UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

Alexandre Ribeiro do Espírito Santo

Cirurgião Dentista

BIRREFRINGÊNCIA DA MATRIZ ORGÂNICA DO ESMALTE DENTÁRIO

Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do título de Mestre em Biologia Buco-Dental na área de Histologia e Embriologia.

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Piracicaba

2005

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecário: Marilene Girello – CRB-8a. / 6159

Es65b

Espírito Santo, Alexandre Ribeiro do.
Birrefringência da matriz orgânica do esmalte dentário. / Alexandre Ribeiro do Espírito Santo. -- Piracicaba, SP: [s.n.], 2005.

Orientador: Sérgio Roberto Peres Line.
Dissertação (Mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Esmalte dentário. 2. Birrefringência. 3. Microscopia de polarização. I. Line, Sérgio Roberto Peres. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

(mg/fop)

Título em inglês: Birefringence of dental enamel organic matrix
Palavras-chave em inglês (*Keywords*): Dental enamel; Birefringence; Microscopy, polarization
Área de concentração: Histologia e Embriologia
Titulação: Mestre em Biologia Buco-Dental
Banca examinadora: Sílvia Regina de Almeida Reis; Sérgio Roberto Peres Line; Pedro Duarte Novaes
Data da defesa: 23/02/2005

UNICAMP

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

A Comissão Julgadora dos trabalhos de Defesa de Dissertação de MESTRADO, em sessão pública realizada em 23 de Fevereiro de 2005, considerou o candidato ALEXANDRE RIBEIRO DO ESPÍRITO SANTO aprovado.

PROF. DR. SERGIO ROBERTO PERES LINE

PROFa. DRa. SILVIA REGINA DE ALMEIDA REIS

PROF. DR. PEDRO DUARTE NOVAES

À minha mãe, Maria Luiza, porque é um exemplo de força, coragem e integridade.

Ao meu pai, José Balbino, que enquanto esteve entre nós, trabalhou incansavelmente para realizar meus sonhos.

Aos meus irmãos, Marinho e Cita, pelo constante incentivo e por expressarem, de forma transcendente, grande felicidade com minhas conquistas.

À Julia Luna, pelo amor inestimável e pelo apoio durante a redação deste trabalho.

AGRADECIMENTOS

Ao Prof. Dr. Sérgio Roberto Peres Line, pela excelente orientação científica e pela grande amizade.

À Profa. Dra. Sílvia Regina de Almeida Reis, pela cuidadosa orientação durante os meus primeiros passos na pesquisa científica.

Ao Prof. Dr. Pedro Duarte Novaes, pela valiosa colaboração no desenvolvimento deste trabalho, especialmente durante a padronização da coleta e do processamento das amostras.

Ao Prof. Dr. José Merzel, por compartilhar o seu conhecimento sobre a formação do esmalte dentário em ratos.

Aos professores do Departamento de Morfologia da Faculdade de Odontologia de Piracicaba (FOP) - UNICAMP, pela carinhosa recepção nesta instituição, pelo agradável convívio durante todo o curso, e pela utilização das instalações deste departamento.

À Maria Isabela Guimarães Campos, pela grande amizade e pelo apoio constante, principalmente durante os primeiros meses de minha estadia em Piracicaba.

À Regina Célia Rocha Peres, pela sincera amizade e por também ter contribuído para a minha formação acadêmica, com o convite de participação em um dos trabalhos de sua tese de doutorado.

Aos alunos do Programa de Pós-Graduação em Biologia Buco-Dental da FOP-UNICAMP, pela amizade e pelo convívio harmonioso, em especial a

Maise, Cristiane Salmon, Maria Cristina, Marisi, Cristiane Borges, Daniel Saito e Silvana Pasetto.

A Eliene, Cidinha e Ivani, funcionárias do Laboratório de Histologia do Departamento de Morfologia da FOP - UNICAMP, pela divertida companhia e pela ajuda em diversos momentos da etapa experimental deste trabalho.

A Eli Cristina, secretária do Programa de Pós-Graduação em Biologia Buco-Dental da FOP-UNICAMP, pela boa vontade e competência na resolução de todas as questões burocráticas.

A Raquel e Érica, secretárias da Coordenadoria de Pós-Graduação da FOP - UNICAMP, pela orientação e ajuda no processo de abertura de defesa desta dissertação.

A Gustavo, Suzete e Joelma, pela atenção e agradável convívio.

À Deise Souza Vilas Bôas, pela eterna amizade e pelo grande exemplo de determinação.

A Maria Eduarda, Mariana, Carol e Clara, minhas sobrinhas, por encherem nossa família de felicidade.

Aos grandes amigos Márcio, Mariana, Marcela, Pedro e Rafael, pela motivação e pelas maravilhosas férias em Salvador.

Aos amigos Maurício, Wagner, Luciana, Poliana, Márcia e Daniela, do Programa de Pós-Graduação em Clínica Odontológica da FOP-UNICAMP, pelos vários momentos de felicidade que me proporcionaram em Piracicaba, compensando a saudade da família e dos amigos distantes.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, pela concessão da bolsa de formação de pesquisador de mestrado (processo nº 131922/2003-3).

SUMÁRIO

RESUMO

A formação do esmalte dentário é um processo biológico complexo, sendo dependente do estabelecimento de uma matriz orgânica bem estruturada. Evidências recentes indicam que os componentes desta matriz formam uma organização supramolecular, cuja significância biológica não é claramente entendida. Este fato é devido em parte a dificuldades em observações morfológicas diretas da matriz orgânica do esmalte estruturada. A detecção de propriedades anisotrópicas de estruturas biológicas tem sido extensivamente utilizada para o estudo de organizações supramoleculares em cortes histológicos comuns. O objetivo deste trabalho foi estudar a birrefringência da matriz orgânica do esmalte durante o desenvolvimento de dentes incisivos e molares de ratos. A amostra constituiu de dentes incisivos superiores e hemimandíbulas de ratos Wistar machos, pesando aproximadamente 300g. Foi realizada perfusão dos animais com paraformaldeído 2% e glutaraldeído 0,5% em solução tampão fosfato 0,2M, pH 7,2. Neste mesmo fixador, as amostras foram então imersas por 16h. A descalcificação foi procedida por imersão dos incisivos superiores e das hemimandíbulas em mistura de ácido nítrico 5% e formaldeído 10% durante 6h e 24h, respectivamente. Realizou-se inclusão em parafina e obtiveram-se cortes longitudinais de 5µm, os quais foram tratados com xilol e hidratados. Curvas de birrefringência de forma foram obtidas após mensurações de retardos óticos utilizando-se fluidos para montagem com diferentes índices de refração. Nossas observações mostraram que a matriz orgânica do esmalte de incisivos e molares de ratos é fortemente birrefringente. A birrefringência começa nos estágios iniciais da fase de secreção e desaparece na fase de maturação. Estes achados revelam que a matriz orgânica do esmalte apresenta uma estrutura supramolecular altamente ordenada. A análise da birrefringência da matriz orgânica do esmalte pode ser utilizada para detectar os efeitos de fatores genéticos e ambientais sobre sua orientação supramolecular e sua relação com defeitos no esmalte maduro.

ABSTRACT

The formation of dental enamel is a complex biological process, which depends on a well-structured organic matrix. Recent evidences indicate that the components of this matrix can interact to form a supramolecular structure. The biological significance of this supramolecular organization is not clearly understood. In part this occurs due to methodological difficulties to perform direct morphological observations of the structured enamel organic matrix. The detection of anisotropic properties by polarizing microscopy has been extensively used to detect and measure the macromolecular organization in ordinary histological sections. The aim of this work was to study the birefringence of enamel organic matrix during the development of rat molar and incisor teeth. Wistar rats with approximately 300g were anesthetized with chloral hydrate and perfused with 2% paraformaldehyde 0.5% glutaraldehyde in 0.2 M phosphate buffer solution, pH 7.2. Upper incisor teeth and hemimandibles were then immersed in the fixative solution for 16h. Decalcification was performed by immersion of upper incisor teeth and hemimandibles in a mixture of 5% nitric acid and 10% formaldehyde for 6h and 24h, respectively. After dehydration, samples were embedded in paraffin and 5μm thick longitudinal sections were obtained. The sections were treated with xylol for removal of the paraffin, and hydrated. Form birefringence curves were obtained after measurements of optical retardations in imbibing media with different refractive indices. Our observation showed that enamel organic matrix of rat incisor and molar teeth is strongly birefringent. The birefringence starts at early secretion and disappears at maturation phase. The results show that enamel organic matrix presents a highly ordered supramolecular structure. The analysis of enamel organic matrix birefringence may be used to detect the effects of genetic and environmental factors on the supramolecular orientation of enamel matrix and its association with enamel defects.

INTRODUÇÃO GERAL

O esmalte dentário é um tecido de origem ectodérmica, altamente mineralizado, que contém os maiores cristais de hidroxiapatita do corpo vertebrado (Eisenmann, 1998; Paine & Snead, 1997). Sua formação ocorre extracelularmente com o auxílio de um suporte protéico temporário que controla o crescimento, a morfologia e a orientação dos cristais (Moradian-Oldak *et al*., 2003; Paine & Snead, 1997). Este suporte temporário constitui a matriz orgânica do esmalte, que é sintetizada, secretada e organizada por células especializadas do órgão dentário chamadas ameloblastos. A referida matriz é depositada com pequena quantidade de mineral durante a fase de secreção da amelogênese, tem sua degradação iniciada na fase de transição, e é extensamente degradada e quase completamente substituída por componente mineral na fase de maturação (Robinson *et al*., 1979; Bronckers *et al*., 1995). Dessa forma, o esmalte maduro é constituído por cristais de hidroxiapatita incluídos em pequena quantidade de material orgânico.

Grandes avanços foram obtidos na identificação e clonagem das proteínas estruturais e proteinases que constituem a matriz orgânica do esmalte em desenvolvimento (Fincham *et al*., 1999).

Quatro genes de proteínas da matriz do esmalte já foram clonados e caracterizados em diversas espécies. São os genes das proteínas amelogenina (Cheng *et al*., 2004; Snead *et al*., 1983; Gibson *et al*., 1992), tufelina (Mão *et al*., 2001; Deutsch *et al*., 1991; Bashir *et al*., 1997; Dodds *et al*., 1996), enamelina (Hu *et al*., 1997; Hu *et al*., 1998; Hu *et al*., 1998b) e ameloblastina (Toyosawa *et al*., 2000; Krebsbach *et al*., 1996; Simmons *et al*., 1998), também conhecida como amelina ou bainhalina. Tufelinas e enamelinas juntas, ameloblastinas e amelogeninas representam 2%, 5% e mais de 90% das proteínas da matriz orgânica do esmalte, respectivamente (Termine *et al*., 1980; Simmer & Hu,

2002). Elas interagem entre si e com os cristais de hidroxiapatita em formação para guiar o crescimento e modular a morfologia dos mesmos (Fincham *et al*., 1999).

As principais proteinases da matriz do esmalte são a enamelisina (MMP-20) e a calicreína 4 (KLK4) (Simmer & Hu, 2002). Estas enzimas catalizam o rompimento de ligações peptídicas, estão expressas na matriz em diferentes estágios de desenvolvimento do esmalte e apresentam funções distintas. Primeiramente, a MMP-20, presente nos estágios iniciais e intermediários da amelogênese, altera as interações proteína-proteína e proteína-cristal, protegendo os cristais de fusões prematuras e permitindo o crescimento deles em altura e não em largura (Moradian-Oldak *et al*., 1994; Tanabe *et al*., 1992). Posteriormente, a KLK4, presente no estágio de maturação, degrada as proteínas do esmalte, facilitando a remoção das mesmas (Moradian-Oldak *et al*., 1998). Dessa forma, ocorre um rápido crescimento dos cristais em largura, levando ao endurecimento do esmalte.

As funções dos componentes da matriz orgânica do esmalte têm sido evidencidas até hoje por estudos *in vitro* e por mutações genéticas em camundongos e humanos (FINCHAM *et al*., 2000; MORADIAN-OLDAK *et al*., 1994; MORADIAN-OLDAK *et al*., 1998; GIBSON *et al*., 2001; PAINE *et al*., 2003; RAVASSIPOUR *et al*., 2000; LENCH & WINTER, 1995; COLLIER *et al*., 1997; PAINE *et al*., 2000; CATERINA *et al*., 2002). Investigações recentes indicam que os referidos componentes podem interagir para formar uma estrutura supramolecular (RAVINDRANATH *et al*., 2004; FINCHAM *et al*., 1999; WEN *et al*., 1999). O significado biológico desta organização supramolecular não é claramente entendido, em parte devido a dificuldades para a condução de observações morfológicas diretas da matriz orgânica do esmalte estruturada. Portanto, tornam-se relevantes métodos que permitam a análise *in situ* da organização

supramolecular da matriz orgânica do esmalte nas diversas fases da amelogênese.

A detecção de propriedades anisotrópicas de estruturas biológicas por microscopia de polarização tem sido extensivamente usada para o estudo de organização macromolecular em cortes histológicos comuns. Dessa forma, o presente trabalho teve como objetivo estudar a birrefringência da matriz orgânica do esmalte durante o desenvolvimento dos dentes molares e incisivos de ratos. O desenho experimental deste estudo foi aprovado pela Comissão de Ética na Experimentação Animal do IB/UNICAMP (protocolo nº 744-1) (ANEXO 1).

O primeiro artigo, uma revisão de literatura, considera o estágio atual de conhecimento sobre a biologia estrutural da matriz orgânica do esmalte.

O segundo artigo mostra que a matriz orgânica do esmalte é altamente birrefringente, apresentando uma estrutura supramolecular ordenada, e sugere novas possibilidades no estudo da biologia do esmalte.

CAPÍTULO 1

ESPÍRITO SANTO AR, LINE SRP. The enamel organic matrix: structure and function.

Artigo submetido à revista ***Brazilian Journal of Oral Sciences*** (ANEXO 2).

The enamel organic matrix: structure and function

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ABSTRACT

Dental enamel is the most mineralized tissue in the vertebrate body and contains the largest known biologically formed hydroxyapatite crystals. Its formation occurs extracellularly through the collaboration of a proteic transient framework (the enamel organic matrix), which controls hydroxyapatite crystal growth, morphology and orientation. This matrix is deposited with a small amount of mineral during the secretory stage of amelogenesis. The organic components begin to be degraded in the transition stage and are extensively corrupted, and almost entirely replaced by the inorganic crystallites during maturation stage. The present paper reviews current knowledge on the structural biology of the enamel organic matrix.

KEY WORDS

Dental enamel, extracellular matrix, amelogenesis, dental enamel proteins, dental enamel proteinases.

Introduction

Dental enamel is originated from ectoderm and is the hardest, most mineralized tissue in the vertebrate body, containing the largest known biologically formed hydroxyapatite crystals[1,2]. Enamel is noncollagenous and does not undergo resorption and remodeling[3]. This striking example of a highly mineralized structure is exquisitely adapted to absorb essential mechanical and abrasive stresses throughout the lifetime of the organism. Enamel formation occurs extracellularly through the collaboration of proteins that assemble in order to form a transient framework, which is important to control hydroxyapatite crystal growth, morphology and orientation[2,4]. This transient protein framework is known as the enamel organic matrix. It is synthesized, secreted and organized by specialized cells of the developing tooth organ, called ameloblasts. As these cells migrate outwards they leave behind a ribbon of secreted proteins in their wake and play active role in transport of ions including Ca^{2+}, HPO_4^{2-}, OH^- and H^+[5,6]. Enamel development is didactically divided into secretory, transition and maturation stages. The enamel organic matrix is deposited with little amount of mineral during the secretory stage, begins degrading in the transition one, and is extensively corrupted and almost entirely replaced by the inorganic crystallites during maturation[6-8]. Thus, despite an embryonic origin in protein, mature enamel is a stiff-brittle-ceramic composed of hydroxyapatite crystallites embedded within a small amount of organic material distributed among the crystallites. These crystallites are roughly organized into bundles called rods or prisms and thus each rod is made of many small individual

hydroxyapatite crystals. This paper specifically presents a review of current knowledge on the structural biology of the enamel organic matrix.

1. Components of the enamel organic matrix

Great advances have been made regarding the identification and cloning of structural enamel matrix proteins and enamel proteinases. The enamel matrix proteins are generally grouped into three classes sharing common features, these are: the amelogenin class of circa 20-kDa hydrophobic proteins; the enamelin class of circa 65-kDa acidic proteins including tuftelin; and the non-amelogenin, non-enamelin class, represented by ameloblastin (also called amelin/sheathlin)[3,9-11]. All three protein classes are proline rich[12]. Enamel matrix proteins assemble, interacting with each other and with the hydroxyapatite crystallites. These biochemical interactions are important to guide hydroxyapatite crystals growth and modulate their morphology. Two major enamel proteinases have been identified: matrix metalloproteinase-20 (MMP-20), also known as enamelysin, and kallikrein 4 (KLK4), which is a serine proteinase[12-17]. These proteinases are expressed in the developing enamel at different times and have different functions. Their roles are to modify and/or to eliminate enamel matrix proteins, which affects the way enamel proteins interact with each other and with the developing enamel crystallites[12]. Sulphated proteins, serum albumin and lipids have also been reported as residents of the enamel organic matrix[3,10]. Detailed information about these matrix components and their roles is presented in the following sections.

1.1. Proteins

Four genes of enamel structural proteins have already been cloned and characterized in several species, these are: amelogenin[18-20], tuftelin[21-24], enamelin[25-27] and ameloblastin[28-30].

1.1.1. Amelogenins

Amelogenins are the most studied enamel matrix proteins. They account for more than 90% of the matrix proteins in the secretory stage of enamel formation[9], cromprising the major components of the supramolecular transient framework, which is absolutely necessary for normal enamel crystals growth and architecture. This condition may be an explanation for significant advances in the knowledge on characteristics and roles of the amelogenins.

Amelogenins may exhibit various forms due to three main reasons: there are two distinct copies of their gene localized in X and Y chromosomes, different amelogenin mRNAs are produced by alternative splicing and they are proteolytic processed after being secreted by ameloblasts. There is an important debate in the literature regarding the way enamel proteins are arranged in order to interact with each other and with the developing hydroxyapatite crystals[4,12,31]. Recent knowledge on amelogenin's primary and quaternary structures has allowed the development of in vitro experimental systems for the study of amelogenin-mineral interactions and interpretation of the function of this structural protein during amelogenesis[4,32-34]. Supramolecular assembly of amelogenin has been assumed to be

critical for a competent enamel organic matrix formation. Full-length amelogenins contain two clearly definable self-assembly domains: the amino-terminal hydrophobic domain-A comprising amino-acid residues 1-42; and the carboxy-terminal hydrophilic domain-B comprising amino-acid residues 157-173[2]. Amelogenin binds apatite crystals through its hydrophilic domain-B[35,36], also known as leucin-rich amelogenin polypeptide (LRAP)[36]. MORADIAN-OLDAK et al.[37] have recently found that the apatite-binding domain can be narrowed down to the C-terminal 12 or 13 amino acids and further digestion from the C-terminal does not affect this binding affinity. It seems that the function of domain-B is crucial only during the early stage of enamel formation since it is cleaved soon after the protein is secreted into the extracellular space[4]. Prevention of premature crystal-crystal fusion at the very early stage of mineral formation is the most likely function for the full-length amelogenin containing the C-terminal[32,38]. While the presence of this domain appears to be critical for the apatite binding affinity of amelogenin, it does not seem to be necessary for the specific modulating effects on crystal morphology. Some studies indicate that the lack of hydrophilic C-terminal affects proteins assembly and results in the fusion of the hydrophobic nanospheres, which are suggested to arrange in order to provide the supramolecular structural framework for the controlled growth of enamel crystals[4,39]. These findings are in agreement with previous report[40], which shows that amelogenin is found in all compartments throughout the entire thickness of developing enamel and that intact amelogenins and their C-terminal cleavage products are only detected within 40 μm of the enamel matrix surface. This finding indicates that full-length amelogenins containing the

hydrophilic C-terminal and their hydrophobic N-terminal proteolytic products have distinct functions.

The ablation of amelogenin gene in knockout mice results in severe disruptions in enamel crystallite architecture, even though a functional mineralized enamel does form[41,42]. This is also true in the enamel of patients with the genetic disease amelogenesis imperfecta in which particular mutations of amelogenin have been identified[43]. These observations imply that the presence of this protein is not an absolute requirement for enamel mineralization[42]. However, other evidences indicate a critical dependency of amelogenin self-assembly on the microstructural organization of enamel. This process involves amino-terminal hydrophobic domain-A and carboxy-terminal hydrophilic domain-B. Amelogenin's amino acid sequence is highly conserved across evolution and this conservation is particularly obvious among the amino-terminal residues 1-51 and again in the carboxyl-terminal residues 160-180[6,44,45]. Conservation of amino acid sequence often implies physiologic relevance. The physiologic relevance of the highly conserved amelogenin's amino-terminus was identified by LENCH and WINTER[46] and confirmed by COLLIER et al.[47]. These authors focused their genetic studies on two unrelated human pedigrees for amelogenesis imperfecta and identified single amino acid changes, occuring within the highly conserved amino-terminus of amelogenin, as the causative factor for the phenotypic changes in the resulting enamel. Some experiments with transgenic animals, containing domain-A, domain-B or both domains deleted, indicated that a highly organized enamel organic matrix (essential to normal enamel formation) results from undisturbed amelogenin self-assembly[42,48]. Mutations in human amelogenin gene located on the X chromosome induce

amelogenesis imperfecta (AIH1) with enamel phenotypes broadly characterized as hypoplastic or hypomineralized. Documented cases of AIH1 with mutations in the C terminus of amelogenin have resulted in a hypoplastic phenotype, whereas mutations located within the N terminus have resulted in hypomineralized enamel[50,51]. Single amino acid in vitro mutations, identical to those appearing in AIH1, were able to reduce amelogenin self-assembly[52]. According to PAINE et al.[49], a possible explanation for this genotype/phenotype relationship observed in vivo may be related to a reduction in the rate of amelogenin hydrolysis[53] and the subsequent events of mineralization. The proline residue at position 169 of mouse amelogenin (M180) seems to play a significant role in amelogenin hydrophobic self-assembly, since this process does not occur after removal of the C-terminal including the referred amino acid[42]. However, the importance of this amino acid has not been well established and some reasons for this fact are the conflicting results of investigations which show that mutating proline-169 within M180 to either a lysine or threonine does not influence amelogenin's domain-A assembly[42].

Both native and recombinant amelogenins are reported to hemagglutinate mouse red blood cells[3,54]. This hemagglutination is inhibited by monomers, dimers and tetramers of N-acetylglucosamine (GlcNAc) but not by N-acetylgalactosamine or related sugars. This activity is retained by TRAP (tyrosine-rich amelogenin polypeptide 45 residues), which results from N-terminal cleavage of amelogenin by enamelysin (MMP-20). It was shown that [^{14}C]GlcNAc binds to the N-terminal sequence of TRAP (-PYPSYGYEPMGGW) but not when the three tyrosyl residues are substituted with phenylalanine or if the third proline is substituted with threonine[3]. This latter modification mimics the point mutation identified

in a case of human X-linked amelogenesis imperfecta[47]. This activity of the TRAP motif of amelogenin is known as lectin-like property and may be functionally involved in interactions with enamel matrix glycoproteins (enamelin, tuftelin or ameloblastin), promoting structural stability of the matrix[3] or, alternatively, functioning in a signaling role through recognition by cell surface glycoproteins[55]. Recent studies indicate that amelogenin may interact with ameloblastin to form a heteromolecular assembly as a consequence from the presence of GlcNAc-mimicking peptides (GMps) at intermittent sites of ameloblastin and from the recognized amelogenin-trityrosyl-motif-peptide (ATMP), which is a GlcNAc/GMp-binding domain in amelogenin[56]. *In vitro* experiments conducted by BOUROPOULOS and MORADIAN-OLDAK[57] strongly suggest that the 32-kDa enamelin and amelogenins cooperate to promote nucleation of apatite crystals and propose a possible novel mechanism of mineral nucleation during enamel biomineralization.

1.1.2. Enamelins

The enamelins comprise a class of enamel acidic proteins that include enamelin and tuftelin. They account for about 2% of all enamel matrix proteins[12].

The major secretory product of the human enamelin gene has 1103 amino acids and is post-translationally modified, secreted and processed by proteases shortly after being secreted[58]. Unlike other enamel proteins, no enamelin isoforms that are translated from alternatively spliced RNA transcripts have been observed[3]. Porcine enamelin proteolytic products were first isolated and characterized by FUKAE and TANABE[59]. Intact enamelin is only found on the enamel surface, within a micrometer of the ameloblast cell membrane,

and many enamelin cleavage products appear to be rapidly degraded and are only found in the outer enamel layer[12]. However, stable enamelin proteolytic products are found throughout the entire thickness of developing enamel. The best-studied stable enamelin cleavage product is the 32-kDa enamelin[3,60-63]. The porcine 32 kDa-enamelin has 106 amino acids (residues 174-279), which include two phosphoserines and three glycosylated asparagines[62-64]. Variable glycosilation contributes to the heterogeneity of this enamel protein[3]. 32-kDa enamelin is concentrated in the rod and interrod enamel and is absent from the sheath space[12]. The functional significance of this specific spacial organization in the enamel layer is not yet understood. Recent *in vitro* evidences indicate that 32-kDa enamelin and amelogenin cooperation promotes nucleation of apatite crystals[57]. The importance of enamelin for normal enamel formation is indicated by an investigation that shows correlation between a mutation in its gene and an autosomal-dominant form of amelogenesis imperfecta (AI)[65]. The mutation is a single-G deletion within a series of 7 G residues at the exon 9-intron 9 boundary of the enamelin gene. Another publication reports that mutations in this gene cause a severe form of autosomal-dominant smooth hypoplastic AI that represents 1.5%, and a mild form of autosomal-dominant local hypoplastic AI that accounts for 27% of AI cases in Sweden[58].

A long recognized structure in enamel is the tuft[6,66], which may represent residual enamel matrices that include proteins responsible for crystals nucleation[3,67,68]. Enamel tufts occur at the dentine enamel junction (DEJ) and appear as 'tornado-like swirls' rising from the DEJ and into the aprismatic enamel[6,68]. Protein was recovered from these tufts by micro-dissection and characterized[69]. Sixteen years later, a cDNA clone with amino acid

sequences that matched the composition of the tuft protein[69] was isolated and characterized[22], and so the name tuftelin was adopted. Since tuftelin is an acidic enamel protein, it joined the enamelin class of enamel protein[6]. Full-length mouse tuftelin cDNA has been characterized[24]. The function of this enamel protein remains unknown but a predicted function, as we referred at the beginning of this paragraph, is to nucleate hydroxyapatite crystal formation. This prediction is made because of tuftelin's anionic character, its localization to the DEJ and its expression prior to amelogenins during development[6,22,70,71]. Tuftelin can be divided into two domains with distinct physical-chemical properties: the carboxyl-terminal domain mediating self-assembly and the amino-terminal domain containing a pronounced anionic motif, which is consistent with tuftelin's proposed role as a crystal nucleator[6]. The bovine tuftelin gene was also cloned and characterized by BASHIR et al.[23], showing a cDNA sequence that was different from the one reported by DEUTSCH et al.[22] at its carboxyl-terminus. The former reported a C-terminus with only 42 amino acids[23] and the second with 92[22]. Tuftelin thus exists as at least two isoforms: the tuftelin A form[22] and the tuftelin B form[23]. The self-assembly properties of bovine tuftelin A and B isoforms and mouse tuftelin have been confirmed by PAINE et al.[72]. Tuftelin interacting proteins (TIPs) have been discovered[73,74] and one TIP protein, encoding a 39-kDa protein (TIP 39), shows enrichment to the secretory surface of Tomes' processes[72], being a candidate molecule linking the ameloblast secretory surface to the assembling enamel organic matrix. The abundance of TIP 39 and tuftelin at the DEJ suggests that these two proteins may also participate in forming specialized enamel at the DEJ.

In an attempt to better define a physiological function for tuftelin during amelogenesis, a recent investigation was carried out with transgenic mice that overexpress tuftelin in ameloblasts and subsequently in the enamel matrix[75]. Overexpression of this protein was shown to impact dramatically upon the enamel crystallite and enamel prismatic structure, resulting in gross imperfections in enamel, which apparently reflects from loss of restricted growth of enamel crystallites along their a-axis and b-axis.

1.1.3. Non-amelogenins, non-enamelins

This third class of enamel proteins is represented by ameloblastin (also called amelin/sheathlin)[3,9-11], which accounts for about 5% of total enamel proteins[12]. Its amino and carboxyl ends are biochemically different and were discovered separately during investigations of pig enamel proteins[3]. The carboxyl end was represented by two polypeptides with apparent molecular weights of 27 and 29 kDa, isolated during a search for enamel proteins that bound calcium[59]. The amino-terminal end of ameloblastin was discovered in the same research that isolated and characterized enamelin cleavage products[76]. Ameloblastin's amino-terminus is represented in the enamel matrix by a group of low molecular weight proteolytic products in the range 13-17 kDa with aggregative properties[76]. Two research groups independently cloned and characterized cDNAs encoding the rat homologue of the protein that had been studied by FUKAE and TANABE[59,76]. The protein was termed ameloblastin[29] and amelin[77]. Later, the cDNA from pig was cloned and

designated as sheathlin[25]. A cDNA encoding the mouse homologue was cloned and also called ameloblastin[30], which is the name used in this paper. Human ameloblastin has already been cloned and characterized[28,78]. HU et al.[25] observed that ameloblastin transcripts undergo a limited amount of alternative splicing, generating two isoforms that differ by the deletion or inclusion of a 15 amino acid segment, which is absolutely conserved between the pig and the rat. Ameloblastin also is proteolyticly processed after secretion into enamel matrix. Intact ameloblastin and its cleavage products containing the C-terminal half of the protein are only found in the outer developing enamel, concentrated among the crystallites in the rod and interrod enamel[12,79]. Proteolytic products containing the amino-terminal side of the protein are found at all depths within the enamel layer, but they are not distributed randomly, being concentrated in the sheath space[12]. Ameloblastin self-assembly could not be demonstrated[74], but interactions between ameloblastin and amelogenin have already been suggested[56]. While amelogenin and tuftelin appear to be restricted in their expression to ameloblasts engaged in forming enamel, ameloblastin is expressed by ameloblasts during amelogenesis, as well as by cells of Hertwig's epithelial root sheath during cementogenesis[80,81]. This spatial pattern of expression suggests that ameloblastin protein participates in the genesis of both tissues[74,80,81].

The significant role for ameloblastin during amelogenesis has been indicated by experiments that place its gene *loci* in the critical region of autosomal-dominant forms of amelogenesis imperfecta[82]. Ameloblastin overexpression in mice has recently been shown to influence enamel crystallite and enamel rod morphology, implicating the role of ameloblastin gene *locus* in the etiology of a number of undiagnosed autosomally dominant

cases of amelogenesis imperfecta[83]. Mutant ameloblastin transcripts have also been reported to be expressed in human ameloblastomas[28].

1.2. Proteinases

Proteinases are present in low abundance in the developing enamel matrix and are not likely to participate directly in the mineralization process[12]. They cleave enamel proteins by catalyzing the hydrolysis of peptide bonds. The nature and sequence of proteolytic activities during enamel biomineralization are critical and somehow unique to this mineralizing tissue[3]. First, these activities cause changes in structural and physicochemical properties of amelogenins, affecting the way they interact with each other and with the developing enamel crystallites[15,84]. Second, the almost complete protein degradation appears to be essential for rapid crystal growth, leading to enamel hardening[85,86]. Several proteinases have been detected in the enamel extracellular matrix[87]. However, two enzymes, enamelysin (matrix metalloproteinase-20, MMP-20) and enamel matrix serine proteinase 1 (EMSP1), which is now officially designated kallikrein 4 (KLK4), are major enamel matrix proteinases[12].

1.2.1. Enamelysin (MMP-20)

Enamelysin mRNA has been cloned from pig[88], human[89], cow[90] and mouse[91]. It exhibits two forms (41 and 45 kDa) in zymograms[87,88,92]. Catalytic domain fragments (21 and 25

kDa) of enamelysin have also been described[90]. This proteinase is expressed during the early through middle stages of enamel development[12,93] and seems to participate in the proteolytic events that allow the crystals to grow in length but not in width or thickness[87]. In vitro, enamelysin catalyzes all of the amelogenin cleavages that are known to occur during the secretory stage in vivo[12,92], and it is probably the enzyme responsible for the processing of all enamel proteins[12]. Enamelysin's main function seems to be the gradual removal of amelogenin C terminus, changing the physicochemical properties of this protein. This cleavage generates the hydrophobic tyrosine-rich amelogenin polypeptide (TRAP)[94], which is important in the arrangement of amelogenin nanospheres directly involved in the regulation of enamel crystals elongation, controlling their growth in width and thickness[4]. Experiments with recombinant enamelysin also indicate that this enamel proteinase can degrade itself[94,95].

The essential role of enamelysin during enamel formation was evidenced by a recent investigation that showed an amelogenesis imperfecta phenotype caused by enamelysin gene deletion in mice[93]. The mice homozygous for this mutation did not process amelogenin properly, showed altered enamel matrix and rod pattern, had hypoplastic enamel and deteriorating enamel organ morphology as development progressed. These alterations, however, were not beared by heterozygous mice (enamelysin$^{+/-}$), indicating that the phenotype observed for mutations in the enamelysin gene is autosomal-recessive. These findings are in accordance with previous data of THOMPSON et al.[96], which indicates that the most autosomal-recessive anomalies are caused by mutations in enzyme genes. The lack of amelogenin processing in the enamelysin null mice observed by CATERINA et al.[93]

likely eliminates necessary changes in the physicochemical properties of amelogenin that are essential for the proper enamel development.

1.2.2. Kallikrein 4 (KLK4)

Kallikrein 4 (KLK4) has been cloned from pig[97], human[98] and mouse[99]. The first KLK4 cDNA was isolated from a pig-tooth-specific cDNA library and designated as enamel matrix serine proteinase 1 (EMSP1)[12,97]. This proteinase is found in the enamel matrix exhibiting these two forms: 30 and 34 kDa[12,100]. Different from enamelysin, KLK4 degrades enamel proteins during early maturation stage of amelogenesis, which facilitates their removal from the matrix and makes way for hardening of the enamel layer[12]. *In vitro* incubation experiments using a fraction rich in KLK4 activity have demonstrated complete degradation of the recombinant amelogenin substrate to peptides as small as 147 Da[3,101]. Theoretically, if KLK4 failed to function enamel matrix would not be reabsorbed efficiently and the crystallites would not be able to thicken fully, resulting in a hypomaturation form of amelogenesis imperfecta[12]. Experiments with engineered KLK4 knockout mice, which can prove this hypothesis, have not been published yet.

1.3. Other enamel matrix components

Serum albumin has been detected in the enamel organic matrix especially throughout the secretory and transition stages of amelogenesis[102-105]. However, this protein is not

synthesized or secreted by ameloblasts[106]. Radiolabeled serum albumin injected into rabbits did not incorporate into the enamel layer, suggesting a physiological barrier between the extravascular fluid and the enamel matrix[107]. Furthermore, ingress of albumin into enamel from dentin is restricted, particularly during the secretory stage[108]. As SHAPIRO and AMDUR[109] demonstrated that red pigmentation observed in extracted developing bovine teeth was probably derived from hemoglobin adsorbed post mortem from the dental sac fluid, FINCHAM et al.[3] suggest that albumin would be likely to exhibit the same behavior.

Lipids have been demonstrated to represent some 0.2% of the developing enamel matrix[3]. Entombed membrane fragments of the Tomes' processes in the matrix were suggested to contribute to the overall lipid content in this tissue[110]. A group of short-lived sulfated proteins (49 and 25 kDa), which are rapidly degraded after secretion, has also been identified in the enamel matrix and suggested to interact functionally with nascent amelogenins[111]. The role of lipids and sulfated proteins in the enamel organic matrix remains poorly understood[3].

2. Concluding remarks

Enamel formation is a highly complex process. The major enamel organic matrix proteins and proteinases have already been cloned and characterized, and their association with several cases of amelogenesis imperfecta has evidenced their importance in the development of enamel. However, there is an important debate in the literature about the enamel proteins processing and the way they arrange in order to interact with each other

and with the developing enamel crystallites. Amelogenin supramolecular self-assemblies appear to be essential for normal enamel formation. On the other hand, the mechanisms by which non-amelogenin proteins and enamel proteinases contribute to this formation should be better established.

Acknowledgements

Alexandre Ribeiro do Espírito Santo was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq - Brazil.

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CAPÍTULO 2

ESPÍRITO SANTO AR, NOVAES PD, LINE SRP. Anisotropic properties of the enamel organic extracellular matrix.

Artigo que será publicado na revista ***European Journal of Oral Sciences***, como trabalho completo apresentado no *The Enamel VII Symposium (Seventh International Symposium on the Composition, Properties and Fundamental Structure of Tooth Enamel)*, entre 10 e 14 de abril de 2005, na cidade de Brewster, Massachusetts, USA (ANEXO 3).

Anisotropic properties of the enamel organic extracellular matrix

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Running title:

Anisotropy of the enamel organic matrix

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Anisotropic properties of the enamel organic extracellular matrix

Eur J Oral Sci

Abstract

Enamel biosynthesis is initiated by the secretion, processing and self-assembly of a complex mixture of proteins. This super molecular ensemble controls the nucleation of the crystalline mineral phase. The detection of anisotropic properties by polarizing microscopy has been extensively used to detect macromolecular organizations in ordinary histological sections. The aim of this work was to study the birefringence of enamel organic matrix during the development of rat molar and incisor teeth. Incisor and molar teeth of rats were fixed in 2% paraformaldehyde 0.5% glutaraldehyde in 0.2 M PBS, pH 7.2 and decalcified in 5% nitric acid 10% formaldehyde. After paraffin embedding, 5μm thick sections were obtained, treated with xylol and hydrated. Form birefringence curves were obtained after measurements of optical retardations in imbibing media with different refractive indices. Our observation showed that enamel organic matrix of rat incisor and molar teeth is strongly birefringent and so presents a highly ordered supramolecular structure. The birefringence starts at early secretion and disappears at maturation phase. The analysis of enamel organic matrix birefringence may be used to detect the effects of genetic and environmental factors on the supramolecular orientation of enamel matrix and their effects on the structure of mature enamel.

Key Words: enamel, birefringence, polarizing microscopy

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Introduction

The formation of dental enamel is a complex biological process. It begins with the secretion of a protein-rich matrix. This matrix is proteolytically cleaved and replaced by mineral ions, which deposit to form hydroxyapatite crystals. Enamel mineralization is further completed with the elongation of the hydroxyapatite crystals, to give rise to the most mineralized tissue of the organism (1, 2). These events take place in a time- and space-restricted pattern, as distinct zones of secreting, maturing and mature enamel are clearly distinguished (3).

The current knowledge on the biochemical and morphological events that occur during the formation of enamel is largely based on *in vitro* studies and genetic mutations in mice and humans (4-13). These studies indicate that the organization and growth of the hydroxyapatite crystals are regulated by interactions with the components of enamel organic matrix. Enamel organic matrix is composed mainly by amelogenins (14), but several other protein components, present in smaller quantities, can also influence its biological properties. Recent evidences indicate that the components of enamel organic matrix can interact to form a supramolecular structure (15-17). The biological significance of this supramolecular organization is not clearly understood. In part this occurs due to methodological difficulties to perform direct morphological observations of the structured enamel organic matrix. The field would, therefore, greatly benefit from methods allowing *in situ* analysis of the supramolecular organization of enamel organic matrix in the diverse phases of amelogenesis.

The detection of anisotropic properties of biological structures by polarizing microscopy has been extensively used to study the macromolecular organization in

ordinary histological sections. The aim of this work was to study the birefringence of enamel organic matrix during the development of rat molar and incisor teeth.

Material and methods

Wistar rats with approximately 300g were anesthetized with chloral hydrate and perfused with 2% paraformaldehyde 0.5% glutaraldehyde in 0.2 M phosphate buffer solution, pH 7.2. Upper incisor teeth and hemimandibles were then immersed in the fixative solution for 16h. Decalcification was performed by immersion of upper incisor teeth and hemimandibles in 5% nitric acid and 10% formaldehyde for 6h and 24h, respectively. After dehydration, samples were embedded in paraffin and 5µm thick longitudinal sections were obtained. The sections were treated with xylol for removal of the paraffin, and hydrated.

Form birefringence curves were obtained after determining optical retardations of the area that showed the highest birefringence brightness, as a function of each refractive index (n) of the following imbibing media: water, 30%, 40%, 60% and 80% aqueous glycerin, 100% glycerin, nujol and immersion oil (Leica Microsystems Inc., Wetzlar, Germany). These fluids were used in the sequence they are quoted. Their correspondent refractive indices are reported in figure 2. Sections were immersed in the imbibing media for 30 min before measuring optical retardations, which were determined in nanometers (nm). Leica DM LP microscope (Leica Microsystems Inc., Wetzlar, Germany) equipped with polarizing filters, Brace-Köhler compensator (Wild Leitz GMBH Inc., Wetzlar, Germany) and polychromatic light was used.

When a change of the imbibing medium had to be secured, the section was air dried, rinsed in the next fluid to be used and finally covered with it for the time reported above, before measurement. After 100% glycerin was removed from sections, they were rinsed twice in distilled water for 10 min and then carefully air dried in order to be ready for immersion in nujol. The sections were rinsed with absolute ethanol after the period they were imbibed in nujol and immersion oil.

Measurements of optical retardations were performed in early secretory enamel, late secretory and maturing enamel (3). All the measurements in the present work were always carried out at the same point of the tissue in order to avoid variations on optical retardations due to thickness fluctuation. Forty measurements were performed in each region.

Hemimandibles from a newborn (0 day), 4 day-old and 9 day-old Wistar rats were also fixed and decalcified as described above. After dehydration, samples were embedded in paraffin and 5µm sectioned. The sections were used to investigate the birefringence of enamel organic matrix during the development of rat molar tooth.

Results

The analysis of rat incisor enamel matrix showed that it exhibits a positive birefringence. The maximum birefringence occurs when prisms are aligned at 45^0 with the polarizer and analyzer (Fig. 1). The analysis of optical retardation in upper incisor enamel matrix as a function of each refractive index (n) of the imbibing media is shown in figure 2. The highest and smallest retardations for the reported refractive indices correspond respectively to n=1.435 (80% aqueous glycerin) and n=1.518 (Leica oil). Optical

retardation values increased progressively from water to 80% aqueous glycerin and decreased progressively when the sections were immersed in 100% glycerin, nujol and Leica oil.

The arithmetic means of optical retardations on early secretory enamel, in the middle portion of secretory, and intermediate zone of enamel matrix imbibed in 80% aqueous glycerin are shown in Figure 3. The birefringence brightness begins to be evident in the early secretory stage, in the region corresponding to the distal root of the third molar (Fig. 4A). It appears initially as a thin layer near dentin-enamel junction and expands progressively towards enamel surface. The highest values of birefringence were found in the late secretory stage in a region that corresponds to the distal root of the second molar. In this region enamel has already reached full thickness (Fig. 4B). Birefringence decreases in maturation stage of amelogenesis, in a region that corresponds to the middle portion of the second molar. In this area the birefringence of enamel organic matrix exhibited a granular pattern and its maximum brightness was observed when the prisms were aligned at 90º with the polarizer (Fig. 4C). The birefringence starts to disappear near enamel surface and progressively extends to the dentin-enamel junction. Analysis of extracted teeth showed that the loss of birefringence occurred in the so called intermediate maturation zone when an opaque boundary can be distinguished on developing enamel after a 5 min drying time (18). These aspects are compatible with the degradation and removal of enamel organic matrix that occur in this area. No birefringence was observed in the region that corresponds to the mesial root of the second molar, even though enamel organic matrix is still present. This indicates that disorganization of enamel organic matrix precedes its nearly complete removal during maturation stage.

The analysis of rat first molar showed that the enamel matrix of 4 day-old animals also exhibited a strong birefringence in the areas of secretory enamel (Fig. 5). The enamel

of 0 day-old animals, which had a thin layer of enamel; and of 9 day-old animals, which was already in the maturation phase did not exhibit any birefringence.

Discussion

Anisotropic substances exhibit double refraction (19). Hence, when passing through these substances polarized light propagates as two wave fronts. Birefringence is the anisotropy caused by the difference between the two refractive indices of a substance. The relative displacement of these two polarized wave fronts is called optical retardation. Optical retardation is given as a length in nanometers and is directly proportional to the thickness of the sections. Then, thicker sections of the same sample show higher optical retardations. Sections of biological structures formed by filamentous, orderly arrayed and parallel macromolecules, exhibit a significant birefringence when examined through polarized light microscopy (20, 21). Birefringence reveals ordered supramolecular organization. In biological samples birefringence is observed in filamentous actin (F-actin), intermediate filaments, myosin and collagen (20-22). During secretory phase the enamel organic matrix is composed mainly by amelogenins, which account for more than 90% of the protein content in this tissue (23). These proteins can form globular structures known as nanospheres (5). Evidences indicate that nanospheres align with the side faces of hydroxyapatite crystallites during enamel development (24). This polymerization of amelogenin nanospheres may account for the birefringence of the enamel organic matrix detected in the present study. In this sense, the organization of enamel matrix may be similar to F-actin, whose birefringence is imparted by an ordered polymerization of globular molecules of G-actin (21, 25).

There are few data in the literature about the appearance of the enamel organic matrix when examined through polarized light microscopy. Previous studies reported that demineralized developing enamel ground sections were shown to exhibit a maximum birefringence ranging from 0.0003 to 0.0009 (26, 27). Considering that birefringence (B) is expressed by the formula B=OR/E, where OR= optical retardation, and E= thickness of the section in nm, the enamel matrix analysed in the present study was about 4-fold more birefringent than the 0.0009 value reported by ANGMAR-MÅNSSON (27). In fact, this author considered that the organic matrix of developing enamel was poorly crystalline. It was concluded that enamel organic matrix was formed by a disordered protein gel filling the spaces between apatite crystallites. The discrepancy found between the present and previous studies may be explained by the fact that the birefringence of this tissue is extremely vulnerable to fixation and demineralization processes. Rat incisor teeth should be rapidly extracted and immediately immersed in fixative solution. Rat hemimandibles should be perfused for proper preservation of the enamel organic matrix. The rapid loss of birefringence may result by the activity of the enamel proteases, which are present during all stages of amelogenesis. The fixing solution used in the present study (10% paraformaldehyde, 0.5% glutaraldehyde) provides a good structural preservation and an effective enzyme inactivation (28). Demineralization was also critical for enamel organic matrix preservation. Demineralization in 5% nitric acid, 10% formaldehyde allowed rapid removal of the mineral contents and preservation of the protein scaffold.

The results presented here indicate that enamel organic matrix is highly birefringent, presenting an ordered supramolecular structure. This finding opens new possibilities in the study of enamel biology. The analysis of enamel organic matrix birefringence may be a useful method to investigate the effects of genetic and environmental factors on enamel organic matrix and its association with enamel defects.

Acknowledgements

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq - Brazil.

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Figure legends

Figure 1. Birefringence brightness in secretory region of the enamel organic matrix of Wistar rat incisor imbibed in 80% aqueous glycerin. Analyzer and polarizer are signalized by crossed bars. A. Position of maximum birefringence (arrow at 45^0 with the polarizer and analyzer). B. The same section rotated 45^0, showing the minimum birefringence (extinction position).

Figure 2. Optical retardations (nm) of the highest birefringence in unstained 5µm thick sections of the enamel organic matrix of the Wistar rat incisor, as a function of each refractive index (RI) of the imbibing media. Measurements of optical retardations were made with Brace-Köhler compensator with polychromatic light. Results are expressed as mean ± standard deviation (SD). Each point in the curve is the average of 40 measurements. Vertical bars represent SD. Note that the highest and the smallest retardations for the series of refractive indices correspond respectively to n=1.435 (80% aqueous glycerin) and n=1.518 (Leica oil).

Figure 3. Form birefringence of enamel organic matrix of rat incisor. Eighty percent aqueous glycerin was used as the imbibing medium. Measurements of optical retardations (OR) were made with Brace-Köhler compensator with polychromatic light, on three different areas corresponding to early secretory, late secretory and maturation stage of enamel development. Results are expressed as mean ± standard deviation (SD). Each point in the curve is the average of 40 measurements. Vertical bars represent SD. Note that the highest birefringence brightness was observed in middle secretory enamel. The

lowest OR was observed in transition stage where extensive corruption of the organic matrix begins to occur.

Figure 4. Birefringence of enamel organic matrix of rat incisor. A. Early secretory stage. B. Late secretory stage. C. Maturation stage . Arrows indicate dentin-enamel-junction (DEJ). Note that birefringence starts near DEJ in early secretory stage and disappears progressively during transition phase of enamel development.

Figure 5. Birefringence of the enamel organic matrix of molar tooth from 4 day-old Wistar rat. Analyzer and polarizer are signalized by crossed bars. Arrow at 45^0 with the polarizer and analyzer indicates the position of maximum birefringence.

Figures

Figure 1

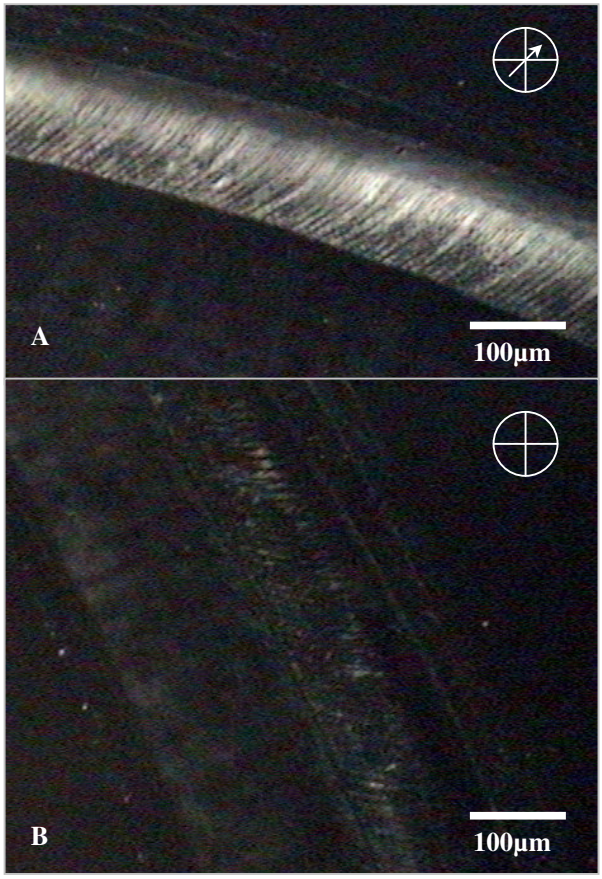
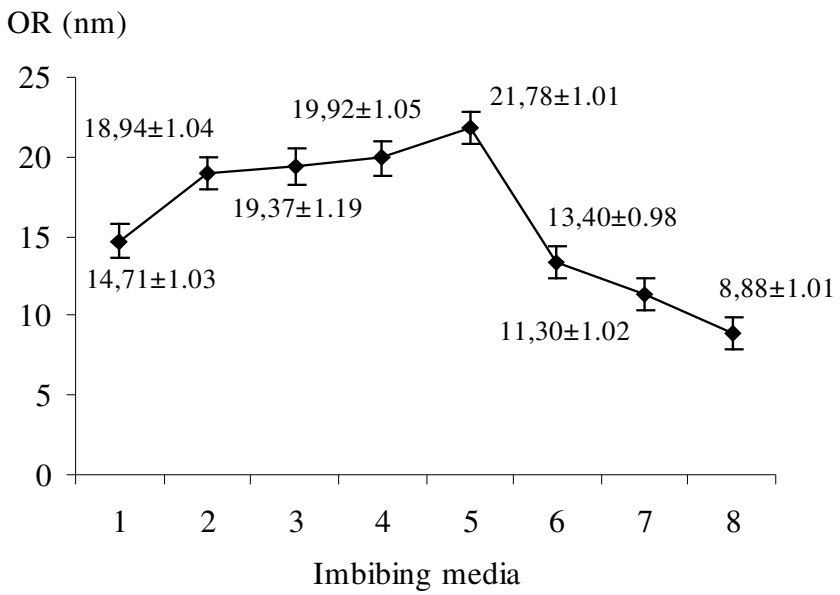


Figure 2



OR=optical retardation.
1=water (n=1.333*), 2=30% aqueous glycerin (n=1.372*), 3=40% aqueous glycerin (n=1.386*), 4=60% aqueous glycerin (n=1.413*), 5=80% aqueous glycerin (n=1.435*), 6=100% glycerin (n=1.461*), 7=nujol (n=1.478*), 8=Leica oil (n=1.518).

n=refractive index.
*According to Vidal BC et al. (1975).

Figure 3

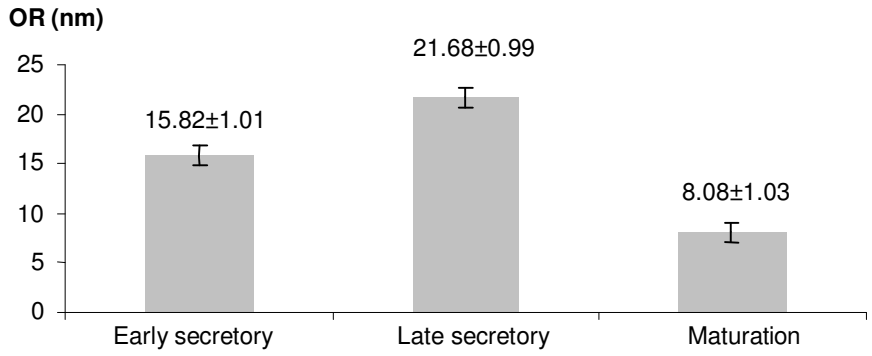

OR (nm)
25
20
15
10
5
0
15.82±1.01
21.68±0.99
8.08±1.03
Early secretory
Late secretory
Maturation

Figure 4

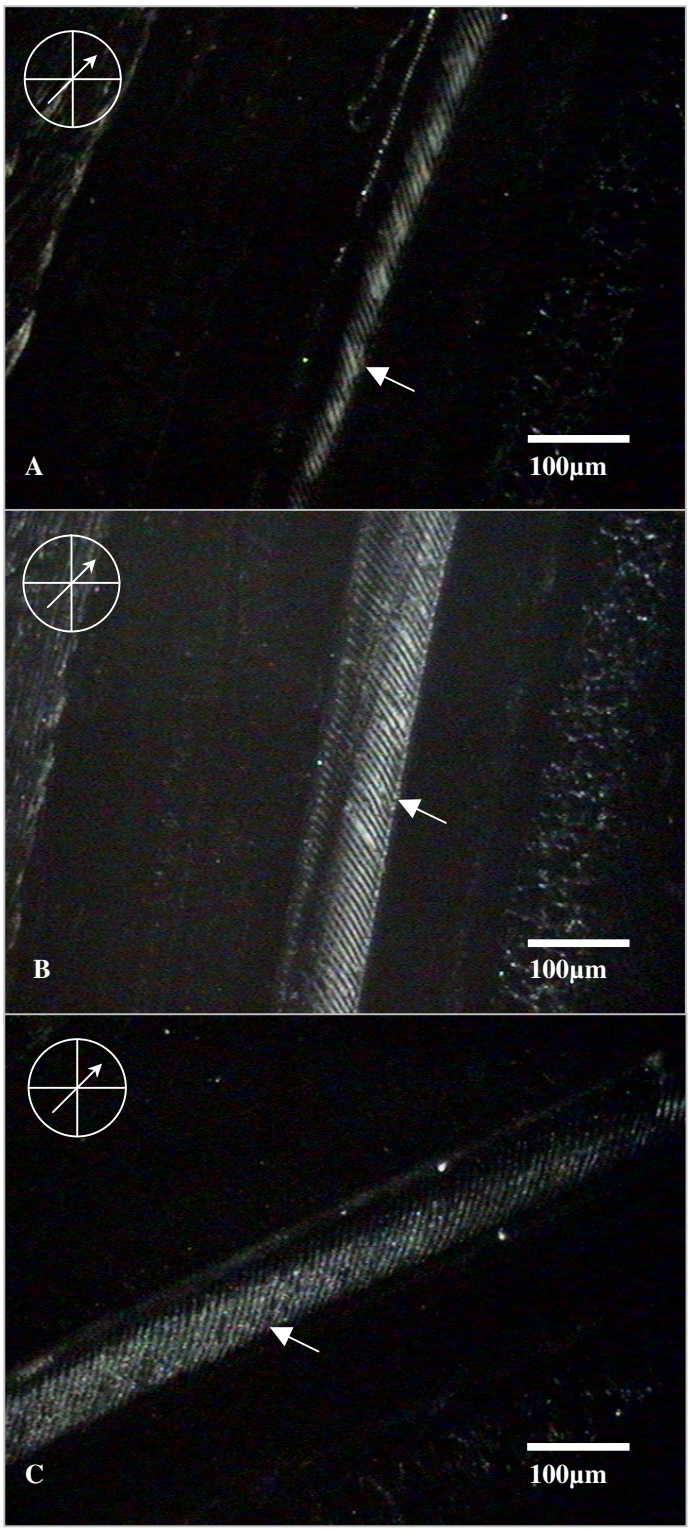
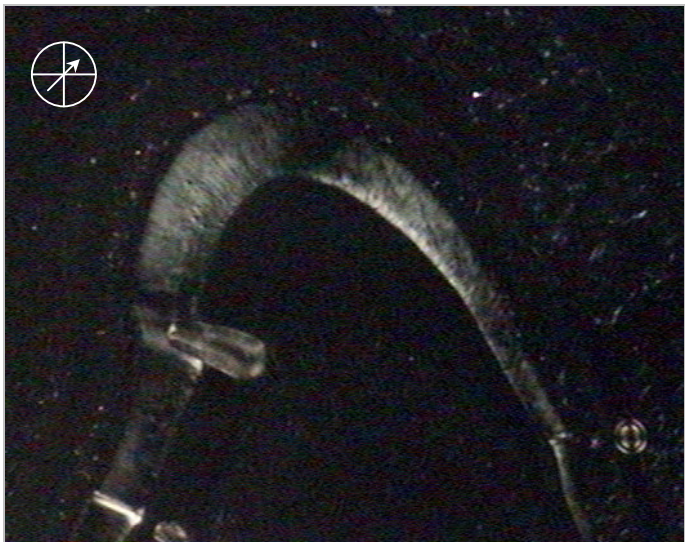


Figure 5



CONCLUSÃO GERAL

Os achados deste estudo mostram que a matriz orgânica do esmalte de molares e incisivos de ratos é altamente birrefringente, apresentando uma estrutura supramolecular ordenada. Esta descoberta abre novas possibilidades no estudo da formação do esmalte dentário, pois permite investigar os efeitos de fatores genéticos e ambientais sobre a organização supramolecular da sua matriz orgânica e correlacionar com defeitos em sua estrutura madura.

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ANEXO 1

UNICAMP

Universidade Estadual de Campinas
Instituto de Biologia

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

C E R T I F I C A D O

Certificamos que o Protocolo nº **744-1**, sobre **"BIRREFRIGÊNCIA DA MATRIZ ORGÂNICA DO ESMALTE DENTAL"** sob a responsabilidade de **Prof. Dr. Sérgio Roberto Peres Line / Alexandre Ribeiro do Espírito Santo** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 04 de novembro de 2004.

C E R T I F I C A T E

We certify that the protocol nº **744-1**, entitled **"BIREFRIGENCE ON THE DENTAL ENAMEL ORGANIC MATRIX"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on November 4, 2004.

Campinas, 04 de novembro de 2004.

Profa. Dra. Liana Verinaud
Presidente - CEEA/IB/UNICAMP

Fátima Alonso
Secretária - CEEA/IB/UNICAMP

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ANEXO 2

BRAZILIAN JOURNAL OF ORAL SCIENCES

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Dear Dr. Alexandre

Thank you for submitting your manuscript entitled. "The enamel organic matrix: structure and function." to the *Brazilian Journal of Oral Sciences.*

Please refer to manuscript number **145** on all further correspondence.

We will contact you again as soon as we have the necessary information for an editorial decision.

Sincerely yours,

Sergio Roberto Peres Line
Editor

ANEXO 3

----- Original Message -----
From: "Enamel7" <enamel7@forsyth.org>
To: "Sergio R. P. Line (E-mail)" <serglin@fop.unicamp.br>
Sent: Monday, December 06, 2004 5:55 PM
Subject: Enamel VII - Notification of Abstract Status

> December 6, 2004
>
> Dear Enamel VII participant,
>
> We are delighted to advise you that your abstract has been accepted for
> presentation at the Enamel VII Symposium to be held April 10-14, 2005 in
> Brewster, Massachusetts. We are in the process of organizing the symposium
> sessions, but for now please plan to prepare a 4 by 4 foot poster for
> presentation during the meeting. Following each poster session, presenters
> will give a 5 minute oral summary outlining the most important findings,
> using PowerPoint or 35 mm slides. At the end of each session, we plan to
> have a panel discussion of the presentations, which will be recorded for
> inclusion in the publication with the submitted manuscripts.
>
> **We also request that you bring to the conference a manuscript based on**
> **your abstract.** Instructions for manuscript preparation will be forthcoming.
>
> We are looking into options for transportation from the airport to the
> Ocean Edge Resort, and will post this information on the conference
> website shortly (www.enamel7.org).
>
> In order to assist us with conference planning, we encourage you to pay
> for meeting registration on the Enamel VII website using PayPal. You may also
> send a check, as indicated. Although this payment is not officially due until
> Jan. 15, 2005, earlier payment will assist us greatly in conference planning. The
> deposit for the Ocean Edge Resort (room and meals) is due by Jan. 30, 2005 through
> the resort website.
>
> Based on the variety and quality of abstracts that have been submitted,
> this is sure to be a very exciting conference, and we look forward to your
> participation.
>
> Finally, please reply to this message and confirm with us the name of the
> presenting author, since this was not clearly indicated in some abstracts.
>
> Best wishes,
>
> Henry and Carolyn
> Co-Chairs for Enamel VII