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Protein profile of pepsin-digested carious and sound human dentine

KEN-ICHI TONAMI[1] & DAN ERICSON[2]

[1]*Oral Diagnosis and General Dentistry, Dental Hospital, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan,* [2]*Department of Cariology, Faculty of Odontology, Malmö University, Malmö, Sweden*

Abstract
The purpose of this study was to describe the protein profile of pepsin-digested carious and sound dentine using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Carious and sound dentine powder was decalcified using 10% EDTA at pH 7.4 for 48 h. The decalcified pellet was digested using pepsin at pH 2 under sequenced conditions: at 4°C for 24 h, a further 24 h at 23°C, and finally for 24 h at 37°C. After every step, the soluble fraction was separated by centrifugation and analyzed in 15% SDS-PAGE. Two bands at 56 and 62 kDa could be observed in carious dentine digests and were considered specific carious bands. Similar bands could be observed in sound dentine samples, but only after pepsin digestion at higher temperatures (23°C and 37°C). Pepsin digests non-helical collagen and the triple helix structure of collagen is lost when the temperature rises. The bands at 56 and 62 kDa in sound dentine specimens thus represent pepsin-cleaved collagen. There is a possibility that the specific carious bands in carious dentine represent collagen decomposed in a manner similar to the way pepsin digests native dentine collagen at 23°C and 37°C.

Key Words: *Carious lesion, collagen, demineralization, dentine matrix, SDS-PAGE*

Introduction

Ever since early reports by Armstrong [1,2], the process of dentine caries progression is considered to include decalcification of the mineral phase by bacterial acids, thus exposing the collagen matrix that is subsequently degraded by enzymes. However, the process of degradation or denaturation is still unclear and several models and theories have been put forward. Recently, it was suggested that the host enzyme took part in denaturation of the matrix. Dung et al. [3] examined breakdown of the organic matrix of decalcified sound dentine using trypsin and matrix metalloproteinase-1, while Tjäderhane et al. [4] demonstrated a pH-dependent activation mechanism using host matrix metalloproteinase-2, 8, and 9. The addition of metabolites and glycoxidation products to matrix collagen has also been suspected to occur in the caries process [1,2,5,6]. The Maillard reaction between sugar and protein has been reported to be the cause of discoloration in carious dentine [1,2]. Kleter et al. [5,6] showed that amino acids in dentine collagen are modified by the Maillard reaction during the carious process and also that the reaction increases resistance to proteolysis, which supposedly induces arrestment of caries. Several models of dental caries progression have therefore been reported, but the consistency of the models with protein profiles of native carious dentine has not been confirmed.

The hypothesis of this study was that if enzymes are responsible for matrix denaturation, molecules of uniform size should be detected in carious dentine, because enzyme cleaves matrix collagen at unique locations. Dentine matrix collagen can be extracted by limited pepsin digestion and analyzed using sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) [7,8].

Our aim was to investigate the protein profile in carious dentine compared with sound dentine after continuous pepsin digestion.

Material and methods

Dentine powder

Twenty permanent human teeth extracted for odontological reasons with moderately active caries lesions (neither very soft nor very hard) were selected. The

Correspondence: Ken-ichi Tonami, Oral Diagnosis and General Dentistry, Dental Hospital, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, 113-8549, Tokyo, Japan. Tel: +81 3 5803 5889. Fax: +81 3 5966 7106. E-mail: ken1.gend@tmd.ac.jp

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teeth were donated to the Department of Cariology, Faculty of Odontology, Malmö University and stored in phosphate buffer solution at 4°C until use. After drying in room temperature, carious dentine and sound dentine were carefully removed from the teeth using round steel burs (nos. 1, 2, and 3) on a low-speed handpiece. The powdered carious dentine and sound dentine were pooled separately and stored at room temperature until use.

Chemicals

Acetic acid, Coomassie brilliant blue (CBB), bromphenol blue, di-sodium ethylenediamine tetraacetate (EDTA), glycine, 2-mercaptoethanol, methanol, polyethylene glycol of molecular weight 6000, sodium chloride and tris (hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Glycerol and sodium dodecyl sulfate were purchased from the Eastman Kodak Co. (Rochester, N.Y., USA). Ammonium persulfate, N,N,N,N′-tetra-methylethylenediamine, 30% acrylamide/bis solution and SDS-PAGE broad range molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, Calif., USA). Pepsin (3443F, 10,900 unit/mg) was from ICN Biomedicals (Aurora, Ohio, USA). All reagents and solutions used in this study were analytical grade.

Experimental procedures (Figure 1)

Dry carious and sound dentine powders in 10 mg aliquots were demineralized in 3.0 ml of 10% EDTA pH 7.4 for 48 h (Figure 1). The soluble fraction of carious and sound dentine, respectively, was separated by centrifuge at 10,000*g* for 10 min and dialyzed in distilled water for 16 h using dialysis membrane (Biotech Membranes MWCO: 8,000, Spectrum Laboratories Inc., Dominguez, Calif., USA) and concentrated at 4°C by polyethylene glycol. The final concentrate volume was 100 µl. To 20 µl of each concentrate, 80 µl sample buffer was added and analyzed using SDS-PAGE (hereafter CDSF for carious dentine and SDSF for sound dentine). The demineralized dentine samples were washed three times in 1.5 ml distilled water and digested with pepsin in 0.25 ml of 0.5 M acetic acid at 4°C, using 1090 U pepsin per mg of wet demineralized dentine [7,8]. After 24 h, the digestion was stopped with the addition of 0.2 M tris-HCl buffer, pH 8.6. The soluble fraction was separated from the insoluble fraction by centrifugation at 3,000*g* for 5 min. To precipitate protein in the soluble fraction, sodium chloride was added to a final concentration of 0.9 M. The precipitate was harvested by centrifugation at 10,000*g* for 30 min, dissolved in 20 µl sample buffer and applied to SDS-PAGE (hereafter CD04 for carious dentine and SD04 for sound dentine). The insoluble fraction from the digestion at 4°C was used for the next pepsin digestion at 23°C for 24 h and separated into a soluble fraction and an insoluble fraction as described above. The precipitate obtained from this second digestion was dissolved and applied to SDS-PAGE (hereafter CD23 for carious dentine and SD23 for sound dentine). The insoluble fraction remaining from the digestion at 23°C was used for the third digestion at 37°C for 24 h and treated as above, and the precipitate was dissolved and applied to SDS-PAGE (hereafter CD37 for carious dentine and SD37 for sound dentine). Pepsin (10 mg/ml in sample buffer) was used as reference in the SDS-PAGE. Gel for SDS-PAGE (15%) was prepared according to Laemmli [9]. Samples of 20 µl were run for 50 min at 200V. The gels were then stained with 0.125% Coomassie brilliant blue in 50 : 40 : 10% water : methanol : acetic acid. The complete experimental procedure was repeated twice.

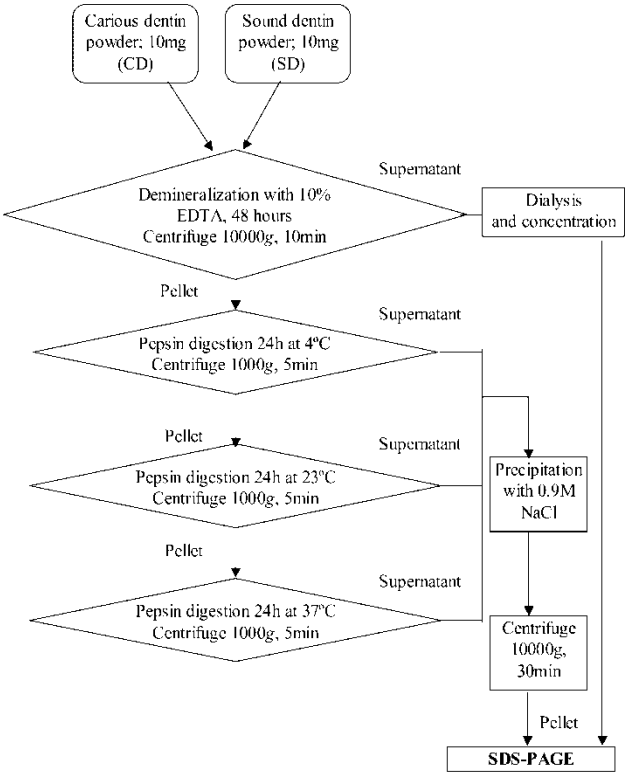

Figure 1. Experimental procedure.

Results

The result of SDS-PAGE is shown in Figure 2. Bands corresponding to collagen α chains were observed at around 100 kDa in all pepsin digests of sound dentine and CD23 (*arrow a*). For carious dentine digests, two bands were observed at molecular weights of 56 and 62 kDa (*arrow b*). These bands were also observed in sound dentine after pepsin digestion at higher temperatures (SD23 and SD37). Bands indicating pepsin were seen at around 40 kDa and lower (*arrow c*). After pepsin digestion for 72 h, the sound dentine sample was completely dissolved; the carious dentine

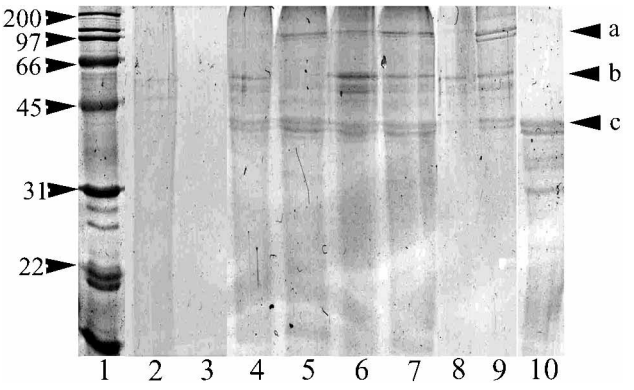

Figure 2. Protein profile of carious and sound dentine examined with SDS-PAGE. Lane 1: molecular weight marker, 2: CDSF, 3: SDSF, 4: CD04, 5: SD04, 6: CD23, 7: SD23, 8: CD37, 9: SD37, 10: pepsin. (a) Collagen α1(I) and α2(I) bands could be observed in sound dentine samples and CD23. (b) The bands corresponding to 56 and 62 kDa were observed in the carious dentine of every condition and in SD23 and SD37. (c) Pepsin band around 40 and lower kDa appeared in every digesting condition.

digest, however, left a pellet of insoluble tissue. The results were the same in repeated experiments.

Discussion

The protein profiles examined in this study would represent the total protein profile of 20 caries lesions. Even though the lesions were similar in consistency, areas of arrested and more active caries are likely to be included in the samples. Limited pepsin digestion is a well-known method for solubilizing collagen for analysis [7,8]. Pepsin acts only at the non-helical end of the collagen molecule, which contains inter- and intra-molecule cross-links formation. Collagen polymers were therefore split into monomers. The monomers of collagen could be detectable as α, β and γ chains with SDS-PAGE [7,8,10]. With this procedure, Takagi et al. [11] examined the deciduous dentine from a patient of dentine dysplasia and reported alteration of the protein profile in dentine matrix. Pepsin digestion followed by SDS-PAGE is thus a suitable method for investigating alteration of the dentine matrix protein profile. In the present study, two bands were observed at around 100 kDa for CD23, SD04, SD23, and SD37. Ninety percent of the protein in dentine matrix consists of type I collagen [12]; therefore, these bands were considered to be collagen α1(I) and α2(I) chains. In addition, two other bands could be observed at molecular weights of 56 and 62 kDa for CDSF, CD04, CD23, CD37, SD23, and SD37. These two bands were considered caries specific, because they appeared in the supernatant of demineralized carious dentine and through the sequence of pepsin digestion of carious dentine. The bands could not be observed in the supernatant of demineralized sound dentine (absent in SDSF, SD04). Two possible origins for these bands may be suggested; one is denatured dentine collagen matrix and the other the organic material outside the tissue, e.g. bacteria products from oral cavity. We believe that the former is more likely because the same two bands could be observed in sound dentine samples only after more extensive pepsin digestion at 23°C and 37°C. This indicates that the bands from sound dentine were pepsin-cleaved collagen and not of bacterial origin, as they appeared in sound dentine and only after a long time and repeated pepsin digestion at 23°C and 37°C. Though the helical region of collagen molecules is resistant to enzymatic proteolysis [7], the triple helix is lost when temperature rises [8]. For native calfskin collagen, which is the same type as dentine collagen, the temperature of the midpoint of this transition was 36°C [13]. Structural changes of collagen molecules at higher temperature decrease its resistance to proteolysis, resulting in the decomposition of collagen into smaller units, e.g. 56 and 62 kDa.

No band could be found at around 79 kDa for carious dentine in this study. However, Dung et al. [3] observed this band in their SDS-PAGE analysis using decalcified dentine powder digested by trypsin and matrix metalloproteinase-1. In the present study, the carious dentine sample was prepared from natural dentine caries lesion and the enzyme was pepsin rather than trypsin, which is possibly the reason for the discrepancy in the results.

Sound dentine collagen was completely dissolved after pepsin digestion at 37°C, while an insoluble fraction remained in carious dentine collagen after pepsin digestion at 37°C, that is, pepsin digestion solubilizes demineralized carious dentine partly but not completely. The dentine caries sample used in the present study included inactive or arrested carious dentine. Kleter et al. [5] reported that artificial caries where the Maillard reaction had occurred was resistant to pepsin digestion and claimed that this may partly explain why caries can be arrested. Meng et al. [14], too, indicated a positive relation between the advanced glycosylation end products of the Maillard reaction and pepsin-insoluble collagen of aorta of diabetic rats. Consequently, such Maillard reaction products included in carious dentine might explain the solubility difference between carious dentine and sound dentine after 72 h of pepsin digestion.

The findings might imply a relation between the structural changes of exposed carious dentine collagen in oral cavity and its sensitivity to attack from enzymes, that is, decalcified dentine collagen is degraded by enzymes when the triple-helix structure has disappeared. The Maillard reaction may protect such exposed collagen, increasing the resistance to enzymatic digestion. However, further investigation is necessary to clarify the mechanism of how the caries process modifies dentine.

Recently, the importance of minimal invasion to sound tissue during caries removal is emphasized

in restorative treatment based on understanding of pulp-dentine complex [15]. From the perspective of minimally invasive dentistry, chemomechanical caries removal might be an alternative approach [15]. Most studies have focused on determining characteristics of the tissue remaining (in the tooth) after caries removal [16–18], and although such experiments were appropriate for evaluating the efficacy of the caries removal, they were not appropriate for clarifying the mechanism of action, as the removed material was not examined. Only few studies have addressed the effect of chloramines on carious dentine [19]. It is still not clear how carious dentine is affected. It is hoped that the specific caries bands detected in this study will contribute to studies on the effect of chemical agents on the molecular of caries dentine collagen in future.

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References

[1] Armstrong WG. A quantitative comparison of the amino acid composition of sound dentine carious dentine and the collagenase resistant fraction of carious dentine. Arch Oral Biol 1961;5:115–3.

[2] Armstrong WG. Modification of properties and composition of the dentin matrix caused by human dental caries. Adv Oral Biol 1964;42:309–32.

[3] Dung SZ, Gregory RL, Li Y, Stookey GK. Effect of lactic acid and proteolytic enzymes on the release of organic matrix components from human root dentin. Caries Res 1995;29:483–9.

[4] Tjäderhane L, Larjava H, Sorsa T, Uitto V-J, Larmas M, Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Res 1998;77:1622–9.

[5] Kleter GA, Damen JJM, Buijs MJ, TenCate, JM. The Maillard reaction in demineralized dentin in vitro. Eur J Oral Sci 1997;105:278–84.

[6] Kleter GA, Damen JJM, Buijs MJ, Ten Cate JM. Modification of amino acid residues in carious dentin matrix. J Dent Res 1998;77:488–95.

[7] Miller EJ, Rhodes RK. Preparation and characterization of different types of collagen. In: Cunningham LW, Frederiksen DW, editors. Methods in enzymology, vol. 82. New York: Academic Press; 1982. p. 33–64.

[8] Kuboki Y. Preparation of collagen from hard tissue. In: Nagai Y, Fujimoto D, editors. Collagen experimental method. Tokyo: Kodansha; 1991. p. 21–30.

[9] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[10] Veis A, Payne K. Molecular structure and functions of collagen. In: Nimni ME, editor. Collagen, vol. I. Biochemistry. Florida: CRC Press; 1988. p. 73–94.

[11] Takagi T, Sasaki S, Kashima K, Shioiri S, Shioda S, Nakamura S, et al. Biochemical and structural characterization of a family with dentin dysplasia. Jpn J Oral Biol 1990;32: 591–9.

[12] Linde A. Dentin matrix proteins: composition and possible functions in calcification. Anat Rec 1982;249:154–66.

[13] Beier G, Engel J. The renaturation of soluble collagen. Products formed at different temperatures. Biochemistry 1966;5:2744–55.

[14] Meng J, Sakata N, Takebayashi S, Asano T, Futata T, Araki N, et al. Advanced glycation end products of the Maillard reaction in aortic pepsin-insoluble and pepsin-soluble collagen from diabetic rats. Diabetes 1996;45:1037–43.

[15] Bjørndal L, Mjör IA. Pulp-dentin biology in restorative dentistry. Part 4. Dental caries – characteristics of lesions and pulpal reactions. Quintessence Int 2001;32:717–36.

[16] Hossain M, Nakamura Y, Tamaki Y, Yamada Y, Jayawardena JA, Matsumoto K. Dentinal composition and Knoop hardness measurements of cavity following carious dentin removal with Carisolv. Oper Dent 2003;28:346–51.

[17] Arvidsson A, Liedberg B, Moller K, Lyven B, Sellen A, Wennerberg A. Chemical and topographical analyses of dentine surfaces after Carisolv treatment. J Dent 2002;30: 67–75.

[18] Splieth C, Rosin M, Gellissen B. Determination of residual dentine caries after conventional mechanical and chemomechanical caries removal with Carisolv. Clin Oral Invest 2001;5:250–3.

[19] Tonami K, Araki K, Mataki S, Kurosaki N. Effects of chloramines and sodium hypochlorite on carious dentin. J Med Dent Sci 2003;50:139–46.