Production and Characterization of Recombinant Amelogenin Phosphorylation Mimics

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Abbreviations

ACP	Amphormous Calcium Phosphate
AMG	Amelogenin
cDNA	Complementary DNA
D or Asp	Aspartic acid
DLS	Dynamic light scattering
E or Glu	Glutamic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMP	Extracellular matrix protein
HAc	Acetic acid
HAP	Hydroxyapatite
KLK4	Kallikrein-4
MMP-20	Metalloprotenaise-20
PdI	Polydiversity index
R_H	Hydrodynamic radius
rH	Recombinant human
SAXS	Small-angle x-ray scattering
TRAP	Tyrosine- rich amelogenin peptide

1. Abstract

Amelogenin is an extracellular matrix protein which has an important role to play in enamel formation during tooth development. The need for amelogenin for research purposes in order to be utilized for its many potential applications have resulted in the rise of the production of recombinant amelogenin. So far, the most optimal expression system used for the production of recombinant amelogenin is *Escherichia coli*. The only difference between native amelogenin and recombinant amelogenin expressed using *E.coli* is that the latter lack methionine at the N-terminus and phosphate on Serine-16.The aim of this study was to produce recombinant amelogenin phosphorylation mimics and to investigate the effects of phosphorylation on amelogenin. A total of 16 recombinant amelogenin phosphorylation mimics were successfully constructed by site-directed mutagenesis where serine-16 was mutated to either aspartic acid or glutamic acid. There were difficulties encountered during the purification of the higher positively charged recombinant amelogenin phosphorylation mimics which indicated that factors other than optical density may have a vital effect on their production. No effect of phosphorylation was found on solubility as the phosphorylated amelogenins shared similar solubility profiles to their non-phosphorylated analogues on the pH range of 4 to 8. The solubility of the recombinant amelogenin fusion and phosphorylation mimics were greater than that of the recombinant native amelogenin and its phosphorylation mimic analogue. The analysis of the recombinant amelogenin phosphorylation mimics by dynamic light scattering showed that they too can form nanospheres. There were minor variations in hydrodynamic radii and polydispersity indices of the recombinant phosphorylated and non-phosphorylated amelogenins at both 20^{o}C and 37^{o}C which indicated that phosphorylation had not affected amelogenin self-assembly. It was found that the recombinant amelogenin phosphorylation mimics as well as their non-phosphorylated analogues had similar strong apatite-binding affinity which also showed no effect of phosphorylation on the binding affinity of amelogenin.

2. Introduction

Amelogenin is an extracellular enamel matrix protein (EMP) involved in biomineralization of the enamel which is the hardest tissue of the human body. Amelogenin, both in the native and recombinant form can be used for numerous applications such as periodontology, wound healing, bone formation and regeneration, implantology and endodontics (Lyngstadaas et al., 2009). Another interesting trait is that recombinant amelogenin can be easily modified and also purified based on its solubility properties with little use of chemicals (Bonde et al., 2012). Bonde et al. showed that amelogenin can be used as a purification tag for production of other proteins by using the same method used for amelogenin purification. This shows great potential for amelogenin in biomedical/ biotechnological applications.

Several expression systems have been used in attempt to produce recombinant amelogenin with great similarity to the native protein. However, *Escherichia coli* expression system has provided most promising results in which extra amino acids are not added. In contrast to its native analogue, recombinant amelogenin expressed using *E.coli* lack methionine at the N-terminus and phosphate on Serine-16 (Simmer et al., 1994). This was first reported by Simmer et al. in 1994; it was reported that murine cDNA encoding M180 was cloned and expressed in *E.coli* which resulted in the production of rM179. The loss of N-terminal methionine and phosphorylation on Serine-16 was suggested to be as a consequence of *E.coli* aminopeptidase. So far, there have been no reported adverse effects caused by the loss.

This present study aims to produce recombinant amelogenin phosphorylaton mimics and to investigate the effects of phosphorylation of amelogenin on its properties. Therefore, site-directed mutagenesis of Serine-16 to aspartic acid (Asp, D) or glutamic acid (Glu, E) was performed in order to promote negative charges and to mimic the effect of the phosphorylated serine in the native amelogenin. The mutagenesis was carried on eight recombinant amelogenin constructs which were (i) rH174, (ii) rH163, (iii) rH146 (iv) rH174+3, (v) rH174+6, (vi) rH174+9, (vii) rH174+12, and (viii) rH174+15 in order to produce recombinant amelogenin phosphorylation mimics (Table 1).

The primary recombinant human protein, rH174, used in this study is expressed from a gene encoding a 175 amino acid long amelogenin of which the N-terminal methionine is cleaved off during its expression in *E.coli* cells. In vivo amelogenin is proteolytically degraded by proteinase to form amelogenin cleavage products. In this study, the recombinant cleavage product equivalents used were rH146 and rH163. The recombinant amelogenin fusion proteins (rH174+3, rH174+6, rH174+9, rH174+12 and rH174+15) are a combination of rH174 and protein fusion tag added to the C- terminal end of the protein. The tag is comprised of the protein sequence KTKR (Lys-Thr-Lys-Arg) of which three of the amino acids are positively charged and hydrophilic (Lys and Arg).Thus, the addition of one tag to the C-terminal end will create an extra three positive charges. Following mutation, cultivation and purification, various properties of several of the recombinant amelogenins were characterized in order to investigate the effect of the phosphorylation on amelogenin.

2.1 Amelogenesis

Amelogenesis is the process during tooth development where the enamel is formed in the extracellular enamel matrix (ECM) (Fincham et al., 1999). The mature enamel is composed primarily over ninety-five percent of an organic content that is carbonated hydroxyapatite (HAP) and negligible amounts of matrix protein. The mature enamel is composed of long, narrow calcium HAP crystals with a length of about 100 µm and a width and thickness of about 70 x 30 nm (Daculsi et al.,1978 & 1984).These crystals are packed into parallel arrays referred to as enamel rods with a diameter of about 5 µm. The rods are separated by interrod enamel which is also composed of HAP intersecting with the enamel rods at 60 ^{o} (Simmer et al., 1995).

The ECM contains enamel matrix proteins, such as amelogenin, non-amelogenin proteins and inorganic ions (e.g. Ca^{2+}, $PO^{4-)}$ necessary for enamel formation. During amelogenesis, amelogenin is secreted from oral epithelium cells called ameloblasts, to regulate the growth of hydroxyapatite crystals of the enamel. 90 % of the organic content of the developing ECM contains amelogenin and the remaining 10 % are non-amelogenin proteins (Fincham et al., 1999). The ameloblasts also express and secrete other enamel extracellular protein e.g. metalloprotenaise-20 (MMP-20, also known as enamelysin) and kallikrein-4 (KLK4, also known as enamel matrix serine protease 1, EMSP1 (Simmer et al., 1998; Fincham et al., 1999; Hu et al., 2002).

During the early secretory stage of enamel formation, long, thin ribbons of enamel minerals are formed shortly after the secretion of EMP by the ameloblasts (Smith, 1998). During enamel formation, amelogenin is gradually digested by proteolytic enzymes resulting in the formation of mature enamel. The proteolytic degradation is carried out by MMP-20 which is expressed during the secretory stage of' enamel formation (Barlett et al., 1998) and KLK4 which is expressed during the maturation stage in the late stage of the enamel formation (Hu et al., 2002). MMP-20 initiates the breakdown whereas KLK4 completes the degradation process hence completely removing amelogenin from the enamel matrix (Simmer and Hu, 2002).During the maturation stage, the EMP are almost completely removed and the mineral ribbons thicken and widen to form the enamel (Smith, 1998).

2.2 Structure of Amelogenin

In vivo amelogenin polypeptides can have a mass from 5 to 25 kDa which are produced as a result of proteolytic degradations, alternative splicing and due to the localization of two distinct single copy of the gene on either X or Y chromosome (Lau et al. 1992; Ribeiro do Espírito Santo et al., 2005). The human amelogenin gene consists of seven exons and most of the proteins formed originate from the X chromosome such as HX 175. Amelogenin and its cleavage products consist of mainly of proline (25%), glutamine (14%), leucine (9%) and histidine (7%).

Amelogenin has three domains which are as follows: (1) a 45 amino acids long tyrosine rich N-terminal hydrophobic domain (TRAP) (2) a hydrophobic central region which mainly consists of Xxx- Yyy- Pro repetitive motifs and (3) an 11 amino acids long C-terminal hydrophilic domain (Fincham et al., 1999; Toyosawa et al., 1998; Margolis et al., 2006). The primary structure of the N and C terminal ends are

highly conserved among species but there exist variations in the middle section which are primarily deletions and insertions of Xxx-Yyy-Pro (Brooks et al., 1994).

Although the three-dimensional structure of amelogenin has not been determined, numerous techniques have been used to provide vital data on the structure of amelogenin. Numerous studies have shown that secondary structures of amelogenin consist of a large fraction of β sheets, turns and random coils whereas minor fraction is of α-helix (Zhang et al., 2011; Margolis et al., 2006). The N-terminus consists primarily of β sheets; the mid-section is rich in polyproline II and β turns such as β spirals and the C-terminus consist of random coils (Goto et al., 1993; Matsushima et al., 1998).

2.3 Characteristics of Amelogenin

2.3.1 Solubility of Amelogenin

Amelogenin has low solubility at physiological pH around 7 which may be accounted for by its tendency to form nanospheres in vivo. However, the solubility is higher at weak acidic and alkaline pH (Tan et al., 1998 (b)). Several of amelogenin cleavage products have been studied and it was found that the removal of hydrophilic C-terminus causes the solubility of amelogenin to be lower under alkaline conditions than when the C terminus is present (Tan et al., 1998). In general, the solubility of amelogenin cleavage products are very high at acidic pH but TRAP exhibited an exception; instead, TRAP has low solubility from pH 4 to 9 (Tan et al., 1998(a)). Furthermore, the results in Tan et al., 1998 (b) showed native phosphorylated amelogenin and non-phosphorylated amelogenin have a difference in solubility. More specifically, the phosphorylated amelogenin has a lower solubility in alkaline pH in comparison to the phosphorylated amelogenin.

2.3.2 Biomineralization

In vitro studies have been carried out using HAP to investigate the interaction between amelogenin with enamel crystals. These studies have shown that the nanospheres provide a well-organized structure to modulate the formation of enamel apatite crystals (Lyngstadaas et al., 2009; Lyngstadaas et al., 1995). An early study by Aoba et al., 1987 has showed that the full-length intact amelogenin has a higher affinity for HAP than the proteolytically degraded amelogenin fragments. Likewise, a similar finding has also been reported by Moradian-Oldak et al., 2002 for recombinant amelogenin. More specifically, Bouropoulos et al., 2003 reported that full-length amelogenin nanospheres adsorb unto the apatite crystals' surfaces as binding units with specific adsorption sites. Amelogenin covers 64% of the total area unit of the apatite binding surface which supports the hypothesis that amelogenin selectively bind to certain sites on the apatite surface. Additionally, Moradian-Oldak et al., 2002 reported that the removal of the C-terminal of amelogenin has been shown to decrease amelogenin HAP binding affinity (Moradian-Oldak et al., 2002).

Wiedemann-Bidlack et al., 2011 has found that phosphorylation of amelogenin has a vital impact on calcium phosphate formation in vitro. Unlike the non-phosphorylated analogue, the phosphorylated full-length amelogenin was able to stabilize amphormous calcium phosphate (ACP) for long period time before its transformation

to HAP crystals (Kwak et al., 2009; Wiedemann-Bidlack et al., 2011). This finding is noteworthy because studies have found that during enamel formation ACP is the first mineral phase formed that subsequently transforms to apatite crystals (Beniash et al., 2009). In contrast, a recombinant amelogenin cleavage product had no effect on calcium phosphate. However, when a phosphorylated analogue of the cleavage product was used, ACP was stabilized for 24 hours but still did not transform to HAP (Kwak et al., 2009; Wiedemann-Bidlack et al., 2011).Therefore, these findings collectively show that phosphorylation of full length amelogenin has an important role to play in biomineralization.

2.3.3 Self-assembly of amelogenin molecules

Self–assembly which is a common property of extracellular organic matrix molecules is the process by which components of a system assemble together to form a larger functional unit which is facilitated by specific intermolecular interactions. Amelogenin self- assembly plays an important role in regulation of the growth and organization of the enamel. There are two hypothetical models to describe amelogenin self-assembly. More specifically, these models describe amelogenin as having the ability to self-assemble into either nanospheres or nanoribbons. These models are described below.

Amelogenin Nanospheres Formation

Amelogenin self-assembles into hydrophobic supramolecular aggregates, referred to as nanospheres that form an insoluble extracellular matrix (Eastoe, 1979, Fincham et al., 1994). A study by Aichmayer et al., 2005 which used a combined approach of DLS and SAXS (small-angle x-ray scattering) to investigate amelogenin self-assembly proposed that nanospheres possess dense hydrophobic cores surrounded by loose shells comprised of negatively charged hydrophilic C-terminus that is exposed to the aqueous surrounding. It has also been suggested that nanospheres can interact via their C-terminus to agglomerate in order to form high order chain-like structures (Aichmayer et al., 2005). A more recent study by Aichmayer et al., 2010 showed that amelogenins deviate from the spherical nanopaticles and forms instead oblate nanoparticles.

A study by Wiedemann-Bidlack et al., 2011 has proven that phosphorylated full length amelogenin can form high-order aggregate structures similar to its non-phosphorylated analogue under physiological conditions of ionic strength (165 mM) and temperature (37^{o}C). It was found that phosphorylation had a small but potentially vital effect on the pH at which the amelogenin begin to form high-order structures. This importance is linked to the fact that high-order amelogenin self-assembly is sensitive to pH. Studies have shown that generally amelogenin tend to form high-order protein assemblies at pH levels near their respective isoelectric points (Wiedemann-Bidlack et al., 2011, He at al., 2008). It is also important to note that Tan et al. 1998 (b) found that amelogenins tend to have the lowest solubility near their isoelectric point.

Studies have shown that both N- and C- terminal are important for amelogenin self assembly (Paine et al., 2003). The removal of either N- or C- terminal ends affect amelogenin nanosphere formation which in turn results in a defective structural organization of the enamel. The amelogenin cleavage products lacking the

hydrophilic C-teminal form larger and less ordered agglomerates than full length amelogenin at higher pH levels of around 7.6-7.7 (Wiedemann-Bidlack et al., 2011; Wiedemann-Bidlack et al., 2007). These findings showed that the hydrophilic C-terminus of both native and recombinant amelogenin plays an important role in the formation of higher-order chain-like assemblies, as was found in previous studies (Aichmayer et al., 2005; Moradian-Oldak et al., 2006).

Dynamic Light scattering (DLS) was used in this study to investigate the effect of phosphorylation on amelogenin self-assembly under two temperatures at a constant pH. DLS measures the size of the aggregate particles in solution that is reported as hydrodynamic radii (R_H) which is the mean particle size and polydispersity (PdI) which is based on the standard deviation around that mean.

Amelogenin Nanoribbons Formation

Studies carried out by Martinez-Alva et al. have shown that amelogenins can form nanoribbons which are facilitated by the interaction of their hydrophobic N-termini (Martinez-Avila et al. 2011 & 2012). The assemblies of nanoribbons are formed by gradually combining and elongating together to form bundles structurally similar to the mineral crystallites in mature enamel. The amelogenin molecules combine together in anti-parallel arrangement with the hydrophilic C-terminus oriented towards the ribbon edges. Thus, these arrangements result in the accumulation of electrostatic charges at the edges which give rise to electrostatic repulsion of amelogenin nanoribbons and in turn, cause their parallel arrangement into bundles. However, self-alignment of the dimers was not observed for amelogenin lacking the hydrophilic C-terminus.

Martinez-Alva et al., 2012 also showed that amelogenin nanoribbon formations are affected by pH, presence of calcium and phosphate ions and amelogenin concentration. It was evident that ribbons were able to form between pH 4.0 to 6.0 but were not formed at a pH below 4.0 (Martinez-Avila et al. 2012). However, pH above 6.0 resulted in large amount of calcium phosphate mineral which obscured the observation of amelogenin structures formed. Therefore, there have been no conclusive experimental results which indicate that nanoribbons are formed above a pH level of 6.0.

In addition, it was found that the presence of both calcium and phosphate ions resulted in the instability and disintegration of amelogenin nanospheres but simultaneously the growth of amelogenin nanoribbons within several days (Martinez-Avila et al. 2012). When the mineral ions were added individually, there were no nanoribbons but the formation of nanospheres were promoted. Furthermore, exposure of EDTA to amelogenin ribbons suspensions resulted in the disintegration of the ribbons but formation of nanospheres. This further supports that calcium is needed for the stability of amelogenin nanoribbon structure

2.3.4 Amyloid Fibril Formation

Amyloid fibrils are ordered aggregates of proteins which organize themselves in a fibrillar structure (Nilsson, 2004). As previously described, studies have shown that amelogenin form filaments that adopt a β-structure (Goto et al., 1993; Matsushima et al., 1998). These structures are the same as those found in amyloid fibrils.

Three criteria need to be satisfied by a protein which forms amyloid fibrils. These include: (i) green birefringence upon staining with Congo red, (ii) fibrillar morphology and (iii) β sheet secondary structure (Nilsson, 2004). The most common methods utilized to identify the presence of amyloid fibrils are Thioflavin T (ThT) fluorescence, Congo Red binding, transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). In this study, we tested amyloid formation for amelogenin nanoribbons using ThT assay. This assay is not one of the criteria but it is relatively accepted as an indicator of the presence of amyloid. ThT assay functions by measuring the changes in fluorescence intensity upon binding with the amyloid fibrils and this enhanced fluorescence can be viewed by fluorescence microscopy or spectroscopy.

3.Materials and Methods

3.1 Site Directed Mutagenesis of Amelogenin

3.1.1 Expression

Site-directed mutagenesis of amelogenin was performed by replacing serine-16 with either D or E in each of eight non-phosophorylated amelogenin constructs. A total of sixteen amelogenin phosphorylation mimics were produced by the mutation which are all considered as phosphorylated amelogenin in this study (Table 1).

Table 1. Recombinant amelogenin phoshorylation mimics successfully produced by site-directed mutagenesis of serine-16 to aspartic acid and glutamic acid.

Template	C-terminal Modification	Mutation	Mutant
rH174		Ser16→Asp	rH174D
rH174		Ser16→Glu	rH174E
rH146		Ser16→Asp	rH146D
rH146		Ser16→Glu	rH146E
rH163		Ser16→Asp	rH163D
rH163		Ser16→Glu	rH163E
rH174+3	KTKR-fusion	Ser16→Asp	rH174+3D
rH174+3	KTKR-fusion	Ser16→Glu	rH174+3E
rH174+6	KTKRKTKR-fusion	Ser16→Asp	rH174+6D
rH174+6	KTKRKTKR-fusion	Ser16→Glu	rH174+6E
rH174+9	KTKRKTKRKTKR-fusion	Ser16→Asp	rH174+9D
rH174+9	KTKRKTKRKTKR-fusion	Ser16→Glu	rH174+9E
rH174+12	KTKRKTKRKTKRKTKR-fusion	Ser16→Asp	rH174+12D
rH174+12	KTKRKTKRKTKRKTKR-fusion	Ser16→Glu	rH174+12E
rH174+15	KTKRKTKRKTKRKTKRKTKR-fusion	Ser16→Asp	rH174+15D
rH174+15	KTKRKTKRKTKRKTKRKTKR-fusion	Ser16→Glu	rH174+15E

The site-directed mutagenesis was performed using polymerase chain reaction (PCR).Two sets of primers were designed to mutate serine-16 to D or to E. The following primers were used:

PhosMimic_Asp_Forw	5'- CAT CCA GGT TAT ATC AAC TTC GAT TAC GAG GTC TTG ACC CCT-3'
PhosMimic_Asp_Rev	5'-AGG GGT CAA GAC CTC GTA ATC GAA GTT GAT ATA ACC TGG ATG-3'
PhosMimic_Glu_Forw	5'- CAT CCA GGT TAT ATC AAC TTC GAA TAC GAG GTC TTG ACC CCT-3'
PhosMimic_Glu_Rev	5'-AGG GGT CAA GAC CTC GTA TTC GAA GTT GAT ATA ACC TGG ATG-3'

The annealing temperature of 62 °C was found by testing the designed primers using conventional PCR. For a single amino acid mutation, the 50 µl PCR reaction contained 0.5 µl of plasmid with rH174 gene (Bonde et al., 2012), 1µl of 2.5 units/µl Pfu DNA polymerase, 0.5 µl of 0.5 mM dNTP, 0.5 µl each of 1 µM forward and reverse primers, 42 µl of deionised water and 5µl of 1x PFU buffer. The PCR cycles were initiated at 95 °C for 5 minutes and followed by 16 amplifications cycles which consisted of 95 °C for 30 seconds, 62 °C for 30 seconds and 72 °C for 12 minutes. The final extension was done at 72 °C for 5 minutes.

3.1.2 Transformation

The PCR products were treated with 1 µl Dpn I at 37°C for one hour. An aliquot of 10 µl PCR product was used to transform previously prepared *E.coli* TGI competent cells, by heat shock at 42 °C for 60 seconds. The cells were incubated at 37°C for 45 minutes in LB medium. The transformed cells were spread on LB plate containing 100 µg/ml ampicillin and incubated at 37 °C overnight.

3.2 Colony Screening

3.2.1 Colony PCR and Sanger DNA Sequencing

The purpose of colony PCR was to screen for plasmids harbouring the desired DNA insert in the plasmid constructs from the bacterial colonies. Colony PCR was performed in parallel to making master plates by streaking selected bacterial colony on an agar plate followed by rinsing the inoculation loop with the transformant in PCR reaction mix. Following PCR amplification, the PCR products are analyzed by agarose electrophoresis to select the positive colonies. The positive colonies were selected from the different constructs for plasmid purification and were verified by Sanger DNA sequencing.

3.3 Cultivation and Expression

Each construct was transformed into the expression host, *E.coli* BL21 (DE3).The expression host cells were inoculated in 10 ml LB-media supplemented with 200 ampicillin and were grown overnight in a shaking incubator at 37°C. These cultures were then used to inoculate TB-media supplemented with 200 ampicillin, on the basis that, 100ml TB-medium should be inoculated with 0.5 ml overnight culture of OD_{600} of 3. Once the cultivations reached an optical density of

1.8-2.0, the cultures were induced by adding IPTG to reach a final concentration of 1 mM. The cultivations were carried out overnight in a shaking incubator at 37°C and rotation speed of 150-170 rpm.

3.4 Purification of Amelogenin- Constructs

3.4.1 Acid/heat Treatment

Cells cultivated for 24 h were harvested by centrifugation (5000g, 5 min) and were washed with 150 mM NaCl followed by another centrifugation (5000g,5 min). The cell pellets were resuspended in 3% acetic acid (HAc), which was followed by 20 minutes of heat treatment at 80 °C in a water bath. The cell suspension were centrifuged (20 000 g, 40 min) and the supernatants containing soluble amelogenin were collected. The supernatants were dialyzed against 0.05% HAc.The dialyzed amelogenin samples were sterile filtered and aliquoted into 1.5 ml cryotubes. All recombinant amelogenin variants were lyophilized for proper storage.

3.5 Amelogenin Concentration

The total protein concentrations were determined by using Bradford assay (1ml Bradford reagent which was diluted 5x and 10 µl protein sample). The protein standard curve was made using 50 mg/ml rH174 which was diluted to concentration of 1.0, 0.75, 0.50 and 0.25 mg/ml.

3.6 Characterization of Amelogenin Constructs

A few of the amelogenin variants were chosen for characterization. These included: (i) rH174, (ii) rH174D, (iii) rH174+3, (iv) rH174+6, (v) rH174+9, (vi) rH174+9D, (vii) rH174+9E, (ix) rH174+12, (x) rH174+12D, (xi) rH174+15 and (xii) rH174+15D. The properties of the aforementioned phosphorylated (ii, vi, vii, x, xii) and non-phosphorylated (i, iii, iv, ix, xi) amelogenins were compared to evaluate the effects of phosphorylation on amelogenin properties.

3.6.1 SDS-PAGE

During the cultivation and acid/heat purification, samples were collected from the uninduced cells (500 µL), induced overnight culture (250 µL) and the amelogenin supernatant (50 µL). The OD_{600} of the induced cells and overnight cultures were measured in order to calculate the amount of SDS-loading buffer necessary for resuspension. The cells were harvested by centrifugation (8000 rpm, 1 minute). Subsequently, the cell pellets were resuspended in SDS-loading buffer to acquire a cell density equal to an OD_{600} of 10. In addition, the lyophilized proteins were also analyzed by SDS-PAGE. The lyophilized proteins were first dissolved in 0.05% HAc by incubating them in ice for three days. Prior to analysis on a 4-20 % SDS-PAGE gel, the samples were first boiled for 5 minutes.

3.6.2 Solubility Test

Solubility was tested in the presence and/or absence of either calcium and/or phosphate at pH range of 4-8. Amelogenin solutions were prepared by dissolving

lyophilized proteins (10 mg) in 0.05% HAc to a final concentration of 50 mg/ml and then incubated overnight on ice at 4 °C. Buffer stock solutions with a concentration of 100 mM were prepared at pH4 and pH5 using HAc, pH6 using MES and pH 7 and 8 using HEPES. Calcium and phosphate stock solutions were prepared using $CaCl_2$ and KH_2PO_4 to a concentration of 3M each, which in turn, were diluted to final concentration of 33.4 mM and 20.9 mM respectively using the buffers at various pH. Mixtures of a total volume 75µl were prepared by adding 33.8 µl of diluted calcium or phosphate buffer solutions and/or buffer to 7.5 µl of amelogenin, followed by three days of incubation at 37 °C. The mixtures were centrifuged (13 000 rpm, 4 min) and the protein concentration of the supernatant was measured at 280 nm using sub-microliter cuvette (Implen).

3.6.3 Dynamic Light Scattering (DLS)

Amelogenin solutions were prepared as previously described above. Amelogenin samples were further diluted to a final concentration of 0.5 mg/ml using PBS buffer (pH 7.4) and water. A pH of 7.4 was chosen for this study because it corresponds closely to the pH found for the enamel fluid phase at the secretory stage (Aoba and Moreno, 1987). The samples were centrifuged for 3 minutes at 13000 rpm and then incubated at 37 °C for 4 hours. After 4 hours of incubation, DLS measurements were made. Subsequently, the amelogenin samples were incubated at 20 °C. The particle size measurements of the amelogenin constructs were carried out both at 37 °C and 20 °C using Zeta Nano S (Malvern Instruments Ltd, England).

3.6.4 Adsorption of Amelogenin onto Hydroxyapatite

Calcium hydroxyapatite (HAP) stock suspensions were equilibrated in PBS to final concentrations of 50 and 25 mg/ml. To obtain the HAP crystals, the stock suspensions were centrifuged and supernatant were discarded. Amelogenin (1mg/ml in PBS) were mixed with the HAP crystals. Samples were collected at various times and centrifuged to obtain the supernatant for analysis. The protein concentrations were measured using Bradford assay.

3.6.5 Amyloid Formation in Amelogenin Nanoribbons

Amyloid formation was studied in the presence and/or absence of either calcium or phosphate at pH 5. The protein stock was prepared by solubilising lyophilized amelogenin in 10mM HCL to a final concentration of 50 mg/ml. The 100 µl protein assembly sample was prepared by adding the components in the following order 2mg/ml amelogenin stock solution, deionised water, 20.9 mM potassium dihydrogen phosphate, 100 mM HAc (pH 5), 33.4 mM calcium chloride and 0.4 mM potassium hydroxide (KOH).The protein assembly samples were incubated at 37°C and 10 µl samples for testing were taken at various times (1h, 4h, 18h, 2 days and 3 days). The stock solution of thioflavin T (ThT) was prepared as described by Nilsson 2004. On the day of fluorescence measurement, the ThT was diluted 50, 25, 10, 5 times. Ten µl of amelogenin protein assembly sample and 90 µl ThT were mixed in a cuvette for the analysis. The fluorescence intensity was measured by excitation at 440 nm and emission 460 nm to 520 nm.

4.Results

4.1 Site-Directed Mutagenesis

In this study, a total of 16 novel recombinant amelogenin proteins were constructed which we refer to them as recombinant amelogenin phosphorylaton mimics. Two recombinant native amelogenin phosphorylation mimics, four recombinant amelogenin cleavage product phosphorylation mimics and ten recombinant amelogenin fusion phosphorylation mimics were produced from the site-directed mutageneses which are listed in table 1.

To check whether site-directed mutagenesis was successful, agarose electrophoresis was used to analyze a few of the PCR products. It can be observed from the agorose gel that there were distinct bands in each lane which indicated that the amelogenin gene is present (data not shown).All sequences of the constructs were compared to the expected sequence using sequence alignment in order to verify that two sequences are the same. The results showed that all the sequences matched the expected target sequences which mean that the mutation was successful.

4.2 Expression and Purification

Due to the numerous numbers of recombinant amelogenins, a few amelogenin constructs were chosen for larger scale production and characterization. All the amelogenin constructs migrated appropriately to their size (Fig.2). For the first cultivation, it can be seen from figure 2 that all the amelogenin constructs were expressed at induction optical density between 1.5-1.8. The band showing expression of rH174+15 was the most distinct with little impurities in comparison to the other amelogenin constructs. Although all the proteins were expressed, rH174+15 as well as rH174+15D produced no protein after purification in comparison to the remaining amelogenin constructs. The amelogenin construct, rH174+9D, did not show a high yield in comparison to rH174D, rH174+6D and rH174+9E (Fig. 2).

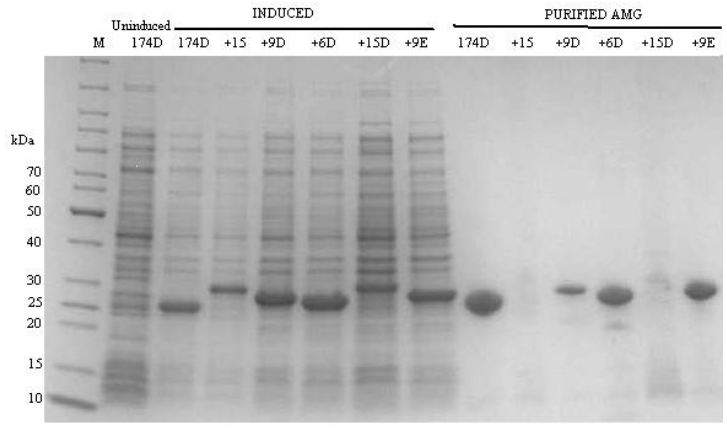


Figure 1. SDS-PAGE analysis of expression and purification of recombinant amelogenin constructs. Lane marked M is the protein molecular weight marker. Uninduced refers to before the addition of IPTG. Induced refers to after the addition of IPTG for induction. Purified AMG represents the amelogenin proteins after acid/heat treatment.

Due to low production of rH174+15 and rH174+15D amelogenin constructs, small scale cultivation was carried out for optimization of their production. To find out the optimal optical density for induction, the culture was induced at five different optical densities which are 1.0, 1.5, 2.0, 3.5 and 4.0. It was observed that rH174+15 and rH174+15D amelogenin constructs were expressed at induction optical densities of 1.0 1.5 and 2.0 (Data not shown). However, at 1.0 and 1.5 protein expression was low in comparison to smaller amelogenin constructs (Fig.3); at 2.0, no proteins were expressed.

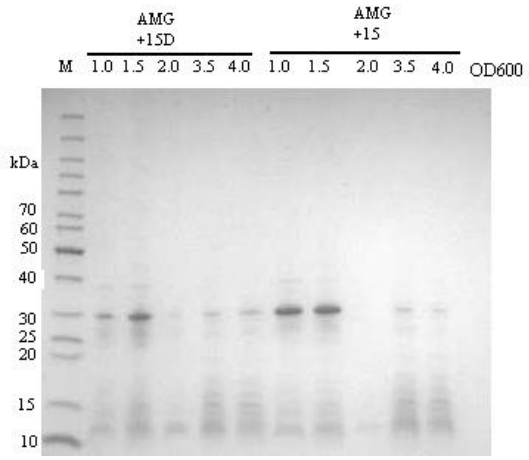


Figure 2. SDS-PAGE analysis of purification of rH174+15 and rH174+15D amelogenin constructs. Lane marked M represent the protein molecular marker. Lanes marked 1.0 to 4.0 represent the induction optical densities tested. AMG stands for amelogenin. The two constructs were analyzed after their purification by acid/heat treatment.

There was another cultivation done to verify the optimal induction optical densities for production of rH174+9D, rH174+15 and rH174 +15D. An additional recombinant amelogenin (rH174+12D) was tested. Surprisingly, rH174+15D, rH174+12D and rH174+15 were successfully induced at induction optical density ranging from 1.8 to 2.0 but with a low yield (Fig.4A). It can be seen that an induction optical density range from 1.4 to 1.8 is suitable for the cultivation of rH174+9D (Fig 4B). This observation suggested that there might be other factors that play vital role in the cultivation of more positively charged recombinant amelogenin.

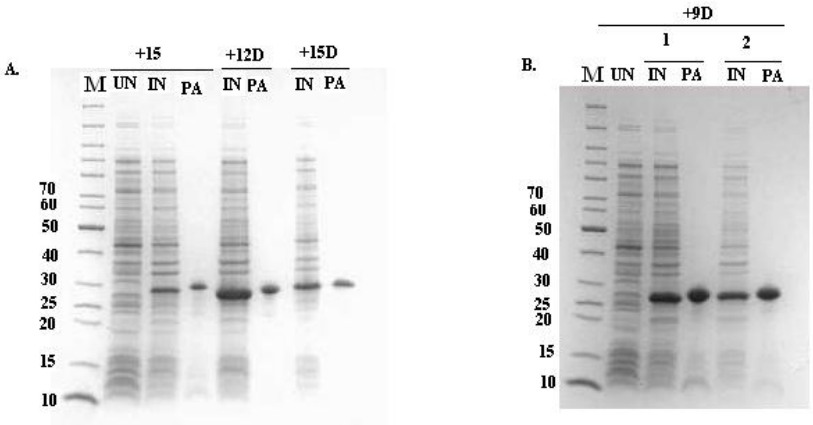


Figure 3 .SDS-PAGE of (A). rH174+12D, rH174+15 and rH174+15D (B). rH174+9D amelogenin constructs, following cultivation and purification. Lane marked M represents protein marker. UN represents uninduced. IN represents induced. PA represents purified amelogenin by acid/heat treatment. Number 1 represents OD600 1.790. Number 2 represent OD600 1.410.

To check the purity of the recombinant amelogenin constructs, lyophilized proteins were solubilised and were analyzed by SDS-PAGE. From figure 5, it can be seen that all the selected recombinant amelogenins for characterization were purified successfully. However, there were minor bands both below and above the major protein bands for all the amelogenins except rH174 and rH174D (Fig 5). Furthermore, the presence of the minor bands became more prominent with increased positive charges of the recombinant amelogenin (Fig.5).

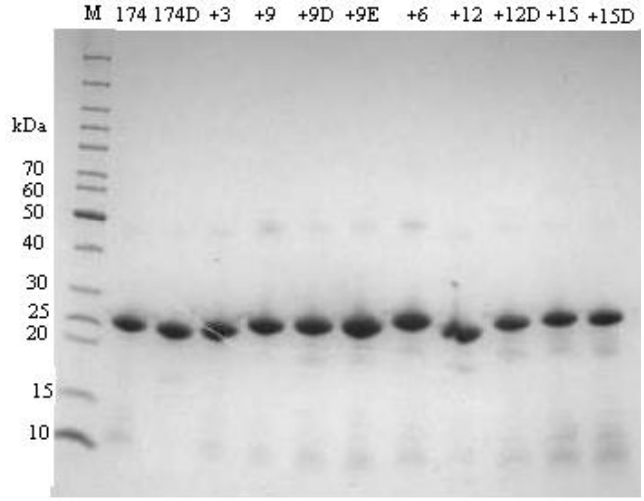
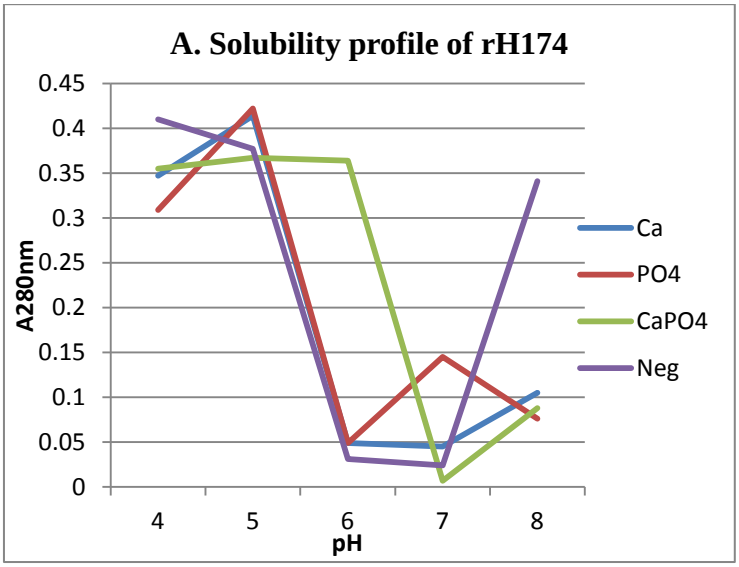


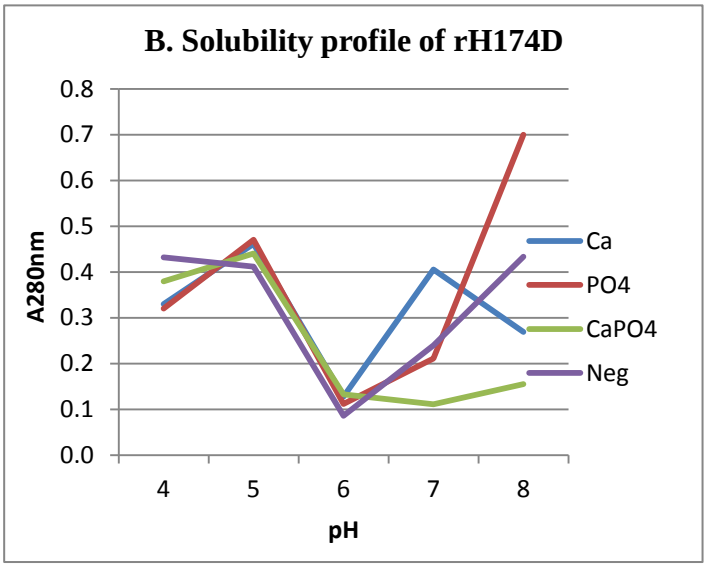
Figure 4. SDS-PAGE analysis of the recombinant amelogenin variants following purification. Lane marked M represents protein molecular marker. Lane 2 represents rH174. Lane 3, 6, 7, 10 and 12 represents amelogenin phosphorylation mimics. Lane 4,5,8,9 and 11 represents amelogenin fusion proteins.

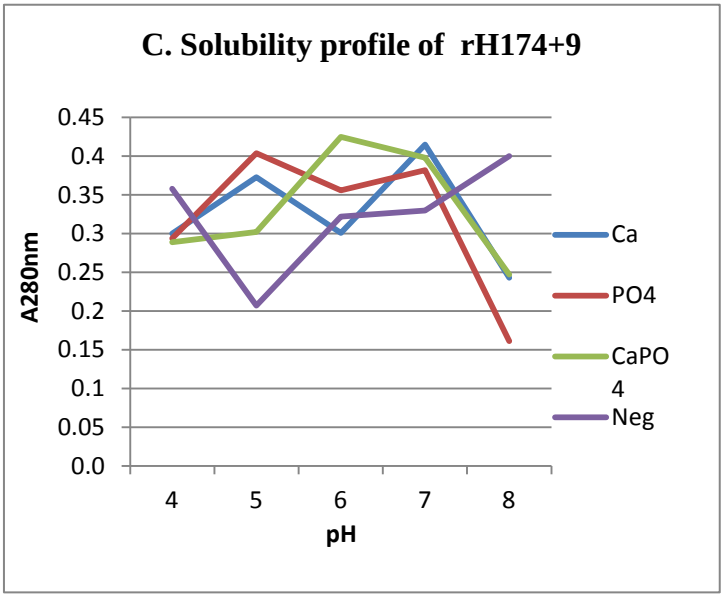
4.3 Solubility Profiles

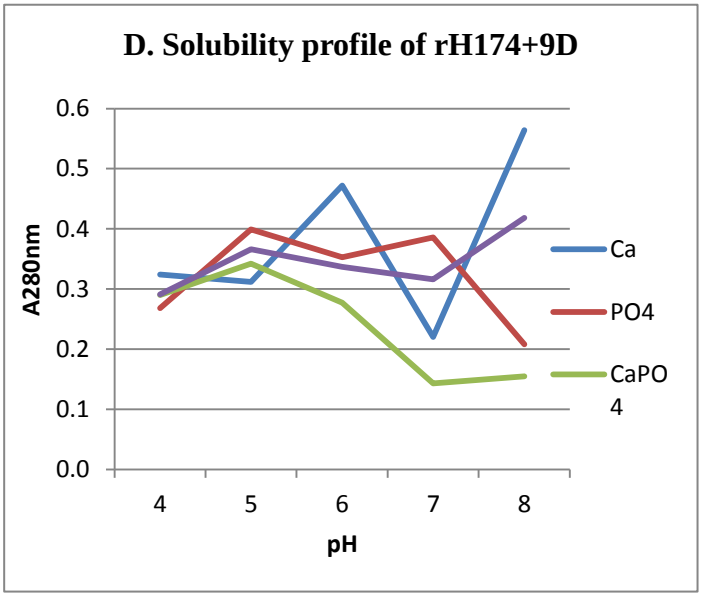
The purpose of the solubility test was to test the effect of the mutation on the solubility of amelogenin. A comparison of the solubility profile of several recombinant amelogenins over the pH range of 4 to 8 in the presence and absence of either calcium or phosphate ions was carried out in order to study the effect of the phosphorylation on amelogenin solubility. Although, the data on solubility of amelogenin was difficult to interpret, yet, efforts were made to draw out some points. The highest solubility for rH174 and rH174D were apparent when the pH was acidic, around 4 to 5 (Fig.6A & B). The solubility of rH174 and rH174D were lowest when the pH was close to neutral (Fig.6A & B). As the pH increased to alkaline, the solubility increased but it was still lower than that of acidic conditions ((Fig.6A & B). Thus, based on the results, it was found that the amelogenin, rH174 and rH174D shared similar solubility profiles.

The solubility of rH174+9, rH174+9D and rH174+9E remained relatively higher than that of the rH174 and rH174D at all pHs (Fig.6 C, D & E) and four mineral conditions (Fig.7). The solubility curves of rH174+9, rH174+9E and rH174+9D all tend to fluctuate slightly over pH range of 4-8 (Fig. 7).Thus, there were no significant differences between the solubility profile of the phosphorylation and non-phosphorylation amelogenins of rH174+9 (Fig.6) for all the conditions tested.









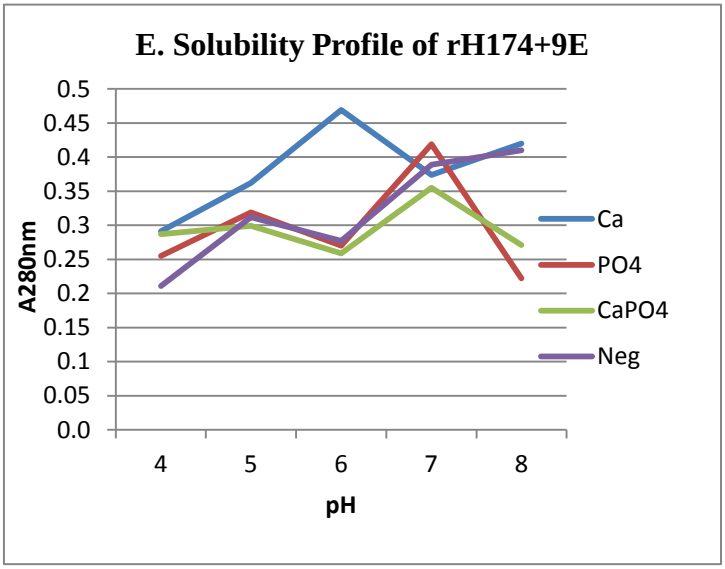


Figure 5. Solubility of recombinant amelogenin proteins at various pH in the presence of calcium (Ca^{2+}, blue), presence of phosphate (PO_4, red), presence of both calcium and phosphate ($CaPO_4$, green) and in the absence of calcium and phosphate (Neg, purple).

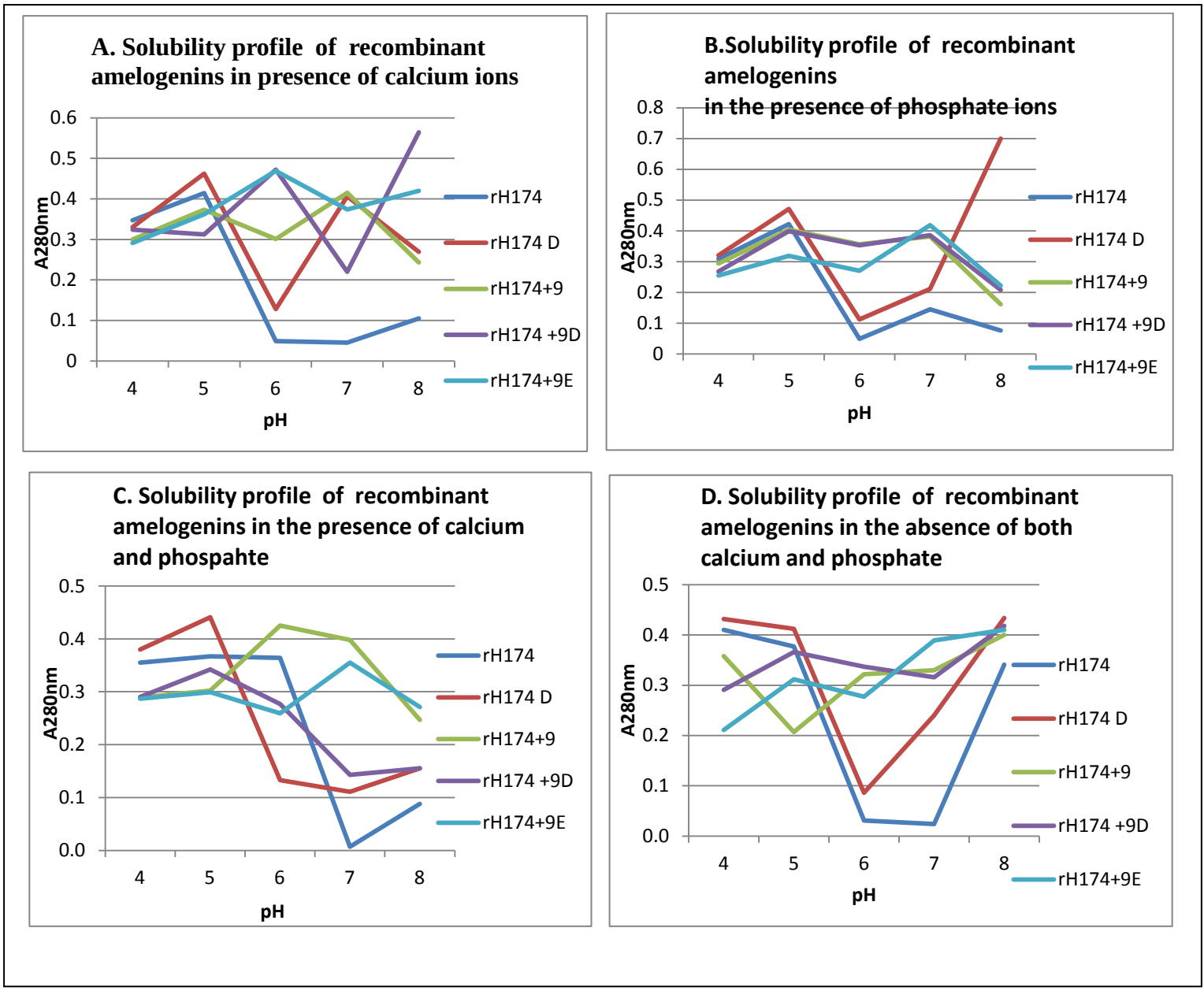


Figure 7. Comparison between solubility profile of recombinant amelogenin in the (A) presence of calcium (B) presence of phosphate (C) presence of calcium and phosphate and (D) absence of calcium and phosphate. Orange, red, green, purple and blue colour represents the recombinant amelogenin proteins.

4.4 DLS Analysis

The hydrodynamic radius (R_H) and polydispersity index (PdI) of the recombinant amelogenins were measured at temperatures of 20^{o}C and 37^{o}C at pH 7.4 in order to check the effect of phosphorylation on amelogenin self-assembly. There were small variations in R_H of each amelogenin variant when compared at 20 ^{o}C and 37 ^{o}C. The phosphorylated proteins of rH174 and rH174+9 had lower R_H than their non-phosphorylated analogues at both temperatures. On the contrary, the phosphorylated proteins of rH174+12 and rH174+15 had higher R_H than their non-phosphorylated analogues (Table 2).

Likewise, the PdI of the amelogenins did not show significant variation at both temperatures. All the amelogenins except for rH174+12D, rH174+15 and rH174+15D are considered to be monodisperse because they have PdIs close to 0.1. The amelogenins constructs, rH174+12D, rH174+15 and rH174+15D, had very high PdIs which were close to 0.4 and significantly higher R_H in comparison to the other amelogenins at temperatures of both 20^{o}C and 37^{o}C (Table 2).

Table 2. The hydrodynamic radius (R_H) and polydispersity index (PdI) of the recombinant amelogenin variants at 20^{o}C and 37^{o}C (pH7.4).

	20 ^{0}C		37 ^{0}C	
Amelogenin Construct	R_H	PdI	R_H	PdI
rH174	17.03	0.037	17.60	0.043
rH174D	16.49	0.198	17.59	0.138
rH174+3	17.28	0.034	18.92	0.114
rH174+6	19.06	0.155	19.63	0.179
rH174+9	17.65	0.053	18.0	0.067
rH174+9D	16.12	0.108	18.47	0.158
rH174+9E	16.16	0.093	18.09	0.082
rH174+12	17.92	0.061	18.51	0.096
rH174+12D	40.23	0.436	38.05	0.417
rH174+15	26.58	0.387	27.41	0.377
rH174+15D	40.76	0.485	41.37	0.477

4.5 Binding Affinity of Amelogenin to HAP

The binding affinity of amelogenin to HAP was tested to investigate the difference in adsorption of phosphorylated and non-phosphorylated amelogenin unto HAP. Following the addition of HAP, the concentration of both rH174 and rH174D decreased sharply from 0 minutes until 1 minute. From 1 minute onwards, amelogenin concentration remained fairly constant, that is, close to zero (Fig.8). This indicated that rH174 and rH174D share almost identical affinity to hydroxyapatite.

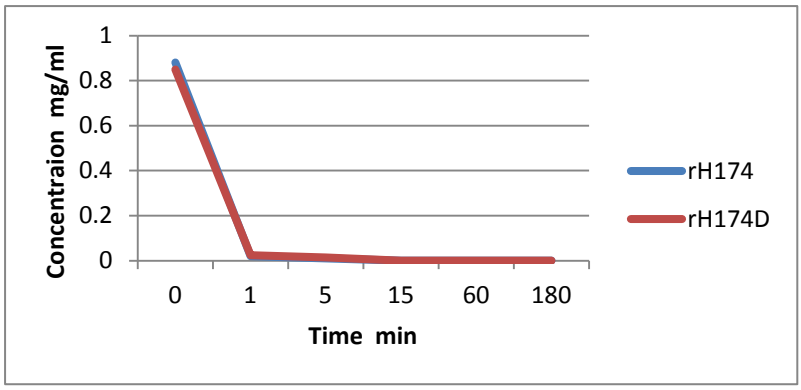


Figure 8 . Binding Affinity Curves of recombinant amelogenin to HAP. The concentration of HAP equilibrated in PBS (pH7.4) was 50 mg/ml. 0 min represent the initial concentrations of amelogenin, without HAP.

From the first binding affinity test it was observed that amelogenin was adsorbed quickly to HAP, thus, the concentration of HAP was changed from 50mg/ml to 25 mg/ml in the subsequent tests. Similar to figure 8, the concentration of the recombinant amelogenins decreased sharply but to approximately 0.3 and/ or 0.4 mg/ml for the first minute (fig. 9 & 10). Then, amelogenin concentration decreased steadily as it approached zero. There was no significant trend among the recombinant amelogenins that can be used to distinguish their affinity to adsorb HAP.

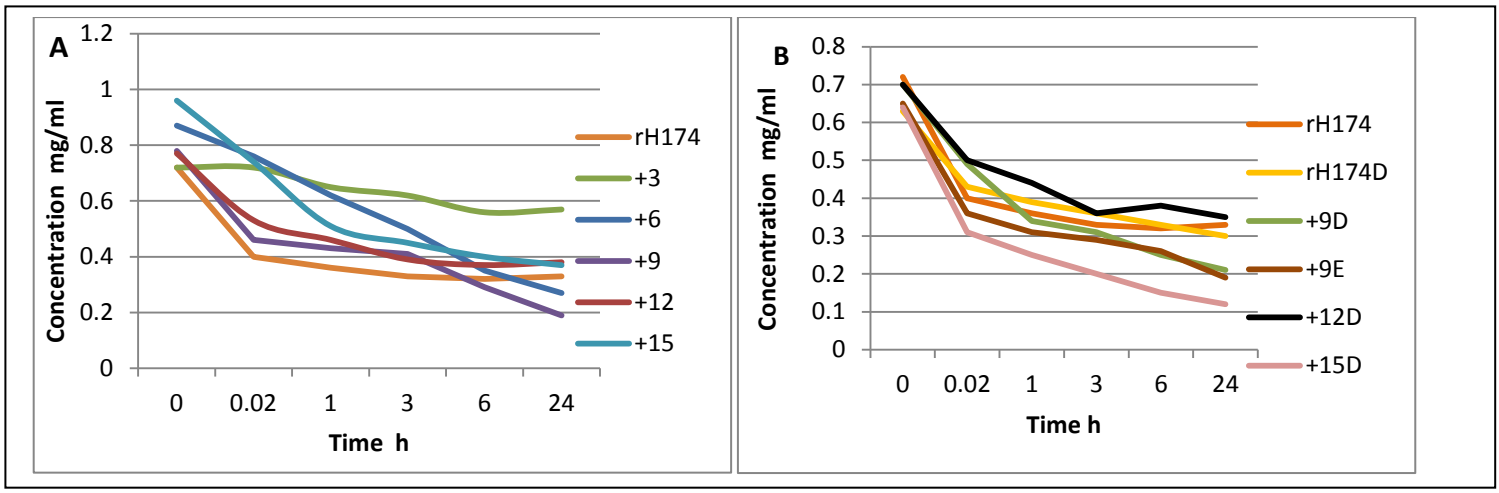


Figure 9. Binding Affinity Curves (A) Comparison between rH174 and the recombinant amelogenin fusion proteins (B) Comparison between rH174 and the recombinant amelogenin phosphorylation mimics. The initial concentration of HAP equilibrated in PBS (pH7.4) was 25 mg/ml. 0 h represent the initial concentration of amelogenin, without HAP.

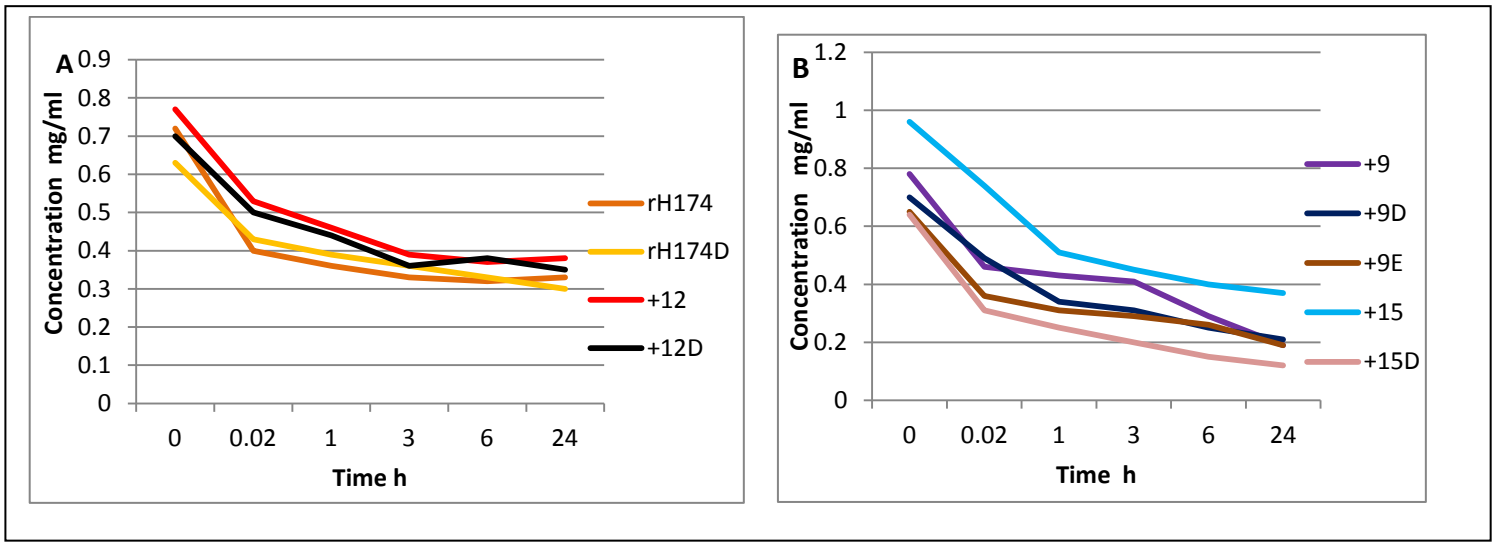


Figure 10. Binding Affinity Curves (A) & (B) Comparison between recombinant non-phosphorylated amelogenins to their phosphorylated analogue. The initial concentration of HAP equilibrated in PBS (pH7.4) was 25 mg/ml. 0 h represents the initial concentration of amelogenin, without HAP.

4.6 Amyloid Formation in Amelogenin Nanoribbons

The results for amelogenin amyloid formation test was not presented here because the data was questionable.

5. Discussion

Most importantly, the site-directed mutagenesis was successful as a total of sixteen recombinant amelogenin phosphorylation mimics were successfully constructed by mutating serine-16 to Asp and/or Glu (Table1). This was confirmed by the positive results obtained from sequence alignments of the amelogenins (data not shown). The recombinant amelogenin phosphorylation mimics are all considered to be phosphorylated amelogenin proteins.

A few of the amelogenin constructs were selected for cultivation and characterization. Among the recombinant amelogenins cultivated for this study, rH174+9D, rH174+12D, rH174+15 and rH174+15D posed the most difficulty for their purification. The results for optimization experiments were inconclusive because an optimal induction optical density could not be found (Fig.2-4). This suggests that there might be other important factors that influence the cultivation yield of these amelogenin which may include number of positive charges of amelogenin, rotation speed and heat exposure. It is noteworthy that with increased positive charges, more minor bands can be seen on the SDS-PAGE picture (Fig. 5).These bands may be as a result of the presence of impurities from synthesis.

It was found that the amelogenin mutants also form nanospheres. Previous studies (e.g Wiedemann-Bidlack et al. 2011) have shown that phosphorylated amelogenin can form nanospheres similar to non-phosphorylated analogues. Therefore the results obtained in this study seem to agree with previous results because there is a small difference between the R_H and PdIs of phosphorylated and non-phosphorylated amelogenin analogues. Analysis of the data presented in Table 2 collectively show that temperature had little effect on the sizes and PdIs of the aggregates of amelogenin fusion and phosphorylation mimic proteins. Although there was a slight increase of R_H of all the amelogenins except rH174+12D at the higher temperature of 37 ^{o} C but no critical effect of temperature was observed. Likewise, the PdI also did not show any changes that can be considered as an effect of temperature. It is noteworthy that the high PdIs exhibited by rH174+12D, rH174+15 and rH174+15D may signify that these amelogenins are more prone to form heterogeneous aggregates or it simply might be the presence of contaminants in the samples.

Amelogenin has been reported to adsorb unto HAP surfaces to guide the organization and shape of the enamel crystallites as they develop in the matrix (Bouropoulos et al, 2003; Simmer et al, 1994). The decrease in amelogenin concentration (Fig. 8, 9 & 10), signifies that the recombinant amelogenin variants bound to the HAP crystals which agrees with previous studies. Both the phosphorylated and non-phosphorylated amelogenin analogues seem to exhibit similar apatite-binding affinity (Fig. 8, 9 & 10). Thus, there were no major effects of phosphorylation found on binding affinity of amelogenin to HAP. In general, all the recombinant amelogenins had very strong binding affinity towards HAP.

There was an initial rapid decrease of amelogenin concentration which most likely suggests that the recombinant amelogenins bound very quickly to the HAP crystals. This was followed by a gradual decrease in amelogenin concentration which probably indicates that all the amelogenin molecules in solution had bound to the HAP crystals up to their saturated point which reduced rate of reaction in the latter stage (Fig.8). A

small difference was observed in the curves when the concentrations of HAP crystals were decreased by half in which there was a slower rate of adsorption for 25mg/ml of HAP crystals (Fig 9 & 10). The higher concentration of HAP crystals (50 mg/ml) has a larger surface area than the lower concentration (25 mg/ml) which resulted in increased exposure of apatite binding sites for amelogenin. The increased exposure in turn increased the probability of amelogenin binding to its specific apatite face (Bouropoulos et al, 2003) which thus increased adsorption of HAP.

There was much difficulty to interpret the results on solubility. The data on solubility could be more reliable if more replicas were done. The similarity of solubility curves between the phosphorylated and non-phosphorylated amelogenins indicated that there were no effects of phosphorylation on the solubility of amelogenin. The results of this present study on the solubility of rH174 and rH174D agrees with previous results that that describe amelogenin solubility to be highest in acidic conditions, low at physiological pH, and high again at basic pH (Tan et al., 1998). The similarity in the solubility profile of rH174 and rH174D (Fig. 6 A & B) also indicates that there is a great probability that rH174E has a similar solubility profile as rH174 and rH174D.The amelogenin fusion protein (rH174+9) and its phosphorylation mimics had higher solubility than rH174 and rH174D (Fig. 7) which indicate that similar findings may be true for the other amelogenins. Furthermore, unlike rH174 and rH174D, the solubility of rH174+9 and its phosphorylation analogues continuously fluctuate over pH range 4 to 8 (Fig.7).These findings may be because the amelogenin fusion protein and the phosphorylation mimics have additional hydrophilic amino acids in comparison to rH174 making them less prone to aggregate.

We wanted to develop a test that could readily check for the presence of various amelogenins based on their property of adopting β structure. Unfortunately, due to the results being inconclusive, the data of the experiment to check for β-amyloid formation in amelogenin nanoribbons were not presented in this study.

The enamel is formed by highly orchestrated extracellular processes which involve enamel matrix proteins to regulate and organize the nucleation and growth of mineral crystals. Although substantial evidence suggests that protein-protein and protein - mineral interactions play crucial roles in enamel formation and organization, yet, the precise mechanism by which this is done is not well understood. The results presented here could give us insight on the role that the properties of amelogenin play in the numerous potential applications of amelogenin.

6. Future Works

It is important to note that there was difficulty in the interpretation of the results on solubility of the amelogenin variants. The results would have been easier to interpret and more reliable, had more replicates been used in the experimental work. Therefore, it is advisable to make several replicates for solubility tests in future studies. In addition, this study showed that amelogenin fusion protein and the phosphorylation mimics generally had higher solubility than rH174 in all conditions tested (Fig. 7). In addition, the solubility of the amelogenin fusion phosphorylation mimics fluctuated over the entire pH scale of 4-8.However in this study, only rH174+9, rH174+9D and rH174+9E were tested and thus it would be better if more investigations was done to include all the amelogenin proteins for more concrete results.

Difficulties were encountered to successfully produce recombinant amelogenin rH174+15, rH174+15D and rH174+9D. An experiment was carried out for optimization of rH174+15, rH+15D and rH174+9D amelogenin constructs but the results were not definitive. Therefore, more experimental works need to be done to optimize the production and/or purification of all the recombinant amelogenin phosphorylation mimics and to verify the optimal conditions for production and purification and to check if the growth conditions are similar to recombinant fusion amelogenin.

In this study, it showed that temperature had little effect on self-assembly of amelogenin fusion and phosphorylation mimic proteins. It was difficult to keep the amelogenin self-assembly samples at the set temperature because of handling of the samples after incubation. This may have possibly altered the structures formed by the amelogenin samples which could have led to more heterogeneous size distribution. It is vital that future experiments work to find a way to keep amelogenin self-assembly samples at the set temperature.

Although from the experimental results it was obvious that the recombinant amelogenin variants have a strong binding affinity for HAP (Fig.8), more replicas should be carried out to investigate whether there are differences among the amelogenin binding affinities. The test should also be carried out under varying conditions (temperature, pH, apatite concentration and amelogenin concentration).

Future testing should be done in an attempt to develop a test to check for amelogenin using amyloid formation as the basis. Both Congo red and ThT can be investigated in order to find which method can be more readily utilized for the test.

As a consequence of lack of time and high numbers of recombinant amelogenin produced, it was not possible to investigate all the 16 amelogenin variants thoroughly in this study. Future studies should aim to compare and contrast various characteristics of all the amelogenin fusion protein to their phosphorylation mimics and to the native recombinant amelogenin.

Generally in this study, the focus was more on performing many experiments to find the differences between phosphorylated and non-phosphorylated amelogenins through testing various properties of amelogenin. Thus, few replicas were used for all the

tests. Preferably, lesser tests and more replicates should have been done for this study in order to obtain more reliable results.

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Popular Science Article

The Effects of Phosphorylation of Amelogenin

We use our teeth daily to eat all sorts of food with varying degrees of texture and hardness. The white part of our teeth called the enamel is the hardest tissue of the human body. Have you ever wondered what makes our teeth so hard and strong that we can chew on a chicken bone? Well, the answer is amelogenin which is the main subject of this study. Amelogenin is one of the many proteins that help build the enamel. However, amelogenin is of greatest importance to enamel formation because in its absence scientists have found that it leads to teeth defects.

Scientists have also discovered that amelogenin can be utilized in many applications such as teeth defects, wound healing, bone formation and regeneration and many others. So far, there are two amelogenin-based products on the market. These products include (i) Emdogain® for the treatment of periodontitis, an enamel defect due to lack of proper amelogenin and (ii) Xelma® used to treat leg ulcers. As a result of the numerous potential applications of amelogenin, thus, there is increasingly more research carried out on amelogenins.

So far, the most optimal production system used for the production of recombinant amelogenin is the bacteria called *Escherichia coli*. The only difference that existed between native amelogenin and recombinant amelogenin expressed using *E.coli* is that the latter lack the amino acid methionine at the N-terminus and phosphate group on Serine-16.The aim of this present study was to produce recombinant amelogenin phosphorylation mimics and to investigate the effects of phosphorylation of amelogenin on its properties. The properties of the novel phosphorylated amelogenin mimics with that of non-phosphorylated amelogenins were compared in order to examine the effects of the phosphorylation.

In this study, a total of sixteen novel recombinant amelogenin phosphorylation mimics were successfully constructed by a type of mutation called site-directed mutagenesis. This mutation was carried out by replacing one amino acid into another amino acid in amelogenin amino acid sequence. More specifically, the amino acid, serine at sixteenth position was replaced with either glutamic acid or aspartic acid in the amelogenin sequence. The site-directed mutagenesis of Serine-16 to aspartic acid or glutamic acid was performed in order to promote negative charges and to mimic the effect of the phosphorylated serine in the native amelogenin. Since the two amino acids used are negatively charged, thus, it was thought that it could be used to substitute the negatively charged phosphate group.

Various properties of amelogenin were tested to investigate the effect of phosphorylation on amelogenin. The study investigated how soluble recombinant amelogenin phosphorylation mimics are in acidic, neutral and alkaline solutions in presence of calcium and phosphate ions. Amelogenin usually change their shape and form spherical particles called nanospheres during enamel formation. Therefore, the radii of these particles and dispersity (how uniform the particles in the sample mixture are) were measured under neutral conditions and at temperatures of 20 and 37^{o}C. The

interaction between the amelogenin and a substance called calcium hydroxyapatite that constitutes ninety-percent of the enamel was also tested.

The phosphorylated amelogenins had similar solubilities to their non-phosphorylated amelogenins in acidic, neutral and alkaline conditions which indicated that phosphorylation had no effect on the solubility of amelogenin. The solubility of recombinant amelogenin phosphorylation mimics was greater than that of recombinant native amelogenin and its phosphorylation mimic counterpart. It was also found that the recombinant amelogenin phosphorylation mimics too can form nanospheres similar to the non-phosphorylated amelogenin. There were minor variations in radii and dispersity of the recombinant amelogenins at 20^{o}C and 37^{o}C which indicated that phosphorylation had not affected the amelogenins. Furthermore, temperature had no critical effect on radii and dispersity of amelogenin. The amelogenin phosphorylation mimics as well as their non-phosphorylated counterparts had a similar, strong interaction with calcium hydroxyapatite.

Although in the study, no exceptional findings were found for the effects of phosphorylation on amelogenin but at least the recombinant amelogenin phosphorylation mimics function to some extent as the native recombinant amelogenins.