessary for calcium binding because it provides a microenvironment for mineral precipitation (Qin *et al.,* 2003 and Gericke et al., 2010).

In vitro, DMP1 acts as a hydroxyapatite (HA) crystal nucleator with very high calcium ion binding capability (He *et al.,* 2003). It plays an active role in nucleating the initial calcium phosphate crystals during the initial stages of dentine and bone formation. (Hao *et al.,* 2004).

DMP-1 is a low abundance protein which is difficult to characterize and it plays a role in the formation of all mineralized tissues during their development (Feng *et al.,* 2003).

It may also be involved in the regulation of phosphate homeostasis through fibroblast growth factor 23 (FGF23), a newly identified hormone that is released from bone and targeted in the kidneys; deletion of the *Dmp1* gene leads to a dramatic increase of FGF23 mRNA in osteocytes (Feng *et al.,* 2006).

The importance of DMP1 in biomineralization has been shown through mice and human genetic studies. In mice, a lack of DMP1 results in poor mineralization of bone and dentin, whereas mutations in the DMP1 gene in humans result in osteomalacia (Ye *et al.,* 2004, 2005; Feng *et al.,* 2006). Interestingly, DMP1 expression was also observed in malignant tumor cells (Fedarko *et al.,* 2001; Fisher *et al.,* 2004; Ogbureke *et al.,* 2007).

It has also been implicated in the transcription activity of other dentine specific matrix genes like DMP-2 (Narayanan *et al.,* 2003) and DSPP (Narayanan *et al.,* 2006) the candidate gene for dentinogenesis imperfecta TYPE II and III genetic disorders.

The extracellular matrix (ECM) of bone and dentin contains fragments originating from intact DMP1, namely, a 37-kDa fragment from the NH2-terminal region and a 57-kDa fragment from the COOH-terminal region of the DMP1 amino acid sequence (Qin *et al.,* 2003). NH2- terminal fragment of DMP1 in the ECM of bone and dentin also occurs as a proteoglycan (Qin *et al.,* 2006). The proteoglycan variant, referred to as DMP1-PG, possesses a single glycosaminoglycan side chain linked to the core protein via Ser74 in the rat DMP1 amino acid sequence.

In vitro mineralization studies have demonstrated that the COOH-terminal fragment promotes mineralization by acting as a nucleator for hydroxyapatite formation (Tartaix *et al.,* 2004; He *et al.,* 2005; Gajjeraman *et al.,* 2007; Gericke et al., 2010). Information regarding the biological functions of the NH_2-terminal fragment and DMP1-PG is lacking.

When DMP1 applied in situ, it induces differentiation of dental pulp stem cells into odontoblasts (Almushayt, 2006).

The aim of our study was to produce DMP-1 protein by transfecting FS 293 cells with DMP-1 cDNA which would then be used *in vitro* and *in vivo* dental and bone injuries and experiments.

2. Materials and Methods:

Materials:

1. Competent DH5α *E.coli* (Invitrogen)
2. Human cDNA clone (Trueclone, catalog No TC303479, Origene)

Accession No: NM_004407.1. Homo sapiens dentin matrix acidic phosphoprotein (DMP1). Vector: pCMV6-Neo Vector size ~ 5.8 kb Insert size (Not1 digest): 1.5 kb.

3. All chemicals for which source is not noted were of analytical grade from Sigma, Fluka, and BDH.

Methods:

1. Reparation of the plates

1%Bacto-Tryptone 10 gm
0.5%Yeast Extract 5 gm
1%Sod.Chloride 10 gm
1.5% Bacto Agar 15 gm

Dissolve in 900 ml dd water and adjust pH to 7.5 with NaOH. Complete the volume to 1000 ml. Autoclave for 25 min. Cool to about 50 ° C before adding antibiotic (1000 µl of 1000x stock /litre).

Note: make sure that the temperature of the solution is about room temp. before adding antibiotic to prevent its destruction.

2.TAE Buffer

Trizma base 48.4 gm
G. Acetic Acid 11.42 ml
Di Na EDTA 7.44 gm

To be dissolved in 1000 ml d. water.

3. LB Media

Trypticase Peptone 10 gm
Yeast Extract 5 gm
NaCl 10 gm

Dissolve in 900 ml dd water and adjust pH to 7.5 with NaOH. Then complete the volume to 1000 ml and autoclave for 35 min.

4.2X YT Media

Tryptone 16 gm
Yeast Extract 10 gm
NaCl 5 gm

Dissolve in 900 ml d. water and adjust pH to 7.4 using NaOH. Then complete the volume to 1000 ml autoclave for 35 min.

5. Antibiotic – Final concentration

Ampicillin 100 µg/ml.

Make 100x stock in water and sterilize through 0.22 µ filter into microfuge tubes.

Store aliquots at -20 ° C

Transformation of DH5α competent cells

Plasmid carrying human DMP-1 gene (Trueclone, catalog no TC303479, Origene) was resuspended in 10 µl deionised water and incubated for10 min at room temperature.

1. Thaw DH5α (commercially competent *E. coli* cells) on ice.
2. Thaw DNA on ice
3. Warm selected antibiotic containing plates (face down) at room temperature for 45 min.
4. To one tube (50 µl) of copetent cells add 0.5 µl plasmid DNA. Incubate on ice for 30 min.
5. Shock at 37° C for 90 sec.

6. Put back into ice for 2 min. and add 100 µl of YT to give 150 µl.
7. To one plate add 10 µl of the above mixture and 40 ul of YT.
8. Add 6 sterile glass beads and rotate for 1 minute until the liquid is gone.
9. Repeat this step in another plate using 25 µl of DNA and 25 µl of YT.
10. Incubate at 37 ^{o} C overnight.

Plasmid DNA Purification (Miniprep).

1. Set up 4 sterile glass tubes, two for each plate.
2. Put 5 ml of LB into each sterile glass tube with lid.
3. Add appropriate antibiotic, 50 µl (100x ampicillin).
4. Using sterile yellow tip pick a single colony from plate and put in the tube.
5. Put the tubes in shaking water bath with vigorous shaking at 37^{o}C for 16-17 hours. .

DNA was then isolated using **Purelink Quick Plasmid Miniprep Kit (Invitrogen Inc.)** according to the manufacturer instructions. This method is comprised of three steps, DNA isolation, DNA binding and DNA elution.

N.B. We used 50 µl sterile water instead of TE buffer for elution of DNA from the column because TE buffer interferes with DNA sequencing and restriction digestion.

Quality assessment of the cDNA: Recovered miniprep DNA samples along with the sample supplied by Origene (as control) were digested with NOT I enzyme (Invitrogen) to release the DMP-1 or control insert.

Restriction digestion mixture: 5.5 ul water , 3 µl DNA, 1 µl 10x buffer (reaction buffer #3) and 0.5 µl enzyme Not 1. Incubate the mixture at 37 ^{o}C for 2 hours in a water bath. Add 1 ul dye to the mixture after digestion.

DNA Electrophoresis: 1% agarose gel electrophoresis was run to the intact DNAs (control) and NOT I digests to confirm the expected insert size. The DNA ladder (Qiagen) was used as a standard. The gel was stained with Ethidium Bromide and visualized by UV light. A picture was taken for records.

DNA Sequencing: One sample of the DMP-1 DNA was sent the sequencing Lab (Department of Biochemistry, University of Alberta) using forward and reverse primers supplied by Origene.

Plasmid DNA purification (Maxiprep)

Transformation of DH5α competent cells: One DNA vector with the correct sequence was retransformed into DH5 α E.coli. and then grown up in large quantities for DNA maxipreps.

I. First day

1. Set up 4 sterile glass tubes, two for each plate. 2. Put 5 ml of LB into sterile glass tubes with lid.
3. Add appropriate antibiotic (50 µl amp.)
4. Using sterile yellow tip pick a single colony from plate and put in the tube.
5. Put the tubes in shaking water bath for 7 – 8 hours at 37 ^{o}C with vigorous shaking.
6. At 4 pm add 100 ml sterile LB to sterile 500 ml flask, add 100 µl 1000x antibiotic stock. Mix, then add 100 µl of starter culture.
7. Grow over night at 37^{o}c in water bath with vigorous shaking.

II. Second day

1.Turn on Sovall centrifuge and cool down GSA rotor. 2. Transfer bacterial culture to sterile GSA rotor bottle and centrifuge for 30 minutes at 3500 rpm. 3. Pour off supernatant gently and discard.

N.B. Make sure that all supernatant has been drained.

DNA was then isolated using **Purelink Quick Plasmid Maxiprep Kit (Invitrogen Inc.)** according to the manufacturer instructions. This method is comprised of three steps, DNA isolation, DNA binding and DNA elution.

N.B. Air dry DNA pellet for 5-10 min. Redissolve DNA in 400 ul sterile water .

Determine the recovered DNA concentration by adding 5 µl in 495 µl water and read absorbance at 260 nm.

The recovered maxiprep DNA samples along with a sample of miniprep (as control) were digested with NOT I enzyme (Invitrogen) to release the DMP-1 or control insert (as done in miniprep), quantified and stored at -22 ^{o}C.

DNA yield from maxiprep

Sample # 1	1.55 ug/ul DNA
Sample # 2	1.12 ug/ul DNA
Sample # 3	0.88 ug/ul DNA
Sample # 4	0.98 ug/ul DNA

DNA Electrophoresis

1% agarose gel electrophoresis was run to the intact DNAs (control) and NOT I digests to confirm the expected insert size. The DNA ladder (Qiagen) was used as a standard. The gel was stained with Ethidium Bromide and visualized by UV light. A picture was taken for records.

3. FS293 cells (Freestyle ^{TM} 293 Expression System, Invitrogen) transient transfection for protein expression:

FS293 cells were grown in suspension culture according to the manufacturer's protocol (Invitrogen). The viable cell number was determined using Trypan Blue. The required number of cells were transferred from the shaker flask into 50ml sterile tube then centrifuged at 1000rpm for 5 minutes. The medium was discarded and the cells resuspended at a known concentration in FS Expression medium. The cells are vortexed vigorously for 10-30 seconds to break up clumps. Because optimal transfection requires single

cell suspension. The volume of the cell suspension needed is transferred to a sterile shaker flask. FS expression medium was added so that the cell density was approximately 1.1 x 10^6 viable cells / ml. The flask was then placed in the incubator on the shaker.

Initially, a small scale (30 ml) transfection is done to check if the protein is expressed.

A. 30µg plasmid DNA in Opti-MEM I was diluted to a total volume of 1ml and mixed gently.
B. 40µl of 293fectin in Opti-MEM I was diluted to a total volume of 1ml (added to 960µl in a sterile 15ml tube) and mixed gently then incubated for 5 minutes at RT.
C. The diluted DNA was added to the diluted 293fectin to obtain a total volume of 2ml, mixed gently, and then incubated for 30 minutes at RT to allow the DNA-293fectin complexes to form.
D. While the DNA-293fectin complexes were incubating, the cell suspension was removed from the incubator and 28 ml of suspension was aliquoted into each sterile 125ml flask. After the complex formation was complete, the 2ml were added to the shaker flask where each flask will have a 30ml volume with a final density of approximately1x10^6 viable cells/ml.
E. The complex was then incubated at 37^{o}C at 8% CO_2 on the shaker and the cells/medium was harvested 96 hours later. SDS-PAGE blot was performed on the supernatant to confirm the expression of DMP-1 and consequently a large scale (1000 ml) transfection was performed.

4. Partial Purification of DMP 1:

Conditioned medium (600 ml) was applied to a 20-ml DEAE-Sephacel (Sigma) column equilibrated in 0.05 M Tris-HCl, 0.01M NaCl, pH 7.4 (Tris buffer) at 10^{o}C at a flow rate of 53 ml/hr. The column was washed with buffer and then eluted with a linear gradient of 0.01-0.8 M NaCl in Tris buffer. It was washed with 2x30 ml 0.8M Nacl/Tris to ensure all bound proteins were removed. Absorbance of eluted proteins was monitored at 230 nm with 2.4 ml fractions collected. Those containing DMP-1 were identified by Dot Blotting.

5. Dot blotting the fractions:

10 µl of the DEAE fractions were applied to a Biorad Dot Blot apparatus on to a Nitrocellulose membrane. The dry membrane was soaked in PBS until wet and blocked with 4 % non fat milk dissolved in PBS/Tween 20 for 30-45 minutes at RT with shaking. The primary antibody (LF 148 of Takara Inc.) was then diluted to the appropriate concentration in PBS/Tw, added to the paper and was left 1-2 hours at room temperature on the shaker. After washing 4x4 with PBS/Tw. The secondary anti-rabbit IgG alkaline phosphatase conjugated antibody (Sigma) was diluted 1:2500 in PBS/Tw, and added to the paper. It was left for 30-90 minutes on shaker, washed 3 x 4 minutes with PBS/Tw. Proteins were visualized with BCIP/NBT (Roche). It was washed several times with water and then air dried. Based on the results of the dot blotting, the Antibody positive fractions containing DMP 1 were pooled and concentrated using Amicon Ultra-15 centrifugal Filter Devices (Millipore 10,000 NMWL) by centrifugation at 3750 rpm until the volume came down to 500-1000µl. The retentate was transferred to a pre-weighed microfuge tube and weighed.

6. *BCA* (bicinchoninic acid) *protein Assay Kit* (Pierce, Rockford, IL, USA) was used to quantify the amount of protein present in the pooled fractions.

7. Protein visualization using SDS PAGE and Western Blotting:

Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis was performed using NuPAGE Novex gradient 4-12% Bis-Tris gels (Invitrogen). Two staining techniques were used, Coomassie blue to visualize the protein bands and estimate their molecular weight. The gels were washed using 10% methanol for 5 minutes twice, then the stain was added and it was placed on a shaker at room temperature for one hour. Then destaining procedure was done using 10% acetic acid and 5% methanol. The gels were washed with distilled water and scanned.

The second technique is Stains All (SIGMA) was used to differentiate between acidic glycoproteins and phosphoproteins which stain blue and other classes of proteins which stain red. The staining solution is composed of 0.005% Stains All, 10% formamide, 25% isopropanol, 15 mM Trizma, 65% H_2O, HCL is added -pH 8.8. This mixture was kept in the dark for several hours. The gels were then washed with distilled water and scanned.

Following SDS-PAGE, the gel is prepared for electroblotting using a standard tank transfer. The proteins were transferred to PVDF membrane (MilliPore) using 32 volts for 1-1.5 hrs. Transferred proteins were visualized as for dot blots.

7. Final purification using HA column:

Equilibrate a 2ml Hydroxyapatite column (CHT Ceramic Hydroxyapatite, Bio-Rad) with approximately 10 column volumes of buffer A (10mM Na phosphate pH 6.8). Prepare the sample, adjusting the pH and conductivity to those of buffer A. The sample was diluted with buffer A containing 0.3 mM $CaCl_2$, then applied to the column. Wash the column with approximately 5 column volumes of buffer A, unbound material was allowed to pass

through the column. The bound proteins were eluted with approximately 20 column volumes of buffer B (10mM Na phosphate pH 6.8) linear gradient of increasing concentration of phosphate buffer (10-700 mM). After elution clean the column with approximately 5 column volumes of buffer B followed by a sanitation step using approximately 5 column volumes of 1 M NaOH. The same procedure for evaluation of fractions was performed.

3. Results:

Quality assessment of the cDNA product:

Purified full length miniprep DNA and the Not I restriction enzyme digestion were separated by agarose gel electrophoresis where their sizes compared to the DNA ladder (Quiagen).

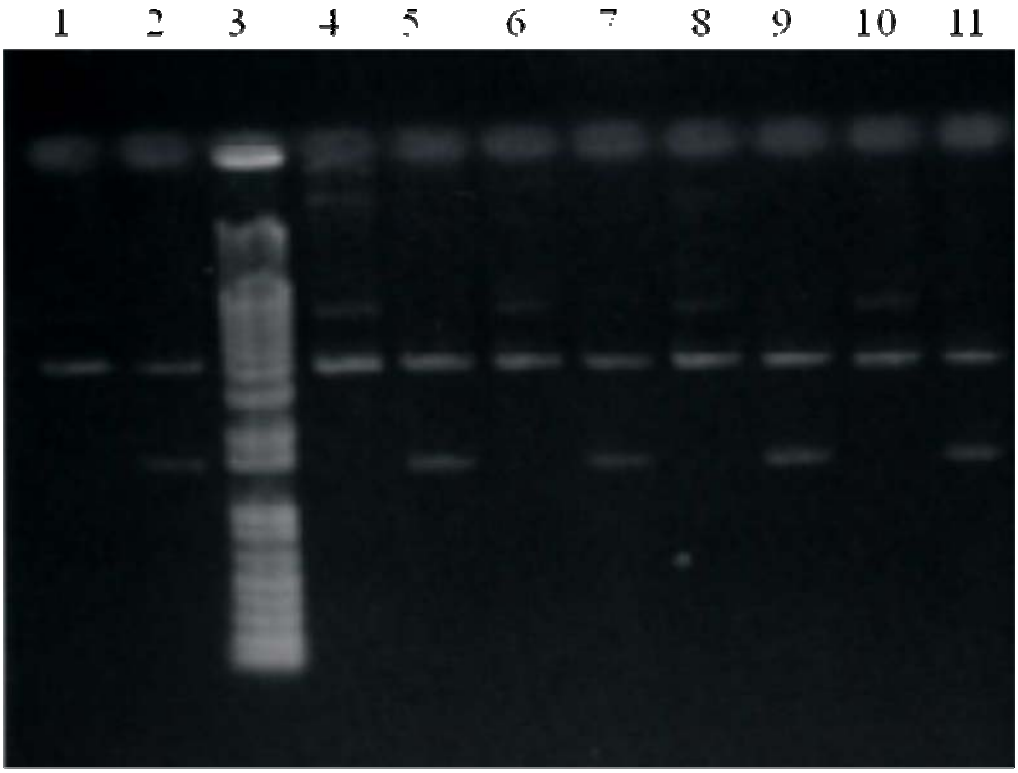

Fig (1): Miniprep products on 1% agarose gel when photographed on ultraviolet lamp using Kodak (camera) and scanned (Epson 1.3) showing lane 1 and 2 represent the intact control sample and its digest , lane 3 represent DNA ladder, lanes 4,6,8 and 10 represent the intact cDNA of DMP 1 while lanes 5, 7,9 and 11 represent the digest of the same samples. Each lane and its digest represent one different single colony. It is clearly obvious the positions of the bands are at the same level mentioned by the manufacture (3.8 and 1.3 kb) which indicates the success of the cloning procedure

DNA minipreps sequencing:

DNA minipreps of the recombinant plasmid containing DMP1 insert were characterized by DNA sequencing in the sequencing Lab (Department of Biochemistry, University of Alberta, Canada) using forward and reverse primers supplied by Origene. The obtained DNA sequence was aligned with Human cDNA clone. Accession No: NM_004407.1. Homo sapiens dentin matrix acidic phosphoprotein. As seen in (Fig.3a,5'→3' seq. &3b, 3'→5'seq.)

5′ -3′ sequence Frame 2

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>ref|NM_004407.1|  Homo sapiens dentin matrix acidic phosphoprotein (DMP1), mRNA
Length=7682

 Score = 1229 bits (665),  Expect = 0.0
 Identities = 717/740 (96%), Gaps = 12/740 (1%)
 Strand=Plus/Plus

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Query  185  AGGTATCAAAATAATGAATCTGAGGATTCTGAAGAATGGAAGGGTCATTTGGCTCAGGCA  244
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Query  245  CCAACACCACCCTTGGAGAGCAGTGAGTCATCAGAAGGCAGTAAAGTTAGCTCAGAGGAA  304
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Query  365  CATCAATACATTTATAGGCTAGCTGGTGGCTTCTCCAGGAGCACAGGAAAAGGAGGAGAT  424
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Query  425  GATAAAGATGACGATGAAGATGACAGTGGAGATGACACCTTTGGTGACGATGACAGTGGC  484
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Query  485  CCAGGGCCCCAAAGACAGACAAGAAGGAGGAAACTCCAGACTGGGAAGTGATGAGGACTCT  544
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Sbjct  817  AAAGAATCTGGAGAAAACAG  836
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3′ -5′ sequence Frame 3

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Length=2682

 Score = 1219 bits (660),  Expect = 0.0
 Identities = 730/761 (95%), Gaps = 20/761 (2%)
 Strand=Plus/Minus

Query  154   GACAGCTGATGCTAATAGCCGTCTTGGCAGTCATTGTCATCTTGGTCCCCAATGGGTTTG  213
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Query  214   TTGTGATAGGCATCAACTGTTAATTTCCGGCTCTCTATCTCAATGTTTTTCAACTGGCCA  273
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Query  274   TCTTCCTCACTGCTTGATTTGCTCTCCGTGGAGTTGCTATCTTCTTTGGATCTGCTGCTG  333
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Query  334   TCTTGAGAGTCACTGTCGTCTTCCTCAGAATGGCTTTCCTCGCTCTGACTCTCTGCTGAG  393
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Query  454   CTTTCCTCTGAGAAGTTGAGGCTCTCACTGGATTCGCTGTCTGCTTGCTCCTCTCTGGAT  513
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Query  574   TGGTCTTCTACATAACTAGTTGTGGGGTCGGGGTTATCTCCCCTGGACTCACTCACCACC  633
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Query  634   TCTACCTGAGACTCACTGCTGTTCTCTTGAGATGACAGGTTGGCCTCTGGGCTGGACTCA  693
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             |||||||||||| |||||||||||| |||| |||| |||||||||||| ||| ||| ||||
Sbjct  1052  TGAGAGTCACCCTTGCTGTCTCTCCTGGGTTGGCTGAGGCCAGTGTCTCTGGAGTTGCTG  993

Query  805   TTTTCTGTA-AGCTC-C-CTTGACCTCT-CCATTGTGGTGTTGNCATCACGCTC-CTTCT  859
             ||||||||| || || | |||||| ||| |||||||| ||||| ||||| |||| ||||||
Sbjct  992   TTTTCTGTAGAG-TCACTCTTGACTTCTTCCATTGTGTTGTTGTCATCAAGCTCGCTTCT  934

Query  860   GTCATCTTCC-CTGAAAT-C-AGAACTTCCTAAAA-TTTTC  896
             ||||||||| |||| || | ||| |||||||||| ||||||
Sbjct  933   GTCATCTTCCTCTGAGATGCGAGA-CTTCCTAAAAATTTTC  894
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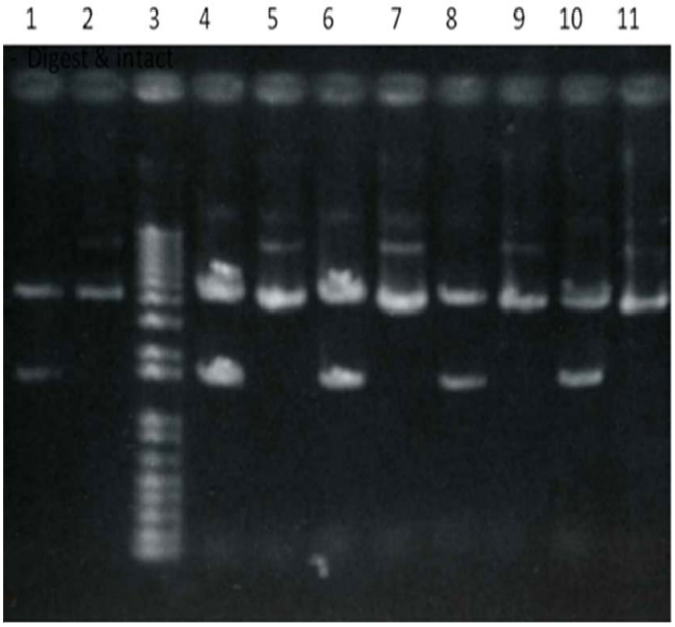

Fig (2): Maxiprep products on 1% agarose gel when photographed on ultraviolet lamp using Kodak (.) and scanned (Epson 1.3) showing lane 2 and 1 represent the intact control sample and its digest , lane 3 represent DNA ladder, lanes 5,7,9 and 11 represent the intact cDNA of DMP-1 while lanes 4, 6,8 and 10 represent the digest of the same samples. Each lane and its digest represent one different single colony. It is clearly obvious the positions of the bands are at the same level mentioned by the manufacture (5.8 kb and 1.5 kb) which indicates the success of the cloning procedure.

Evaluation of the transfection procedure

Western blotting was used to evaluate the conditioned medium collected 96 hours post transfection 20µL (DMP-1) medium and the control (E.V.) medium were blotted into PVDF membrane. The takara® antibody (Fig. 4) was used for immunostaining the conditioned medium from the DMP-1 transfection and showed bands in consistent with the position of the DMP-1 (~57 kda).Medium of the control group didn't show any bands indicating successful transfection and secretion of the DMP-1 protein.

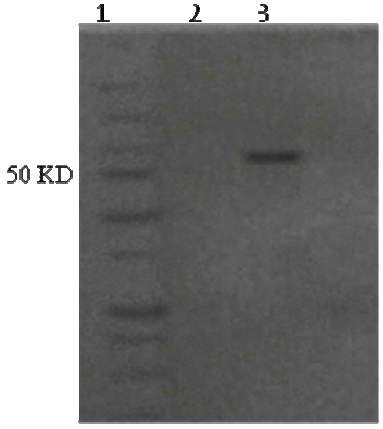

Fig (4): The scanned blotted PVDF membrane using the DMP-1 antibody, showing the presence of DMP-1 (lane 3) in the conditioned medium post transfection while the absence of this protein in the control E.V. group (lane 2). Lane 1 contains protein standard (Bio Rad).

Purification using DEAE column:

The DEAE column was connected to the holochrome monitor 230 nm and 0-2 A. 85 fractions were produced from 600 ml of the conditioned medium. Each fraction contains ~2.4ml of protein in 0.05 Tris. The chromatography showed two peaks: the first from fractions (#1) 10-30 while the second (#2) was from fractions 33-65. Fig (5).

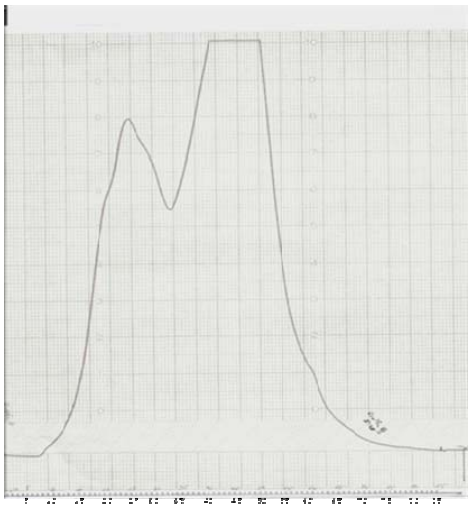

Fig (5) The DEAE elution profile of proteins

Dot Blotting the fractions using *Takara* Antibody was performed in order to exclude all non DMP-1 containing fractions. Fig (6).

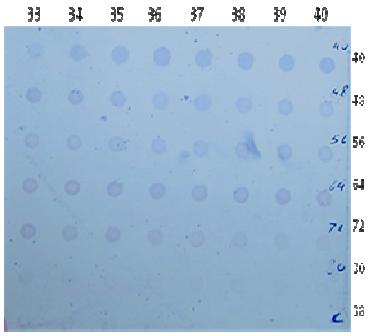

Fig (6): Positive fractions were from number 33 to 68 as seen on the nitrocellulose membrane after blotting the fractions using DMP-1 antibody (LF 148 – Takara inc.)

BCA protein assay was performed to quantify the concentration of protein available in these samples. Table (1) showing the alignment of the samples in response to the standard (D1-E3) with a gradual increase of 1 µl each three wells and the samples (A4-F5) of 2 µl each in three wells each sample. These data gave the concentration µg/2µl. The concentration (µg/µl) was calculated and tabulated in table (2).

Table 1: Calculated concentrations sheet (Raw data of BCA protein assay)

	1	2	3	4	5
A	EMPTY	5.061	9.694	8.109	4.360
B	EMPTY	6.067	9.084	7.316	4.329
C	EMPTY	5.945	11.370	9.115	4.055
D	2.653	5.091	11.827	13.656	1.038
E	2.562	8.353	11.492	12.772	1.312
F	1.891	7.987		14.448	1.495
G	6.250	9.724		EMPTY	EMPTY
H	4.878	9.785		EMPTY	EMPTY

Table 2: Concentration of proteins in samples 1- 4

Sample number	Concentration (µg/µl)
1	4.0895
2	6.607
3	2.135
4	0.587

Two SDS pages were run and stained with Commassie blue (Fig 7) to visualize acidic proteins which stain blue and Stains All (Fig 8) to differentiate between glycoprotein and phosphoprotein which stain blue and other proteins which stain red. Our targeted protein is an acidic phosphoprotein, that's why these two types of stains were chosen, where the results indicated according to the estimated molecular weight, its presence on both types of stains but with a higher concentration in sample 2.

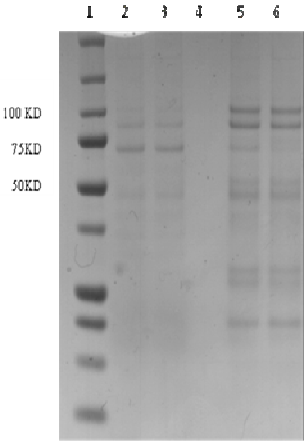

Fig (7): A photograph showing SDS page stained using Commassie blue. Lane 1 standard (Bio-Rad) lane 2 sample 1, lane 3 sample 2, lane 4 was left empty, Lane 5 sample 3 and lane 6 sample 4. Note that the presence of a stain at the level from 50-75 KD in samples 1,2 only.

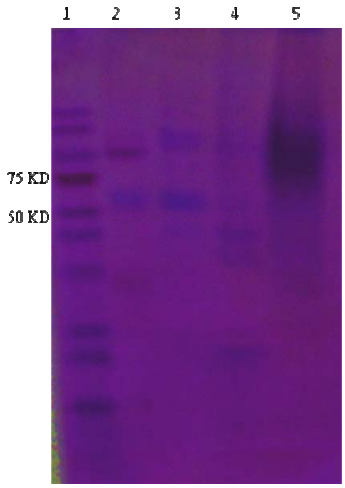

Fig (8). A photograph showing SDS page stained using Stains All. Lane 1 standard (Bio-Rad) lane 2 sample 1, lane 3 sample 2, lane 4 sample 3 and lane 5 sample 4. Note that the presence of a blue stain at the level from 50-75 KD in samples 1,2.

Purification using Hydroxyapatite column: Samples 1 and 2 containing DMP1 were further purified using the Hydroxyapatite column .

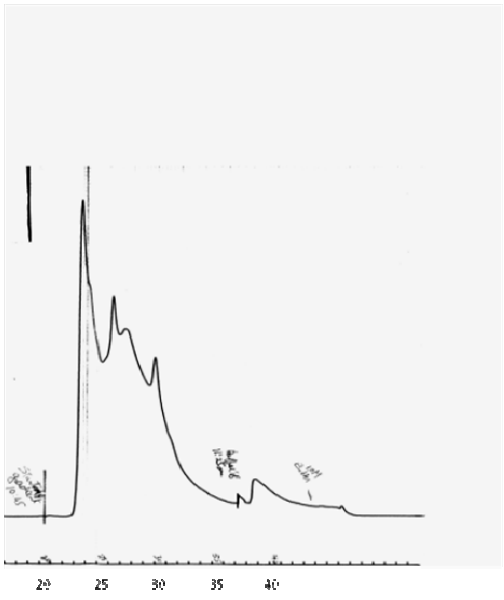

Fig (9)- represents the protein in question relative to the 25-34 fractions.

Dot blotting the fractions (Fig 10) using the *Takarra* Antibody revealed the presence of DMP-1 in fractions (26-34).

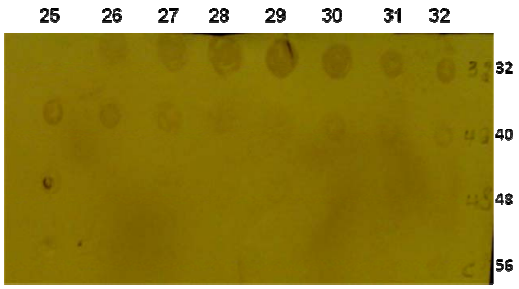

Fig (10): Nitrocellulose membrane of the eluted fractions from the HA column, notice the dots in response to the reaction to the DMP-1 antibody (LF 148 Takara inc.) in 26th to 34th fraction.

4. Discussion:

Dentin matrix protein-1 (DMP1), a member of the Small Integrin-Binding Ligand, N-linked Glycoprotein (SIBLING) family of extracellular matrix proteins (Qin *et al.*, 2003), occurs predominantly as (1) a 37K N-terminal fragment, (2) a 57K C-terminal fragment, and (3) glycosaminoglycans (mainly chondroitin 4sulfate) are linked to the NH_2-terminal 37-kDa fragment of DMP1 via Ser^{74}, located in the Ser^{74}-Gly^{75} dipeptide and referred to as the dentin matrix protein-1 proteoglycan fragment (Qin *et al.*, 2006).

DMP1 was originally thought to be found only in dentin, but later it was detected in bone, cartilage, and non-mineralized tissues such as the brain, pancreas, kidney, and salivary glands (George *et al.*, 1995; Begue-Kirn *et al.*, 1998; Feng *et al.*, 2003; Ogbureke and Fisher 2004,2005,2007).Non-human DMP-1 has been cloned and sequenced from a number of animals (George *et al.*, 1993, Hirst *et al.*, 1997a, Macdougall *et al.*, 1998 Toyosawa *et al.*, 2000) as well as human DMP-1 (Hirst *et al.*, 1997b) . DMP1 is a multifunctional protein that has been found to regulate cell attachment (Kulkarni *et al.*, 2000), cell differentiation (Narayanan *et al.*, 2001 and Kalajzic *et al.*, 2004) to activate matrix metalloproteinase-9,(Fedarko *et al.*, 2004) and has been postulated to play a significant role in biomineralization (Ye *et al.*, 2004). *In vitro*, DMP1 acts as a hydroxyapatite (HA) crystal nucleator with very high calcium ion binding capability (He *et al.*, 2003). DMP1 also is involved in calcium and phosphate metabolism through the kidney (Terasawa *et al.*, 2004 and Gericke et al., 2010).

Recombinant bacterial DMP1 has been used in several studies (Kulkarni *et al.*, 2000, Narayannan *et al.*, 2003, Fedarko *et al.*, 2004), while to our knowledge no one produced it from transfection of a eukaryotic cell line. The FS 293 cells are human embryonic kidney cells that have been adapted (Graham *et al.*, 1977) to serum free suspension culture producing high levels of protein. (*Invitrogen* Catalogue, 2002). Western blotting for the

conditioned medium of this transfected cell line revealed the presence of DMP-1 which indicated the success of the transfection procedure while DMP-1 was not produced by cells transfected with the empty vector). Two types of transfection are commonly known, the permanent one (stable transfection) and the transient type. The stable transfection produce a consistent amount of protein while in the transient one, the level of protein is unknown. The consistent protein release from the stably transfected cell line (HT1080 unpublished data) can be used as co-culture in cell culture experiments. Our results indicated the success of both types of transfection and their consistency.

Column chromatography is one of the most common methods of protein purification. The acidic nature of the DMP-1 making it easily isolated using the DEAE column. Further purification of DMP-1 containing fractions detected by antibody staining was performed using a hydroxyapatite column and the results were in accordance to another study which used the same type of column in purifying the DSP. (Yamakoshi *et al.,* 2005)

Two types of stains were used to stain the SDS gels, the first was the Comassie Blue which detects proteins by their visualization as blue bands. The second stain was Stains All which differentiates between blue bands representing acidic glycoprotein and phosphoproteins (DMP-1), and red bands representing other proteins. (Kim *et al.,* 2006)

DMP1 is present in bone and dentin as proteolytically processed fragments; those are the 37-kDa fragment from the NH2-terminal portion and a 57-kDa fragment from the COOH-terminal region (Qin *et al.,* 2003). Cleavage of rat DMP-1 at the NH2 terminal leaves four aspartic acid residues. This cleavage pattern is similar to that of Dentin Sialo-Phosphoprotein (DSPP) where the fragment having two aspartic acid residues at the NH2 terminal is dentin sialoprotein DSP, and that from the COOH terminal is dentin phosphoprotein (Qin *et al.,* 2001). These cleavage explain the different forms observed for the DMP-1 using two types of antibody (Dr Larry Fisher's and Takara's inc.). The (57-KDa) fragment was seen in all our fractions using both types of Antibody. According to studies of in vitro mineralization, the COOH-terminal 57-kDa fragment has been shown to promote mineralization by acting as a nucleator for the formation of hydroxyapatite (Tartaix *et al.,* 2004; Lu et al., 2009). The existence of different forms is also supported by its presence at (95-KDa) as reported by Yu *et al.* (2006).

Further results obtained from the DEAE column seemed to contain a GAG chain which is in consistent with Yamakoshi *et al.,* (2005) who found this type of chain in porcine DSP, a similar acidic glycoprotein extracted from dentin. The availability of these chains may promote the hydration of the tooth as well as facilitate the molecular interaction between the different growth factors during dentin mineralization (Yamakoshi *et al.,* 2005).

It has been reported that in addition to its direct role in the formation and/or growth of hydroxyapatite crystals, DMP1, acting as a transcription factor, may be involved in regulating other genes associated with dentinogenesis and osteogenesis (Narayanan *et al.,* 2003 and Narayanan *et al.,* 2006).

DMP1 is highly expressed in the osteocytes embedded in bone matrix, and is associated with maintenance of the lacunarcanalicular system of these cells (Toyosawa *et al.,* 2001; Rios *et al.,* 2005; Feng *et al.,* 2006). DMP1 was increased in osteocytes in loaded bone, perhaps functioning in the mechanical response (Gluhak-Heinrich *et al.,* 2003; Yang *et al.,* 2005; Foster *et al.,* 2012). DMP1-null background resulted in osteomalacia, abnormal osteocyte maturation. The biological activity of DMP-1 in osteocyte maturation is mediated by its 57-kDa COOH-terminal fragment (Lu *et al.,* 2011).

DMP1 possesses endogenous biological activity that can be released by proteolytic cleavage. Matrix metalloproteinases (MMPs), which are able to cleave DMP1 into peptides of various molecular sizes, represent good candidates for regulating the function of extra cellular matrix (ECM) molecules during dentin development or during pulp repair after an injury such as carious decay. In addition, *in vivo* confirmation of the capacity of the C-ter peptide to promote cell differentiation opens a therapeutic interest of using this peptide to regenerate dentin after an injury. (Chaussain *et al.,2009).*

It was reported that exogenous DMP1 added to exposed dental pulp could act as a morphogen trigger and/or promoter of the differentiation of undifferentiated ectomesenchymal cells in the pulp toward the odontoblast lineage (Narayanan *et al.*, 2006).

Furthermore, it was reported that DMP1 is primarily localized in the nuclear compartment of undifferentiated osteoblasts, implying that DMP1 could act as a transcriptional component for the activation of osteoblast/odontoblast-specific genes, like osteocalcin (Narayanan *et al.*, 2003). Qin *et al.* (2006) studies has revealed the role of DMP-1 as a key player in the control of mineralization and Pi homeostasis, hence, its importance in osteogenesis and dentinogenesis.

The produced DMP1 protein will be used in dental and bone injuries. DMP1 protein osteogenic and dentinogenic effects will be the main goal in our future *in vivo* and *in vitro* studies which will shed new light on the manner in which DMP1 controls

osteogenesis and dentinogenesis in both healthy individuals and those with disease.

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