Effect of tooth bleaching agents on protein content and mechanical properties of dental enamel

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ABSTRACT

This study investigated the effect of two bleaching agents, 16% carbamide peroxide (CP) and 35% hydrogen peroxide (HP), on the mechanical properties and protein content of human enamel from freshly extracted teeth. The protein components of control and treated enamel were extracted and examined on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Marked reduction of the protein matrix and random fragmentation of the enamel proteins after bleaching treatments was found. The mechanical properties were analyzed with Vickers indentations to characterize fracture toughness, and nanoindentation to establish enamel hardness, elastic modulus and creep deformation. Results indicate that the hardness and elastic modulus of enamel were significantly reduced after treatment with CP and HP. After bleaching, the creep deformation at maximum load increased and the recovery upon unloading reduced. Crack lengths of CP and HP treated enamel were increased, while fracture toughness decreased. Additionally, the microstructures of fractured and indented samples were examined with field emission gun scanning electron microscopy (FEG-SEM) showing distinct differences in the fracture surface morphology between pre- and post-bleached enamel. In conclusion, tooth bleaching agents can produce detrimental effects on the mechanical properties of enamel, possibly as a consequence of damaging or denaturing of its protein components.

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1. Introduction

Dental enamel, as the hardest tissue in the body, acts as a protective covering of teeth and can withstand a wide range of functional and non-functional loads. Enamel is a hierarchical bio-composite made of 94–96 wt.% inorganic content, 1 wt.% organic matrix and 4–5% water [1]. The inorganic content is comprised of long thin hydroxyapatite crystals tightly packed together with a protein glue (hydrophobic enamelin) to form enamel rods [1]. These rods are encapsulated by a thin protein-rich organic sheath (primarily hydrophilic ameloblastin) around 0.8–1 μm thick [2]. Although the organic contents of enamel comprise less than 1%, they play a significant role in determining the mechanical behavior of enamel [3]. This organic content helps to define three-dimensional cleavage planes to deflect cracks which prevent fracture from progressing through enamel and allows limited movement between the rods during stress [2,4].

The mechanical properties of enamel are dependent on its composition and its structural organization [5]. The hardness and modulus of elasticity of the rods have been reported to be much higher than that of the organic sheath [6]. The highest values of hardness and modulus of elasticity have been found at the occlusal surface and decrease toward the dentin-enamel junction (DEJ) as mineral density decreases and the organic matrix increases [7,8]. In addition, the fracture toughness of enamel ranges from 0.4 MPa m 1/2 in the outer enamel to 2.37 MPa m 1/2 in the inner region near the DEJ [9,10]. This increase is primarily due to an increase of the organic matrix and different rod orientations (decussation) which causes crack propagation resistance by the formation of protein bridges, crack deflection and microcracks [9].

Tooth bleaching has become a highly popular esthetic dental treatment as it is the easiest and the least destructive procedure for treatment of tooth discoloration. At present, there are three main tooth bleaching techniques being used: in-office bleaching, dentist supervised home bleaching and over the counter (OTC) bleaching products [11]. Contemporary bleaching techniques use
hydrogen peroxide (HP) or its precursor carbamide peroxide (CP) as an active ingredient [11]. Bleaching peroxide penetrates through enamel and dentin eventually reaching the pulp in the course of which it undergoes chemical breakdown forming oxygen free radicals. These free radicals are highly unstable and capable of oxidizing and disintegrating a wide range of organic and inorganic materials including chromophores [12]. Despite their ability to disintegrate pigmented molecules in tooth structure, bleaching agents can also attack the organic and inorganic contents of tooth structure [13].

Morphological alterations of enamel surface after bleaching treatment can be seen as an increase in surface roughness and erosion [14,15], Changes of the chemical composition of enamel and mineral loss has also been reported [15,16]. Micro- and nano-mechanical investigations of enamel subjected to bleaching agents have shown that HP significantly decreases the hardness and modulus of elasticity of enamel [17–19], as well as the fracture toughness and wear resistance [20,21]. These changes in the mechanical properties of enamel could be due to both, loss of mineral content as well as denaturation and degradation of the organic matrix by the oxidation reaction [18,19,21]. However, the mechanism(s) of action by which the bleaching peroxide induces detrimental effects on the mechanical properties of enamel has not been fully identified. Therefore, this study aims to analyze the influence of bleaching agents on the nanomechanical properties of enamel and enamel matrix protein/peptides along with the underlying mechanisms in an attempt to understand the structural alterations that accompany these changes. The nanomechanical properties of enamel were investigated before and after bleaching and according to the manufacturers’ instructions as outlined below.

2. Materials and methods

2.1. Specimen preparation

Twenty-four fully erupted human third molars were obtained from healthy patients aged 18–40 according to protocols approved by the Sydney West Area Health Service (Ref. No. HREC/11/ WMEAD/115). The extracted teeth were cleaned and embedded in a cold-curing epoxy resin (Epofix, Struers, Denmark). Upon curing the occlusal surface was ground flat with silicon carbide papers with grit sizes 240, 400, 600 and 1200 under continuous water irrigation to avoid overheating. Specimens were then polished by specimen polisher (RotoPol-22, Struers, Denmark) with 9 μm and 1 μm diamond polishing pastes then lastly with 0.04 μm colloidal silica. After each polishing cycle, specimens were ultrasonically cleaned (Unisonics, Australia) for 5 min and stored in Hank’s balanced salt solution (HBSS, Sigma–Aldrich, Germany) at room temperature between the bleaching treatments. Prior to indentation experiments, the specimens were examined under optical microscope to exclude excessively scratched and cracked surfaces. One half of the specimen was covered with nail varnish (control) and the other half was treated with one of the bleaching agents.

Two bleaching agents were used in this study: 16% carbamide peroxide (CP) home bleaching gel (Polanight, SDI Limited, Australia); and 35% hydrogen peroxide (HP) in-office bleaching agent (Polaoffice+, SDI Limited, Australia). The whitening treatments of enamel samples were performed at room temperature and according to the manufacturers’ instructions as outlined below.

Briefly, for the CP group, 16% CP gel was applied on the tooth surface for 90 min. Samples were then rinsed thoroughly with water, dried and stored in HBSS. This process was repeated daily for 14 days. For the HP bleaching group, 35% HP was applied on the surface of specimen for 10 min, rinsed under running water and dried. The treatment was repeated 3–4 times in a single session. The mechanical tests were conducted within 24 h after application of bleaching agents.

2.2. Hardness and elastic modulus

The indentation experiment was conducted using an Ultra Micro Indentation System (UMIS-2000, CSIRO, Australia), equipped with a three-sided Berkovich indenter tip calibrated on a fused silica standard sample of known properties. Specimens (n = 12) were nanoindented using a maximum load of 10 mN. A total of 15 indentations separated from each other by 20 μm were positioned on the occlusal surface of each specimen along its buccal aspect for both the bleached and non-bleached halves of each tooth sample. The UMIS system software (Ibis, Fisher-Cripps laboratories, Australia) was used for the subsequent calculation of the elastic modulus and hardness of the samples tested. The nanoindentation hardness (H) is the contact pressure of the indenter divided by the projected contact area (A) of the sample at maximum load (Pmax) which can be estimated from [22]:

\[ H = \frac{P_{\text{max}}}{A} \] (1)

whereas elastic modulus of the specimen \( E \) can be calculated from [22]:

\[ \frac{1}{E_i} = \frac{(1 - \nu_i^2)}{E} + \frac{(1 - \nu_i^2)}{E_i} \] (2)

where \( E_i \) is the indentation elastic modulus (reduced modulus), \( E \) and \( n \) are the elastic modulus and Poisson’s ratio of the diamond indenter 1070 GPa and 0.07 respectively. The Poisson’s ratio, vs, for enamel is 0.3 [23]. The data were analyzed according to Oliver and Pharr [22] and the hardness and elastic modulus values were averaged from the results of 15 indents.

2.3. Load dependent mechanical properties of enamel

To investigate the effect of tooth bleaching agents on subsurface enamel and its depth dependent mechanical properties, a range of loads 5, 10, 20 and 50 mN were applied to control and bleached enamel samples. Hardness and elastic modulus were obtained following the methods described above.

2.4. Creep behavior of enamel

The approach for determination of creep behavior of control and bleached enamel was the same as that used by He and Swain [24], A 250 mN force was applied using the UMIS nanoindentation system with a 900 s holding time at maximum load. Another 900 s hold period was included during unloading at 2% of the peak load to analyze the creep recovery of enamel [24]. One-increment step during loading and unloading was used in order to reduce the viscoelastic effects that result from gradual loading and unloading [25]. The difference between the displacement during creep at maximum load and creep recovery at minimum load represents the amount of unrecovered deformation during a hold period at constant force. In addition, the relative recovery of the material in relation to the deformation was calculated as a percentage by the relationship:

\[ \% \text{Recovery} = \frac{h_2 - h_1}{h_2} \times 100 \] (3)
where $h_1$ is the indentation depth displacement during hold at maximum load and $h_2$ is the displacement recovery during the holding time at 2% of the maximum load upon unloading. This relative recovery indicates the amount of the deformation that occurred during loading that was recovered to its normal state once the loads were almost entirely released.

2.5. Fracture toughness and crack behavior

Fracture toughness ($K_{IC}$) of the material may be calculated from the size of radial cracks about a Vicker's hardness impression using the following expression [26]:

$$K_{IC} = \frac{x(E/H)^{1/2}}{P/c^{3/2}}$$

where $P$ is the applied load, $c$ is the crack length, and $x$ is a geometric constant of the indenter $x = 0.016$, $E$ and $H$ are Young’s modulus and hardness of the enamel.

Hardness was measured with a Vicker’s microhardness tester (Shimadzu, Japan) and a load of 9.8 N was applied. Five indentations were made on the polished occlusal surface of each specimen. Three specimens were used and indentations were performed both on the control and on the bleached surface. The indentations were viewed with a Leica microscope and the crack length was measured using Image J (version 1.44, National Institutes of Health, USA).

2.6. Scanning electron microscopy

On completion of the enamel stress tests, the fracture surface and crack patterns were examined by FEG-SEM. Tooth samples were dehydrated with graded ethanol concentrations, mounted on a sample holder with carbon tape and sputter coated with gold about 15 nm thick. Observation was performed under high resolution FEG-SEM (Zeiss Ultra plus, Germany). Images were obtained using a secondary electron detector at an accelerating voltage of 10.00 kV.

2.7. Extraction and purification of enamel proteins

After bleaching treatments, the enamel from each group was separated from dentin and pulverized. The protein extraction technique was similar to the protocol used previously [27]. The pulverized enamel was demineralized with 10% acetic acid with protease inhibitor (Protease Inhibitor Cocktail P8340, Sigma–Aldrich) for 24 h at 4 °C under constant agitation. The first supernatant was collected by centrifugation at 4000g for 5 min and stored at −20 °C. The precipitated demineralized enamel was suspended in 4 M guanidine-HCl in 0.05 M TRIS with protease inhibitor for 24 h at 4 °C under constant agitation. These extraction procedures were repeated three times. The collected supernatant was purified by running through OASIS-HLB reversed-phase liquid chromatography (OASIS®-HLB Plus, Waters; Ireland) and freeze-dried (Ilshin Lab co., Korea). The protein aggregates from each group were separated in 200 µL MQH2O and the total protein yielded from each group was quantified spectrophotometrically (Bradford protein assay, Thermo Scientific; USA).

The enamel protein extracts were separated by SDS–PAGE on a 16.5% Tricine gel with a broad range standard molecular weight marker (BIORAD; USA). The gel was stained with Coomassie brilliant blue G250 for 30–40 min. The stained gel was scanned and analyzed using Image J (version 1.44; National Institutes of Health).

2.8. Statistical analysis

All data are presented as mean ± SD. Statistical significance was determined by comparison between CP and HP bleached groups and a control group. The data were analyzed using ANOVA and a Tukey/Kramer test with the confidence level set at 5%. In the present study, a $P$-value $\leq 0.0001$ was considered as statistically significant.

3. Results

3.1. Hardness and elastic modulus

The nanoindentation elastic modulus and hardness were calculated as a function of penetration depth from the load–displacement curves. Typical behavior of the bleached versus control enamel is shown (Fig. 1). In the CP group, elastic modulus and hardness were significantly reduced by approximately 6.5% and 13.7% respectively (Table 1). Although the decrease in hardness and elastic modulus is small, the differences were statistically significant ($P \leq 0.0001$). At the same time, the HP group presented a significant ($P \leq 0.0001$) reduction of ~10% in hardness and 5.5% in elastic modulus in comparison with the control (Table 1). There was no significant difference between the two treatment groups.

3.2. Load dependent mechanical properties of enamel

The hardness and elastic modulus of control enamel decrease from about 7.5 GPa and 121 GPa at 5 mN load to 4 GPa and 96 GPa at 50 mN. There is more than 45% reduction in hardness and about 20% reduction in modulus of elasticity as the depth of penetration increases (Table 2). A similar reduction of the mechanical properties with increasing contact depth was also seen in the bleached enamel (Fig. 2). The difference in enamel mechanical properties between the control and the two bleached groups is greater under lower loads. Furthermore, as the load and contact depth increase the difference among the three groups decreases.

3.3. Creep deformation behavior

The nanoindentation creep data of triplicate samples from each group were averaged and plotted (Fig. 3a). The control and CP treated enamel showed a similar creep deformation of 80 nm and 83 nm respectively, while the HP bleached enamel had a significantly higher creep deformation under constant loading of about 96 nm displacement at maximum load.

Conversely, the creep recovery data (Fig. 3b) show the highest values for control enamel with 73% of the creep deformation...
Table 1
The average and standard deviations of the hardness and elastic modulus of enamel with the P-values for the control, CP and HP bleached.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>CP</th>
<th>HP</th>
<th>P-value</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>H</td>
<td>E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Depth</td>
<td>H</td>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elastic modulus</td>
<td>108 ± 2.8</td>
<td>101 ± 6.9</td>
<td>102 ± 4.7</td>
<td>&lt;0.0001</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Hardness</td>
<td>5.8 ± 0.3</td>
<td>5.0 ± 0.7</td>
<td>5.2 ± 1.1</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
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during loading recovered at unloading. On the other hand, HP bleached enamel had the least recovery with about 49 nm unrecovered deformation and only 48% relative recovery. The CP bleached enamel; on the other hand presented 66% relative recovery.

3.4. Fracture toughness and crack behavior

The indentations and the crack patterns of the control and bleached enamel were examined for measurement of crack length. The control enamel yielded an average crack length of 67.9 ± 14.8 μm and average fracture toughness 1.3 ± 0.5 MPa m^{1/2}. The fracture toughness of the CP enamel was significantly reduced (P < 0.0001) to 0.7 ± 0.2 MPa m^{1/2} with the average observed crack length of 100 ± 18 μm. The HP bleached enamel, fracture toughness was also significantly reduced to 0.8 ± 0.3 MPa m^{1/2} (P < 0.0001) with an average crack length of 86.2 ± 20 μm. On the bleached specimens, these cracks were observed to be longer with multiple secondary cracking and detaching of the adjacent enamel. Additionally, in the bleached groups the cracks tended to propagate between the enamel rods along the least resistant inter-rod sheath (Fig. 4).

3.5. Scanning electron microscopy results

The microstructures of fractured and indented samples were examined with FEG-SEM. Cracks arising from the Vicker's indentations of control enamel are shown in Fig. 4a. At higher magnification, crack deflection, crack tip splitting and crack bridges appear as indications of resistance of enamel structure to crack propagation. In the bleached enamel, secondary multiple cracks arise from the indenter impression which propagate through the interrod enamel as indicated by the semi-circular like crack path about each rod (Fig. 4b). This crack pattern suggests a weaker connection between the rods in the bleached enamel.

The fractured surface of control enamel in Fig. 5a illustrates the line of fracture propagating through the rods and inter-rod components, irrespective of their orientation. However, in bleached enamel, fracture propagation appeared in an arch-like pattern predominantly through the inter-rod enamel as shown in Fig. 5b. Cracking and dislodgement of the inter-rod and rod enamel were also seen. Under higher magnification, the SEM image of control enamel clearly shows the tightly packed nature of hydroxyapatite crystals found within both the rod structure and the inter-rod enamel (Fig. 6a). Comparatively in Fig. 6b, the bleached enamel appears more 'loosely' attached, with increased spacing and porosity between the crystals.

3.6. Protein analysis and gel electrophoresis

The protein component of enamel from bleached and control groups was extracted and quantitated by Bradford assay (Table 3) then examined on SDS–PAGE (Fig. 7a). SDS–PAGE gels show a reduction of high and low molecular weight proteins after the application of bleaching agents, with the most remarkable reduction occurring in the lower molecular weight proteins below 6 kDa (Fig. 7a). This is supported by the Image J plot analysis of SDS–PAGE gel staining intensity (Fig. 7b) and the Bradford protein assay results (Table 3).

4. Discussion

4.1. Hardness and elastic modulus

The average elastic modulus and hardness for the control enamel at 10 mN load are consistent with the values reported in previous studies [7,28,29] where the elastic modulus and hardness ranged from 120 GPa and 6.4 GPa at the cusp tip to 47 GPa and 2.7 GPa near the DEJ [7]. In the current study, the area of enamel investigated was just below the occlusal surface and parallel to the rod direction where the highest values of hardness and modulus of elasticity are reported [7].

Both HP and CP bleaching agents resulted in significant reduction of enamel hardness and modulus of elasticity. These changes in mechanical properties are presumed to be due to dissolution of the hydroxyapatite [16-30-32] and destruction of the protein matrix by the peroxide free radicals [16,18,19,21]. Zimmerman et al. claimed this reduction of the mechanical properties after whitening treatment is due to denaturing or loss of the protein structure as evidenced by loss of fluorescence particularly at the surface enamel [19]. In another study by Jiang et al. [18] using Raman/Fluorescence spectroscopy, the authors found significant reduction of fluorescence intensity in the HP treated group suggesting that the organic matrix of enamel may be greatly affected by tooth bleaching [18].

In the current study, there was no significant difference in the changes of the mechanical properties between the two peroxide concentrations used. This is in agreement with the results of De Abreu et al. [33] who found that the tooth bleaching agents produce significant enamel microhardness loss irrespective of the peroxide concentration, pH or time of exposure. Hence, the significant reduction of the hardness and elastic modulus of dental enamel after application of bleaching agents are suggested to be primarily due to loss or destruction of the protein layer and are not as dependent on the concentration or pH of the whitening products.

4.2. Load-dependent mechanical properties

For control and bleached enamel the values of hardness and modulus of elasticity decrease as the load and associated depth
of penetration increase. This load and depth dependence of enamel mechanical properties was attributed to its hierarchical structure. As the load increases, more rods are involved and the volume fraction of the sheath protein increases which leads to reduction of the mechanical properties under the indenter [34].

The load-dependent mechanical properties were also seen in the bleached enamel. This indicates that the reduction of hardness and modulus values is higher for the surface layer of enamel and returns to normal as the depth increases. Similar results were established in a previous study by Ushigome et al. [15] who indented a cross-section of a bleached enamel surface. They found that the reduction in hardness was greatest at the outer 2 µm and was less significant at deeper regions up to 20 µm below the surface. Consequently, we support the perspective that the most deleterious effect of tooth whitening products is limited to the surface layer of enamel.

4.3. Creep deformation and recovery

By considering its anisotropic structure dental enamel has considerable creep deformation under constant load and upon unloading. From the current results, control enamel exhibited steady creep deformation during time held at maximum load. In a recent study comparing the creep behavior of enamel with hydroxyapatite, the authors found that enamel has much greater creep deformation, which indicates that creep behavior of enamel principally originates from its protein components [24]. This deformation behavior can be explained by the Tension-Shear Chain (TSC) model of Ji and Gao [35]. Under constant loading the force of the indenter generates tension on the mineral crystallites that is transferred to the protein between the crystals and in the rod sheath as shear. The shearing of the protein is argued to be due to unfolding of its domain structure and enables limited sliding of the hydroxyapatite crystals [35]. Moreover, during unloading the stretched proteins tend to return to their normal form by refolding of the domain (sacrificial) bonds which results in relative recovery of the deformation upon unloading. Similarly, Schneider et al. [36] have proposed that the creep behavior of enamel is due to flow of the protein in the inter-rod sheath layer under stress application. It has been suggested that the thickness and shear properties of the protein layer are the significant parameters that endow enamel with its inelastic deformation behavior [37].

The higher creep deformation seen in bleached enamel; therefore, can be attributed to the conformational changes that are induced by the oxidation reaction of the bleaching agent. These structural changes of the protein may lead to denaturing or loss of the protein domain bonds which during hold at maximum load, results in increased creep deformation. In addition, loss of the protein can lead to movement of the mineral crystallites due to greater slippage at protein–mineral interface that manifests as higher creep deformation. This was observed in SEM images as increased spacing between the crystals (Fig. 7b).

For the unloading period, bleached enamel manifested limited recovery of the creep deformation in comparison with the control enamel. This may arise because refolding of the protein domain bond does not take place due to conformational changes or damage caused by peroxide free radicals.

![Graph showing hardness and elastic modulus](image)

**Fig. 2.** Plot of (a) hardness and (b) elastic modulus for control and bleached enamel at different indentation loads.
4.4. Fracture toughness and crack behavior

The average fracture toughness of control enamel was 1.3 ± 0.5 MPa m$^{1/2}$ which is within the range of the reported enamel toughness [2,38,39]. Although in this study fracture toughness was determined using Vicker’s microindentation approach based on direct measurement data from crack length, the values obtained were within the range of the results from the most recent energetic methodology [40]. Using continuous stiffness measurements of the depth sensing indentation approach, fracture toughness values reported were in agreement with previous literature [41]. The resistance of enamel to wear and cracking is crucial in order to survive its physiological functions. The toughness characteristics of enamel are attributed to its anisotropic structure, which is made of highly mineralized enamel rods embedded in a protein-rich organic matrix [39]. It has been shown that the fracture toughness of enamel is inhomogeneous and spatially anisotropic. Dental enamel has higher resistance to cracks that are perpendicular to the rod direction than cracks parallel to the rod direction [39]. Cracks initiating at the surface enamel toward DEJ are deflected by the decussated rods and continue growth about the tooth's periphery away from dentin and pulp [42]. By comparing the fracture toughness of enamel with its primary constituent hydroxyapatite (HAP), it has been found that enamel is much tougher than HAP. This was attributed to the structural organization of enamel rods and its organic matrix where the stress about the crack tip is transferred from the harder mineralized crystals to the softer organic layer around them [2,43]. The higher fracture resistance of enamel has been shown to be partly due to stretching of the organic matrix forming protein bridges to resist separation which exert crack closure stress [38]. This mechanism is argued to be formed by unfolding of the structural domains “sacrificial bonds” of these protein bridges between the mineral and protein or between different portions of the protein itself [38,44]. In addition, unfolding of this bond allows a limited slippage at the mineral-protein interface which in turn increases dissipation of fracture energy [35]. This crack resistance characteristic was reduced in bleached enamel which appeared as multiple longer cracking that primarily extended within the inter-rod region. These results were also seen in a previous study where 10% CP resulted in significant reduction of enamel fracture toughness due to alterations of the organic matrix [21]. Similar results have been found by Garrido et al. [20] after application of 38% HP bleaching agent. Their results have also shown increased wear rate and reduced fracture toughness after bleaching. However, a partial recovery of the reduced fracture toughness has been observed after one week storage in artificial saliva [20].

4.5. Scanning electron microscopy analysis

Cracks found in bleached enamel propagate a greater distance in an arch-like pattern as they grow through the inter-rod enamel demonstrating a weaker matrix surrounding the rods (Fig. 4b). In

![Figure 4](image-url)  
**Fig. 4.** Vicker’s indentations of control enamel (a), higher magnification of the cracks (inset) showing the crack bridging, and (b) bleached enamel.

![Figure 5](image-url)  
**Fig. 5.** Fracture surface of (a) control enamel shows the path of fracture (dotted line) including rods (R) and interrod (IR) enamel and (b) fractured enamel surface after bleaching showing the arched-like pattern of the interrod enamel (dotted line) with cracking of the inter-rod enamel (arrows) and dislodgement of the rods.
addition, the fractured surface of bleached enamel (Fig. 5b) clearly shows cracking and dislodgement of the rod and inter-rod enamel indicating changes in the properties of the protein glue that hold the crystals together. By comparing Fig. 6a and b, the bleached enamel shows increased spacing and porosity between the HAP crystals which may be a result of loss or destruction of the protein glue [45]. In contrast for control enamel, the highly tortuous path formed by rod/inter-rod enamel (Fig. 5a) contributes to crack deflection and reduction of stress at the crack tip, thereby acting as a toughening mechanism [38].

The extension of the cracks from the corners of indentations and in the fractured specimens of enamel at the inter-rod region is most pronounced following bleaching. This observation may possibly arise because the protein remnants of the inter-rod are primarily hydrophilic ameloblastin rich [46] and as such the peroxide bleaching could more readily penetrate, interact and fragment such proteins [47]. The protein remnants binding the crystallites within the rods together on the other hand are primarily hydrophobic enamelin [48] and as such the penetration of the peroxide is far more difficult resulting in less complete dissolution and break up of such proteins. This would explain why the extent of fracture toughness degradation (~50%) is far more significant than the reduction in elastic modulus and hardness.

4.6. Protein analysis and gel electrophoresis

Control enamel presents greater protein content than bleached enamel (Table 1). Analysis of the protein extracted from control enamel by SDS–PAGE shows low (~ 5 kDa) and high (~90 kDa) molecular weight proteins. The distinct reduction of protein intensity along with the lower shift of low molecular weight protein was observed in CP and HP bleaching groups compared to the control. This suggests the slightly greater ability of CP bleaching agent to degrade enamel protein (Fig. 7). The urea contained in CP bleaching gel is credibly responsible for this protein degradation by affecting hydrogen-bond of matrix proteins [49].

In mature enamel, the residual matrix proteins play a functional role in mechanical properties of enamel [5]. Dental enamel is much tougher than its major components HAP [2]. The increase in toughness is due to the residual protein components that have profound softening effect and provide a degree of ductility to an otherwise brittle mineralized structure [50]. Mechanically, the residual matrix proteins could prevent crack propagation by defining three dimensional cleavage planes that deflect cracks laterally and allow limited movement between adjacent rods. This could reduce stresses and increase enamel’s resistance to fracture [2]. Moreover, it

![Fig. 6. Higher magnification of enamel structure (a) control enamel showing the hydroxyapatite crystals tightly packed together forming the rod structure surrounded by the inter-rod enamel (b) bleached enamel shows increased porosities and spacing between the crystals (insets).](image)

![Table 3](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Enamel weight (g)</th>
<th>Protein recovered (µg/µl)</th>
<th>% of protein in enamel (wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.86</td>
<td>5.272</td>
<td>0.036</td>
</tr>
<tr>
<td>HP</td>
<td>1.89</td>
<td>1.527</td>
<td>0.016</td>
</tr>
<tr>
<td>CP</td>
<td>2.87</td>
<td>1.044</td>
<td>0.007</td>
</tr>
</tbody>
</table>

![Fig. 7. (a) SDS–PAGE of protein extracts from control enamel, HP and CP bleached enamel. (b) Intensity plot of the SDS–PAGE gel of extracted enamel proteins in (a) as analyzed by Image J (version 1.44; National Institutes of Health). Intensity of low molecular weight enamel proteins recovered from bleached groups is lower compared to control.](image)
has been shown that enamel matrix proteins have the ability to regulate the electrostatic properties of enamel surface and thus can buffer the HAP against acid attack [51]. Therefore, even a slight effect on organic composition of enamel could lead to significant changes in its mechanical properties.

The results from this study clearly show the deleterious effect of tooth bleaching products on the mechanical properties and matrix proteins of dental enamel. In particular, the organic content of enamel has been shown to be the responsible structure in regulating enamel mechanical properties and viscoelasticity [37]. Removal or degradation of enamel organic matrix has demonstrated significant reduction of its mechanical properties [5]. The current results support the notion that H$_2$O$_2$ in tooth bleaching products gives rise to unstable oxygen free radicals that are able to induce structural changes in the matrix proteins located between the crystallites and in the rod sheath area. Tooth bleaching agents have induced significant alteration of the interrod proteins which was seen as increased cracking and reduced fracture toughness. The semicircular crack paths seen under FEG-SEM demonstrate the reduced presence of the hydrophilic sheath proteins to resist fracture.

Accordingly, the ability of enamel matrix proteins to enable shear and stress dissipation as well as subsequent recovery properties is compromised. Therefore, destruction or denaturation of these organic components leads to a decrease in the ability of enamel for stress-redistribution and fracture resistance.

One of the limitations of this study is that the enamel surface of the specimens was ground and polished before bleaching. This procedure was done to provide a more even surface for the accuracy of the indentations. However, sample preparation can induce residual stresses in the surface enamel which consequently alter crack length [52].

Another limitation is the use of HBSS solution for storing teeth after bleaching instead of artificial or human saliva which may induce enamel remineralization and recovery. It has been found that bleaching agents could breakdown or remove salivary protein that tightly bound to enamel lesions which consecutively enhance the remineralization of the early carious lesions [53]. Various salivary proteins have been found to maintain oral health by protecting against tooth wear, controlling microbial adherence, inducing ion transport, and enhance remineralization [54]. In addition, saliva plays an important role in re-establishing the characteristics of the enamel and reduction of its roughness after microabrasion [55]. However, many previous studies have used artificial saliva or remineralizing solution after bleaching to simulate clinical situation and have also found significant reduction of the mechanical properties [56–59]. From this study further research exploring the roles that salivary proteins may potentially play in repair of bleaching damaged enamel would be recommended.

In summary, tooth bleaching products, which have been shown to induce strong oxidation reaction of the protein/peptide components of enamel, compromise its mechanical properties and as a consequence, reduce its ability to resist deformation and withstand contact induced damage during function.

5. Conclusions

Significant reduction of enamel hardness, modulus of elasticity and fracture toughness occur after whitening treatment regardless of the type of tooth whitening gels. These effects, which are consistent with the degradation of the matrix protein by the bleaching agents, result in a significant decrease in the mechanical properties of enamel. In addition, the bleaching agents increased the creep deformation and reduced the creep recovery of enamel under constant loading which is also consistent with damage to the deformable matrix protein by the peroxide free radicals. Changes in protein structures caused by the H$_2$O$_2$ appear to be responsible for modifications in the structure and the altered pattern and characteristics of the fracture surface of bleached enamel. Therefore, tooth whitening products potentially produce detrimental effects on the mechanical properties of dental enamel as a consequence of destruction or denaturing of the matrix proteins. Accordingly, the treatment protocol for tooth discoloration, particularly tooth bleaching should be re-evaluated to prevent the undesirable altered enamel properties.

Disclosures

This study has no conflicts of interest.

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–3, and 7, are difficult to interpret in black and white. The full colour images can be found in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.03.035.

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