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Papain-gel Degrades Intact Nonmineralized Type I Collagen Fibrils

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Summary

Papain-gel has been utilized as a chemomechanical material for caries removal due to its ability to preserve underlying sound dentin. However, little is known about the effect of the papain enzyme on intact type I collagen fibrils that compose the dentin matrix. Here we sought to define structural changes that occur in intact type I collagen fibrils after an enzymatic treatment with a papain-gel. Intact and nonmineralized type I collagen fibrils from rat tail were obtained and treated with a papain-gel (Papacarie) for 30 s, rinsed with water and imaged using an atomic force microscope (AFM). Additionally, polished healthy dentin specimens were also treated using the same protocol described above and had their elastic modulus (E) and hardness (H) measured by means of AFM-based nanoindentation. AFM images showed that the papain-gel induced partial degradation of the fibrils surface, yet no rupture of fibrils was noticed. The distinction between gap and overlap zones of fibrils vanished in most regions after treatment, and overlap zones appeared to be generally more affected. Mechanical data suggested a gradual decrease in E and H after treatments. A significant two-fold drop from the values of normal dentin ($E = 20 \pm 1.9$, $H = 0.8 \pm 0.08$ GPa) was found after four applications ($E = 9.7 \pm 3.2$, $H = 0.24 \pm 0.1$ GPa) ($P < 0.001$), which may be attributed to the degradation of proteoglycans of the matrix. In summary, this study provided novel evidence that intact nonmineralized type I collagen fibrils are partially degraded by a papain-gel.

Keywords

collagen; papain; dentin; AFM; mechanical properties

Introduction

Recent advances in restorative dentistry emphasize the development of materials and procedures that facilitate the preservation of sound dental tissues before restoration, thus favoring minimal invasiveness. Chemo-mechanical methods of caries removal have been increasingly indicated as an option for minimally invasive treatments. Chemo-mechanical agents rely on the action of proteolytic agents, such as papain and sodium hypochlorite, to further degrade the partially demineralized and altered dentin matrix that has been previously exposed to bacterial action (infected dentin), thus facilitating its removal and preventing damage to the underlying remineralizable tissues (affected dentin) (Banerjee et al. 2000). The papain enzyme, particularly, a plant-derived cysteine protease of broad proteolytic activity (Thomas and Partridge 1960), has been used as a chemo-mechanical material since its recent introduction by Bussadori's group (Bussadori et al. 2005). The papain-gel has been suggested to act by exclusively breaking down the partially degraded collagen molecules and contributing to the degradation and elimination of the fibrin "mantle" formed by the carious process, without

damaging intact collagen fibrils (Bussadori et al. 2005). This selective interaction of the enzyme with the affected components of the carious dentin has been suggested to be due to the lack of an antiprotease α -1-anti-trypsin, which inhibits protein digestion in sound collagen-based tissues (Bussadori et al. 2005; Flindt 1979). However, this so-called “protective” action of the antiprotease α -1-antitrypsin against papain is largely based on its effects in soft tissues, particularly the lungs (Flindt 1979; Hogg and Timens 2009), and to date, to our knowledge, has not been identified in dentin.

The proteolytic action of papain has also been investigated in cartilage (Ueda et al. 1981), which, like the lung parenchyma and the dentin matrix, is largely composed by collagen fibrils and proteoglycans. Research performed in cartilage and lungs have shown that papain promotes the digestion of proteoglycans of the extracellular matrix, such as decorin and biglycan (Halpern et al. 1965; Hogg and Timens 2009; Ueda et al. 1981). These proteoglycans are important determinants of the physical properties of the extracellular matrix of tissues in general (Bourdon et al. 1985; Ho et al. 2005). It has been suggested that decorin crosslinks collagen fibrils and that the level of decorin present in the tissue reflects how tightly the collagen fibrils are attached to each other (Hogg and Timens 2009). Therefore, an increase in decorin implies stiffening of the collagen network, whereas a loss leads to increased compliance (Hogg and Timens 2009). Moreover, in load bearing tissues, proteoglycans contribute to the osmotic pressure by providing sites of electrostatic interaction within glycosaminoglycans that guarantee higher hydrophilicity to the tissues (Bourdon et al. 1985; Ho et al. 2005). It is, therefore, believed that the presence of proteoglycans in the dentin matrix and their interaction with the collagen fibrils might play a pivotal role on the mechanical functionality of the tissue. Thus, it is hypothesized that the treatment of dentin with a papain-gel might affect the tissue's mechanical properties. Although the effectiveness of papain-gel in facilitating removal of carious dentin is well described in the literature (Bussadori et al. 2005; Correa et al. 2007, 2008; Piva et al. 2008), no information exists on whether or not papain affects the structure of intact and nonmineralized type I collagen fibrils. We hypothesize that the selective action of the papain-gel in carious dentin is due to its inability to digest collagen fibrils that are protected by mineral in sound dentin, as opposed to the putative “protective” action of the α -1-anti-trypsin in intact collagen fibrils (Bussadori et al. 2005; Correa et al. 2007). Therefore, to give insight into the action of papain-gel on collagen based tissues, this study sought to determine if intact and sound nonmineralized collagen fibrils are degraded by a papain enzyme. To test this hypothesis we used a simple experiment where intact non-mineralized type I collagen fibrils from rat tail were exposed to a papain-gel (Papacáries) digestion and subsequently evaluated the nanostructural changes that followed the treatments using atomic force microscopy (AFM) imaging. Further, in order to observe the possible proteolytic effect of papain-gel on the proteoglycans of healthy dentin, we analyzed representative specimens using nanoindentation to gain insights into mechanical changes that occurred following the papain-gel treatment.

Materials and Methods

Rats were obtained using animal tissue transfer according to guidelines by Committee on Animal Research, UCSF. For topological assessment by AFM, collagen was prepared from rat tail tendon (flexor digitalis tendon) by dissection in saline and stored at 4°C until use. Fibrils were deposited from saline on plain glass slides and allowed to dry in ambient temperature which resulted in firm attachment to the glass slide. The length and width of the samples to be scanned varied according to how much was attached to the glass slide. A minimum of 3 collagen containing glass slides were scanned. The papain-gel (Papacáries®, Formula & Ac-ão, São Paulo, Brazil) was applied to the glass slide fully covering the collagen sheets and allowed to react for 30 s. Specimens were carefully rinsed with de-ionized water for 20 s following manufacturer's directions and allowed to dry before imaging. Images were obtained in tapping mode with a Multi-Mode Atomic Force Microscope (Nanoscope III, Digital Instruments, Santa

Barbara, CA). Additionally, we analyzed representative healthy dentin specimens to gain insight into nanomechanical changes that occurred after enzymatic degradation with the papain-gel. Dentin squares measuring 3.5mm in length and width and 2mm in thickness were cut from the midcoronal region of selected fully formed permanent caries-free third molars. The sections were cut perpendicular to the dentin tubule direction. The specimens were ground with SiC abrasive papers from 600 to 1200 grits, and then polished with diamond suspensions (Buehler, Lake Bluff, IL) of 1.0 and 0.25 μm particle sizes. The dentin specimens were thoroughly cleaned and subjected to 2–4 applications of the papain-gel using a syringe, as provided by the manufacturer. The gel was allowed to react for 30 s followed by rinsing with deionized water for 20 s for each application. Mechanical properties were measured after each papain-gel application. Measurements started after the second application because the manufacturer recommends at least two applications for optimized results. Indentations were made with a calibrated Berkovich diamond indenter on an atomic force microscope with the standard head replaced by a calibrated Triboscope indenter system (Hysitron Inc., Minneapolis, MN) to evaluate the reduced elastic modulus (E) and hardness (H) of the tissues before and after treatments, following protocols previously described (Marshall et al. 2001). Nanoindentations used a trapezoidal force profile with 3 s loading, hold and unloading segments and peak loads between 50 and 250 mN. Each indentation yielded a load–deformation curve, from which the reduced elastic modulus, E, and hardness, H, were determined according to the following equations (Doerner and Nix 1986):

$$E = \sqrt{[\pi/a]} \cdot S/2.$$

$$H = \frac{F_{\max}}{a}$$

where S represents the slope of the unloading curve based on the method of Oliver and Pharr (1992), a is the indentation contact area, and Fmax is the maximum force. Calibration used a silica standard as previously described (Marshall et al. 2004). A minimum of 15 indentations were made in intertubular dentin at approximately 20 μm intervals. AFM images were collected before and after indentations to ensure that they were uniform, well-defined, and within intertubular dentin. A Kolmogorov–Smirnov test showed a normal distribution of data. Data was subsequently analyzed using analysis of variance and Tukey's test with significance level of 5%.

Results

Figure 1(A) shows images of intact nonmineralized collagen fibrils from rat tail before enzymatic degradation with the typical staggered pattern due to the D-banding periodicity of 67 nm. Figure 1(B) shows a 3-D perspective of the same structures. At this stage the fibrils present a smooth surface and well-defined gap (black line) and overlap zones (white dotted outline). Figure 2(A) and (B) shows collagen fibrils after one papain-gel application from a top view and a 3-D perspective of the same structures, respectively. The papain-gel induced degradation of the fibrils surface, yet no rupture of fibrils was noticed. The clear distinction between gap and overlap zones seen in the untreated fibrils vanished in most regions after treatment. Images also suggested that overlap zones were generally more affected as random areas of fibrils still presented gap zones (arrowheads). Regions within the degraded overlap zones presented a generalized roughened surface. Mechanical data (Fig. 3) showed that properties of normal dentin treated with papain tended to decrease gradually as the papain-gel applications were done. Averaged values of elastic modulus and hardness were significantly different from normal dentin ($E = 20 \pm 1.9$, $H = 0.8 \pm 0.08$ GPa) and from each other after 2 ($E = 13 \pm 2.8$, $H = 0.4 \pm 0.1$ GPa), 3 ($E = 15.3 \pm 3.7$, $H = 0.5 \pm 0.2$ GPa) and 4 ($E = 9.7 \pm 3.2$, $H = 0.24 \pm 0.1$ GPa) gel applications ($P < 0.001$).

Discussion

This study sought to identify structural changes in intact nonmineralized type I collagen fibrils induced by an enzymatic treatment with a papain-gel. Our observations provide conclusive evidence that papain promotes superficial degradation of collagen fibrils and that this is independent of a previous partial degradation from bacterial action. These results support our initial hypothesis that the softening of carious dentin after application of papain-gel with limited effects on the remaining sound tissue may be due to the inability of the enzyme to attack the hydroxyapatite-coated collagen, as opposed to the putative ‘protective’ action of the α -1-anti-trypsin molecule in sound fibrils in dentin (Bussadori et al. 2005). α -1-anti-trypsin is a molecule produced by hepatocytes and mononuclear phagocytes, and is extensively found in the lower respiratory tract during the degradation of the lung parenchyma in respiratory diseases, such as pulmonary emphysema (Hogg and Timens 2009). Although the presence of this molecule in normal dentin has not been ruled out, it is likely that the inflammatory process that would release signals to recruit leukocytes, such as those released from hepatocytes and mononuclear phagocytes should be mainly confined to the pulp chamber, where the tooth inflammatory response takes place. Moreover, if α -1-anti-trypsin in sound dentin prevented enzymatic degradation, similar effects might be expected against the proteolytic action of bacteria, which does not appear to be the case. Although, this would depend upon the type of bacteria and the byproducts they were releasing. The proteolytic activity of papain has been well described in the literature, including the degradation of elastin and proteoglycans (Halpern et al. 1965; Johanson and Pierce 1972; Junqueira et al. 1980; Thomas and Partridge 1960; Ueda et al. 1981). Nevertheless, this is the first study providing evidence of degradation of collagen fibrils with a papain-gel. AFM images showed a surface degradation, particularly of the overlap zones, with no evident disruption of fibrils. We add that the apparently more evident degradation of the overlap zones might be due to a geometrical effect due to the tip shape, as it is easier to reach the upper bumps on the overlaps than the crevices in the gaps. Yet, the bumps of the overlap zones might be more easily accessible to the gel than the valleys of gap zones, which could also explain the more pronounced degradation of the former. Other imaging techniques, such as transmission electron microscopy that do not provide a topographical mapping of the tissue, might lack the capability to detect the surface changes seen here. However, high-resolution SEM, though with some level of difficulty, should be able to identify these changes, but to our knowledge this is the first study devoted to analyze the effect of papain enzyme on the degradation of naked type I collagen fibrils. It should also be pointed out that although SEM provides good visual description, it is not a quantitative method and prone to subjective interpretation, moreover, it does not allow the three-dimensional measurements that are possible with the use of AFM. Early studies suggested that collapse of the rabbit ears by papain was due to a marked decrease of proteoglycan in the ear cartilage (Tsaltas and Greenawald 1965). Shepard and Mitchell (Shepard and Mitchell 1977) showed that the rabbit articular cartilage previously treated with papain collapsed as a consequence of the loss of proteoglycans by papain digestion. Proteoglycans have been suggested to work as bridges between contiguous fibrils (Scott 1988); moreover, they are believed to regulate the biophysical properties of load bearing tissues by filling spaces, binding and organizing water molecules. These observations support our mechanical properties results. Although the collagen structure in dentin is coated with mineral, proteoglycans in the matrix are expected to be more exposed to allow their interaction with water molecules. Therefore, they should be more susceptible to enzymatic degradation. Ho (Ho et al. 2005) observed a two-fold decrease in elastic modulus and hardness after the degradation of decorin and biglycan in dentin near the cementum dental junction. The results of this former investigation are in good agreement with the two-fold drop in modulus and hardness we observed after four applications of the papain gel in normal dentin. Furthermore, a recent investigation on the mechanical properties of dentin after complete removal of the carious portion with a papain-gel showed a decrease in comparison with the values of healthy dentin, which also supports our findings (Correa et al. 2007). Yet, a conclusive

explanation for such a decrease in mechanical properties remains to be fully understood. At this point our explanations are an extrapolation of other's observations, thus other factors, such as the gel pH, the action of its other components (toluidine and chloramine) and the actual degradation of the mineralized collagen in healthy dentin cannot be ruled out as contributors to the decrease in properties we observed. Although this study provided evidence of degradation of type I collagen with papain, it does not succeed in describing the effect of this enzyme in collagen from dentin, which represents the major limitation of our observations. It is known that the collagen in unmineralized tissues is cross-linked in a different manner than in mineralized tissues (Knott and Bailey 1998); thus, enzymatic effects that take place in the former might not be exactly replicated in dentin. Further, technical limitations such as the inability of our method to follow changes in individual fibrils with repeated papain treatments, due to the water-rinsing step, make our conclusions limited to rather short-term effects, and at this point we cannot ensure that papain does not induce other morphological changes to the collagen fibrils after longer periods of time.

Conclusion

Within the limitations of this study, we provide evidence that intact nonmineralized type I collagen fibrils are partially degraded by a papain-gel. In addition intact mineralized dentin had reduced mechanical properties after treatments with the papain-gel, presumably due to the effect of the protease on dentin proteoglycans and possibly on the mineralized collagen fibrils as well. This study helped to elucidate the action of papain enzyme-based gels on collagen-based tissues and provided insights into how it may affect the mechanical properties of healthy dentin. Our results strengthen the clinical effectiveness of papain-based gels and suggest that it may become one more promising agent for chemomechanical removal of caries.

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Fig 1.

The intact nonmineralized collagen fibrils from rat tail before enzymatic degradation from the top (A) and a 3-D perspective (B). Fibrils present the typical staggered pattern with a periodicity of 67 nm of gap (black line) and overlap zones (white dotted outline). At this stage the fibrils present a smooth surface and clear distinction between zones.

Fig 2. Collagen fibrils after papain-gel digestion from a top view (A) and a 3-D perspective (B). Images show degradation of the fibrils surface, which appeared roughened, yet no rupture of fibrils was noticed. The distinction between gap and overlap zones vanished in most regions with random preservation of gap zones (arrowheads).

Fig 3.

Averaged values of reduced elastic modulus, E , (A) and hardness, H , (B). For each application the papain-gel was allowed to react with the dentin sample for 30 s and rinsed with de-ionized water for 20 s. Treated specimens were significantly different from normal dentin ($P < 0.001$).