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Protein Expression and Purification 45 (2006) 43–53
Protein Expression & Purification
www.elsevier.com/locate/yprep

High yield of biologically active recombinant human amelogenin using the baculovirus expression system

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Received 29 March 2005, and in revised form 19 May 2005
Available online 21 June 2005

Abstract

The amelogenins are secreted by the ameloblast cells of developing teeth; they constitute about 90% of the enamel matrix proteins and play an important role in enamel biomineralization. Recent evidence suggests that amelogenin may also be involved in the regeneration of the periodontal tissues and that different isoforms may have cell-signalling effects. During enamel development and mineralization, the amelogenins are lost from the tissue due to sequential degradation by specific proteases, making isolation of substantial purified quantities of full-length amelogenin challenging. The aim of the present study was to express and characterize a recombinant human amelogenin protein in the eukaryotic baculovirus system in quantities sufficient for structural and functional studies. Human cDNA coding for a 175 amino acid amelogenin protein was subcloned into the pFastBac HTb vector (Invitrogen), this system adds a hexa-histidine tag and an rTEV protease cleavage site to the amino terminus of the expressed protein, enabling effective one-step purification by Ni^{2+}–NTA affinity chromatography. The recombinant protein was expressed in *Spodoptera frugiperda* (Sf9) insect cells and the yield of purified his-tagged human amelogenin ($rHAM^{+}$) was up to 10 mg/L culture. Recombinant human amelogenin ($rHAM^{+}$) was characterized by SDS–PAGE, Western blot, ESI-TOF spectrometry, peptide mapping, and MS/MS sequencing. Production of significant amounts of pure, full-length amelogenin opened up the possibility to investigate novel functions of amelogenin. Our recent in vivo regeneration studies reveal that the $rHAM^{+}$ alone could bring about regeneration of the periodontal tissues; cementum, periodontal ligament, and bone.

Keywords: Recombinant human amelogenin; Baculovirus; Insect cell culture; ESI-TOF; Mass spectrometry; MS/MS; Peptide mapping

The amelogenins play a major role in the biomineralization and structural organization of enamel [1–3]. The developing extracellular enamel matrix is comprised of a number of proteins of which the amelogenins constitute about 90% [4]. They are hydrophobic molecules that self-assemble into nanosphere structures, which are thought to be involved in regulating the ultrastructural organization of the developing enamel crystallite [5–8]. During enamel development and mineralization, the secreted amelogenins are lost from the tissue, along with most of the other enamel matrix proteins, due to sequential degradation by specific proteases. They are replaced by mineral ions, calcium, and phosphorus, which eventually results in fully mineralized, hard, and mature enamel [2,3,9]. The protein undergoes post-translational modifications and post-secretory processing [1,10–12]. These factors, as well as alternative mRNA splicing, give rise to a heterogeneous mixture of polypeptides in the enamel matrix [3].

The human amelogenin gene has been mapped to Xp22.1–p22.3 and Yp11.2 with 90% of the transcripts expressed from the X- and 10% from the Y-chromosome

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doi:10.1016/j.pep.2005.05.010

[13–15]. The gene contains 7 exons, which undergo alternative mRNA splicing. The most abundant isoform of the native protein secreted into the enamel matrix lacks the internal region encoded by exon 4 [15]. More recently, two additional exons downstream of exon 7 have been identified in a rare alternatively spliced RNA transcript of amelogenin [16,17]. Mutations in the X-chromosomal copy of the amelogenin gene have been associated with the hereditary disease amelogenesis imperfecta, which illustrates the importance of amelogenin in developing enamel [18,19]. Defective enamel formation has also been demonstrated by knock-out of amelogenin expression using antisense oligonucleotides [20], ribozymes [21], and a recent amelogenin null mouse which resulted in enamel characteristic of hypoplastic amelogenesis imperfecta [22].

The amelogenins were thought to be tissue-specific and exclusively expressed by the enamel producing ameloblast cells [6,23], however, various isoforms have since been found in the dentin matrix [24] and the associated odontoblast cells [25,26]. Recent reports suggest amelogenin expression in the periodontal ligament and Hertwig's epithelial root sheath of the tooth attachment apparatus [27–29]. The expression of amelogenin in different tissues suggests that amelogenin may be multifunctional. A major discovery that highlights a new role for enamel matrix proteins was the finding that the application of an enamel matrix protein extract to tooth root surfaces, in sites of diseased periodontium, promotes the regeneration of all the periodontal tissues [30,31]. Since the developing enamel matrix is a mélange of various isoforms of amelogenin and other enamel matrix proteins and their degradation products, proteases and other minor components ([6,32] and Deutsch et al. 2004, personal communication), the production of recombinant proteins free of other matrix elements is a valuable tool to investigate which components are responsible for the regeneration. Amelogenin was suggested to be such a candidate. In addition, low molecular mass amelogenin polypeptides have since been associated with cell signaling and have been suggested to have osteogenic potential [33,34].

The described heterogeneity of the matrix makes isolation and purification of sufficiently large quantities of any one of the amelogenin isoforms from the developing tissue, extremely challenging. This problem has been previously overcome by high expression of mouse amelogenin [35], porcine amelogenin [36], and human amelogenin [37,38] in prokaryotic bacterial (*Escherichia coli*) systems. Here, we describe the expression of a recombinant human amelogenin protein (rHAM$^+$)[1] in the eukaryotic baculovirus system for the first time, which has made possible the investigation of novel functions of amelogenin by providing relatively high amounts of pure amelogenin, free from contamination of other enamel matrix proteins.

Our current in vivo regeneration studies revealed that this recombinant amelogenin protein alone could bring about substantial regeneration of the periodontal tissues: cementum, periodontal ligament, and alveolar bone after induced periodontitis [29,39,40,58].

Materials and methods

Construction of the recombinant amelogenin baculovirus

The recombinant human amelogenin vector was constructed and purified according to standard recombinant DNA techniques [41] as follows: human amelogenin cDNA was amplified by PCR from a recombinant plasmid [37] containing human amelogenin X cDNA (GenBank Accession No. M86932), representing the most abundant amelogenin mRNA transcript in the developing enamel, which lacks exon 4 and codes for a 175 amino acid protein [15]. Specific oligonucleotide primers were designed to include restriction enzyme sites (underlined) *Sfo*I on the sense primer (5′…CTG AAG GGC GCC ATG CCT CTA CCA CCT CAT CCT G…3′) and *Eco*RI on the antisense primer (5′…TCG CCG GAA TTC TTA ATC CAC TTC CTC CCG CTT GGT…3′) for subcloning into the pFastBac HTb donor vector of the baculovirus expression system (Invitrogen). This system adds a hexa-histidine tag and an rTEV protease cleavage site to the amino terminus of the expressed protein. The PCR product was cloned into the donor vector and the recombinant vector was sequenced to confirm correct cDNA insertion using an automated DNA sequencer (ABI prizm 377, Perkin-Elmer, USA). The recombinant vector was then electrotransformed into DH10BAC bacterial cells (Invitrogen) for transposition of the human amelogenin cDNA into the genetically modified baculovirus (*Autographa californica*) genome (bacmid). Positive recombinant bacmids were used to transfect *Spodoptera frugiperda* (Sf9) insect cells for viral particle formation. All procedures for the production of viral particles were performed according to the manufacturer's manual (Bac-to-Bac, Invitrogen). Recombinant amelogenin baculovirus underwent one round of plaque purification and subsequently three rounds of amplification (48 h each) by infecting Sf9 monolayers in 25 cm^2 and then 75 cm^2 T-flasks to generate 100 ml of high titre virus (3×10^7 to 3×10^8 pfu/ml as determined by plaque assay) for rHAM$^+$ expression.

Sf9 cell maintenance

Sf9 cells adapted to serum-free Bioinsect-1 medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin,

[1] *Abbreviations used:* rHAM$^+$, recombinant human amelogenin protein; ESI-TOF/MS, electrospray ionization time-of-flight mass spectrophotometry; CID, collision-induced dissociation; MOI, multiplicity of infection.