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Research Article

Release of Collagen and Protein from Teeth Treated with Over-the-Counter Whitening Strips

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Abstract

The aim was to characterize the effect of over-the-counter whitening strips on proteins in teeth. The active ingredient is Hydrogen Peroxide (H_2O_2). Teeth, either treated or untreated, were dialyzed against 10% EDTA pH 9.0; the dialysis fluid contained all proteins other than collagen and was measured with Lowry assay. Collagen was extracted from the teeth and a modified version of the Lowry assay and hydroxyproline assays were used to measure it. A novel method was developed to measure the protein and collagen released from teeth into the surrounding fluid. The Molecular Weights (MW) of proteins were measured using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE). There was a 15% and 37% loss of non-collagen proteins after one and three rounds of treatments. These values were 32% and 55% for collagen and 49% and 80% for hydroxyproline. The MW (70,11 and 130 kDa) of the proteins in these fractions matched those of proteins in teeth. No protein was released from teeth into the surrounding fluid without application of whitening strips but with strips, there was protein. Non-collagen protein averaged 0.2867mg/ml for one round of treatments and 1.240mg/ml for collagen. The MW of proteins released are slightly smaller than those extracted from teeth. Over-the-counter whitening strips resulted in a decrease of non-collagen and collagen protein in teeth. In addition, both types of protein were released from treated teeth; none was observed in untreated teeth.

Keywords: Whitening Strips; Collagen; Hydrogen Peroxide; Protein

Introduction

Foods and beverages can stain teeth, and the demand for tooth whitening is high. Stains contain organic material with conjugated alkenes. Oxidation and conversion to smaller molecules is required for removal. There are several possible oxidizing agents. Hydrogen peroxide or carbamide peroxide, which degrades to hydrogen peroxide and urea, are two common active ingredients [1]. Hydrogen peroxide penetrates the tooth [1]. The amount used in different treatments varies in the United States, over-the-counter whitening strips typically contain 6.5% while this amount can be as high as 30% for professional treatments done in dentists offices [2]. Hydrogen peroxide can damage proteins. There are proteins found in all layers of the tooth. The outermost layer is enamel; it contains 97% inorganic material and only 3% protein. The enamel is secreted by cells called ameloblasts followed by extensive proteolysis to produce the hard surface [3]. The ameloblasts are only present during the formation of teeth. Underneath the enamel is the dentin, which is made by the odontoblasts. These cells produce dentin throughout the lifetime [4]. The odontoblasts originate in the pulp and the ability to repair dentin thus depends on having healthy pulp tissue. Based on weight, dentin is 70% inorganic material, 20% organic including proteins and the remaining 10% is water [3]. For the organic portion of dentin, collagen comprises up to 90%. There are several types of collagen, but all consist of three intertwined and chemically cross- linked protein subunits. Collagens also contain a distinct amino acid composition including hydroxyproline. It is formed by post-translational modification of proline and is unique to collagen. For this reason, hydroxyproline can serve as a marker of collagen. Dentin contains greater than 90% Type I collagen, which is made of two copies of α-1 subunit and one copy of the α-2 subunit as well [5]. While hydrogen peroxide has been shown effective at whitening teeth, it is capable of damage. High concentrations applied to teeth can cause damage to the pulp [6]. In one study, teeth treated excessively with whitening strips had no decrease in microhardness nor change in structure [2]. In another study, enamel treated with 30% carbamide peroxide showed a decrease in hardness and fracture toughness. There

was an increase in crack length as well as a change in microscopic appearance [7]. When teeth were treated with hydrogen peroxide, there was a decrease in hardness and changes in tooth morphology [8]. The goal of this research was to examine the effect of over the counter whitening strips on proteins in teeth. Teeth were either untreated or treated with one or three rounds of whitening strips while being suspended in artificial saliva. The teeth were placed in dialysis tubing with 10% EDTA pH 9.0 to remove the inorganic components of teeth. All proteins except collagen were released from teeth and found in the dialysis membrane. These proteins, called the non-collagen fraction, were measured using the Lowry assay. The MW of extracted proteins was determined using SDS PAGE. Based on its unique properties, collagen was not extracted with the other proteins but found in the tooth remnants. It was measured using a modified version of the Lowry assay. The cross-linked and tight packing of the subunits of collagen prevents accurate measurements by the Lowry assay. The modified version uses higher temperatures to dissociate the three helices and allow the reagents to react. In addition, amount of hydroxyproline was measured to determine the amount of collagen. The amount of protein released from teeth treated with whitening strips was also measured. Since this contains both collagen and non-collagen proteins, it was necessary to separate the two types of proteins prior to analysis. To characterize the size and amount of protein fragments produced from whitening strips, various types of proteins were treated with hydrogen peroxide and dialyzed to separate by size.

Materials and Methods

Extraction of Proteins from Teeth: For each trial, a total of four intact and caries-free human molar teeth were extracted for this study from local dentists. Patients had previously signed informed consent document. Previously used methods were modified to extract protein from teeth [5,9]. Teeth were placed into pieces of Styrofoam and either untreated or treated with one or three rounds of Crest 3D White © over-the-counter whitening strips following manufacturer's instructions at 37°C (one hour/strip) while suspended in Artificial Saliva (AS). These strips contained 6.5% hydrogen peroxide and each round consists of twenty strips. Teeth were suspended in Artificial Saliva (AS) during treatment at 37°C and artificial saliva was changed daily; unless otherwise indicated, one strip was used per day. Untreated teeth were treated in the same way, with the exception of adding the whitening strips. Artificial saliva was a modified version of previous formulations and it contained: 8.38mM KCl, 0.29mM $MgCl_2$ $6H_2O$, 1.13mM $CaCl_2$ $2H_2O$, 4.62mM H_2HPO_4, 2.40mM KH_2PO_4 and 13.1mM methyl-p-hydroxybenzoate [10]. After treatment, the teeth were removed, weighed and placed in dialysis tubing with molecular weight cutoff (MWCO) of 3.5kDa and dialyzed against 10% Ethylenediaminetetraacetic Acid (EDTA) pH 9.0 for two weeks at 4°C with changes of fluid every 3 days. After this treatment, the fluid within the dialysis tubing was collected; it contains the non-collagen proteins. The tooth remnants were placed in new dialysis tubing and dialyzed against water for two weeks with changes every three days. After this, teeth remnants were removed, suspended in liquid nitrogen, hammered into shards and then placed in a blender for 1 minute. The resulting powder was suspended in 0.5M acetic acid, 0.2M sodium chloride solution and contains the collagen fraction. Collagen and non-collagen fractions were measured in the modified Lowry and hydroxyproline assays for the former and the Lowry assays for the latter. In addition, samples were immediately prepared for SDS PAGE. Fractions were stored at –70°C.

Hydroxyproline Assay: Collagen fraction was mixed with 6M HCl and heated at 95°C for 24 hours in order to hydrolyze all peptide bonds. The remaining solution was neutralized with 6M NaOH and tested for hydroxyproline. This colorimetric assay [3] was modified with the substitution of concentrated hydrochloric acid instead of perchloric acid [11]. Briefly, the sample is mixed with chloramine T reagent at room temperature for 20 minutes. A solution of dimethylaminobenzaldehyde or Erhlich's solution is added, mixed and incubated at 65°C for 20 min. After removal, absorbance is measured at 550nm and a standard curve is constructed from 1mg/ml 4-hydroxyproline in 50% isopropanol. Total mg of hydroxyproline/g of teeth values are calculated. In order to validate the method, known amounts of hydroxyproline were hydrolyzed as described and % recovery was calculated. In addition, samples were spiked with known amounts of hydroxyproline and % recovery was calculated as well.

Lowry and Modified Lowry: The total amount of non-collagen protein was measured using the Lowry assay. Since EDTA interferes with the Lowry assay, it was necessary to remove it by dialysis. Portions of dialysis fluid were placed in Pur-A-Lyzer dialysis tubes (Sigma) and dialyzed against distilled water (volume of distilled water was 200 times that in the tubes) for 4 hours at 4°C. The resulting fluid from the tube was collected and tested in the Lowry assay for protein. 1mg/ml bovine serum albumin was used as a standard. Portions of a 1:1:100 solution of 1% copper (II) sulfate: 2.68% potassium sodium tartrate: 2% sodium carbonate in 0.1M NaOH mixture was added to samples and standards, mixed immediately and incubated at room temperature for 10 minutes. An aliquot of 1N Folin reagent was added, mixed and incubated at room temperature for 30 min before absorbance was measured at 720nm. The total mg of protein/g tooth was calculated [12]. In addition, all samples were spiked with known amounts of chicken egg white albumin and the % recovery was calculated as well. The amount of collagen protein was measured using a modification of the Lowry assay [13,14]. The sample was mixed with reagent A, which contains Na_2CO_3, NaOH and potassium sodium tartrate at 50°C for 20 minutes. Reagent B containing potassium sodium tartrate, $CuSO_4$ $5H_2O$ and NaOH was added and allowed to incubate at room temperature for 10 minutes before adding 1:5 Folin, shaking

and incubating at 50°C for 10 min. The absorbance at 600nm was measured and used to calculate total mg of collagen protein/g tooth. The standard was 2mg/ml gelatin in 0.05M acetic acid or 1mg/ml Type I collagen. All samples were spiked with known amounts of a 2mg/ml gelatin in 0.5M acetic acid and % recovery was calculated.

Hydrogen Peroxide Colorimetric Assay: Standards are made using 0.3% hydrogen peroxide and range from 0 to 3 microgram. Aliquots of samples are removed and mixed with 1mM sodium molybdate in 0.5M sulfuric acid, 50mM hydrochloric acid, freshly made 1M potassium iodide and a 5% solution of starch. After 20 min, the absorbance is measured at 570nm and the standard curve is used to measure the % hydrogen peroxide in various samples [3].

Release of Protein from Teeth: Teeth contain both collagen and non-collagen protein; there is no method to accurately measure the total amount of protein in mixtures of these two. For this reason, known amounts of non-collagen protein (human albumin chicken egg white albumin, porcine hemoglobin or bovine hemoglobin) were mixed with known amounts of Type I collagen. An aliquot was removed, four volumes were mixed with 1 volume of 100% trichloroacetic acid (TCA) and stored at 4°C for 10 min. After, it was centrifuged at 14,000 x g for 5 min at 4°C; the supernatant contains the collagen fraction while the pellet contains the non-collagen protein. The pellet was washed twice with -20°C acetone, centrifuged at 14, 000 x g at 4°C; the final pellet was dried at 95°C for 5 min. The mass of the non-collagen protein was determined by the change in mass. The supernatant was adjusted to alkaline pH with 10M NaOH and tested in the modified Lowry assay. It has been previously noted that alkaline conditions are required for the Lowry assay [15] and the modified Lowry also requires these conditions. The same procedure was applied to measure collagen and non-collagen protein in the fluid surrounding teeth either treated or not treated with over-the-counter whitening strips. Four molars were placed into pieces of Styrofoam as previously described and suspended with 50ml of artificial saliva at 37°C. One set of teeth was treated with Crest Professional© Whitening Strips (active ingredient is 6.5% hydrogen peroxide) following manufacturer's instructions (1 hour/strip and one round contains 20 strips). The artificial saliva was changed daily. This sample was collected and tested for both the level of both non-collagen and collagen proteins as described. In these assays, samples were spiked respectively with known amounts of bovine serum albumin and gelatin for non-collagen and collagen respectively and the percent recovery was calculated.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) of Teeth Proteins: The proteins in both non-collagen and collagen fractions were separated and characterized using SDS PAGE. Prior to use, equal volumes of samples and 2 X sample buffer were heated at 95°C for 5 minutes. Bio Rad mini gels were used and prepared according to the instructions. For the collagen containing fractions, a 6% resolving and 4% stacking gel was prepared and run at constant 150V for 45 minutes while for non-collagen fraction, a 12% resolving and 4% stacking gel was used at 200V for 45 minutes. Bio Rad high molecular weight markers were used for the 6% gels and low MW markers for the 12% gel. The gels were stained using Bio Rad Silver Stain plus kit and imaged using Kodak Gel Imager. Proteins released from teeth were also characterized using SDS PAGE on a 12%/4% gel as described. The samples were concentrated using Nano sep protein concentrations followed by centrifugation at 14 x g for 5 min at 4°C.

Protein Fragments Released by Hydrogen Peroxide Treatment: This experiment was done on the non-collagen and collagen fractions as well as type I collagen and chick egg white albumin. Protein was mixed with varying concentrations of hydrogen peroxide or 1mg/ml trypsin for the non-collagen fraction and 1mg/ml collagenase for collagen fraction and type I collagen at 37°C in pre-soaked snakeskin dialysis tubing with MWCO of 3.5kDa and dialyzed against ultrapure water. After 30 minutes, the Lowry assay was done for non-collagen samples and modified Lowry for collagen samples on the fluid both inside and outside the dialysis tubing. The percent of protein in each fraction was calculated. In another method, a Sephadex column with an exclusion limit of 10kDa was prepared by dissolving 9g of Sephadex G 10 in 0.1 M Tris pH 7.2 and after storing it overnight at 4°C. Then a column was poured. In order to determine the void volume, a solution containing 1% blue dextran was passed though the column. Aliquots of collagen or non-collagen fractions were passed through the column and 5x2ml fraction were collected after the void volume and tested for protein using the modified Lowry or Lowry assay respectively. This was repeated with dialysis fluid that was pretreated at 37°C for 30 min with hydrogen peroxide alone or with trypsin before passing over the column. Finally, 1mg/ml solution of bovine, human or chicken egg white albumin was incubated with hydrogen peroxide at 37°C for 30 min in a microfuge tube, one volume of 100% trichloroacetic acid was added to four volumes of sample, and incubated at 4°C for 10 min. The microfuge tube was centrifuged at 14,000 x g for 5 min and the supernatant, which contains small, released protein fragments, was tested using the Lowry assay and mg protein fragments released was calculated.

Results

Effect of Whitening Strips on Amounts of Proteins Extracted from Teeth: Collagen and non-collagen proteins were extracted from teeth using EDTA which is a metal chelator. Teeth typically lost an average of 3.5% of the original mass. The amount of non-collagen protein was measured using the Lowry assay. It relies of reduction of Cu (II) to Cu(I) by the peptide bond in alkaline conditions and the Folin reagent produces a chromophore based on interaction with specific amino acid residues. The non-collagen fraction contains EDTA and potentially, hydrogen peroxide. While previous reports have demonstrated hydrogen peroxide does not

significantly interfere with the Lowry assay [12], other results suggest otherwise [16]. Various concentrations of hydrogen peroxide were tested in the Lowry assay: high concentrations positively interfere with the assay (concentrations of 2.5 to 12% hydrogen peroxide averaged 1.45mg protein/ml). The amount of hydrogen peroxide tested in the non-collagen fraction, however, was 0mg/ml in untreated teeth and an average 0.0055% in teeth treated with three rounds of whitening strips. This is well below the amount required to positively interfere with the assay (data not shown). Artificial saliva did not positively interfere with the assay but EDTA did. For this reason, samples were dialyzed against water to remove the EDTA. After 4 hours, a dialyzed EDTA solution showed no interference. This shows that 4 hours is enough time to remove EDTA from the protein solution. In addition, samples were spiked with known amounts of a chicken egg albumin solution and there was an average percent recovery of 97.5±3.2. As shown in Table 1, teeth treated with one round of treatment showed nearly a 10% loss of non-collagen protein while three rounds produced 52% loss of protein.

Table 1: Effect of Whitening Strips on Protein and Hydroxyproline in Teeth Proteins.

Teeth treatment	Amount of protein		Amount of collagen		Amount of hydroxyproline	
	mg/g teeth	% loss	Mg/g teeth	% loss	Mg/g teeth	% loss
None	0.1980±0.0372	0%	0.2223±0.0202	0%	1.308±0.240	0%
one round	0.1788±0.0684	9.70%	0.1497±0.0579	32.60%	0.884±0.091	32.60%
Three rounds	0.0949±0.0068	52.10%	0.1075±0.0115	51.60%	0.537±0.100	72.60%

The collagen fraction was extracted from teeth. With its triple helical structure, reagents used in Lowry assay are not capable reacting with the peptide bond; thus, the Lowry assay cannot accurately measure collagen. A modified version includes higher temperatures to dissociate the triple helix. Gelatin was used as a standard and it produced values that were identical to that of Type I collagen (data not shown). Hydrogen peroxide was shown to positively interfere with the assay while artificial saliva did not. A solution of 1% hydrogen peroxide produced a value of 1mg protein/ml in the modified Lowry assay and the value increased to 4mg/ml at 12% hydrogen peroxide. The level of hydrogen peroxide in the collagen fraction was measured and the values were 0% for untreated samples, 0.025% for teeth treated with one round and 0.045% for teeth treated with three rounds. These hydrogen peroxide concentrations are too small to show any positive interference in the modified Lowry assay (data not shown). All collagen samples were spiked with known amounts of type I collagen solution. The percent recovery averaged 98.5±2.7%, which shows the samples do not interfere with the assay. Similar to the amounts of non-collagen proteins from teeth, the amount of collagen decreased in teeth treated with whitening strips. As shown in Table 1, one round of treatment produced a 33% loss of collagen and three rounds produced a 52% loss of collagen. These amounts show a similar loss of collagen compared to the non-collagen protein. Another way to measure the amount of collagen is by hydroxyproline; this modified amino acid is unique to collagen. The colorimetric test is based on oxidizing the hydroxyproline using chloramine T, which converts the alcohol functional group to an alkene. The alkene reacts with Erlich's reagent to produce a chromophore that can be detected [11]. The original method called for the use of perchloric acid, but it has since been shown that concentrated hydrochloric acid can be successfully used in its place [11,17]. Collagen fractions were hydrolyzed to release amino acids from the protein. Known amounts of hydroxyproline were subject to hydrolysis, and the average percent recovery for a range of concentrations was 98.0±2.7. This result demonstrates that hydroxyproline is not lost in the hydrolysis process. When hydrolyzed collagen fractions were spiked with known amounts of hydroxyproline, the average percent recovery was 97%, which demonstrates there is no negative interference. As shown in Table 1, there is a 33% loss of hydroxyproline following one round of treatment and an 73% loss with three rounds.

Measurement of Protein Released from Teeth: Whitening strips cause a decrease in the amount of both collagen and non-collagen protein from teeth. It is not known if the observed decreases are due to release of proteins from teeth or degradation. The amount of protein released in the fluid surrounding teeth was measured from teeth untreated or treated with one round. Any protein released from teeth can be either collagen or non-collagen. It is necessary to accurately measure both types. Collagen and non-collagen interfere in their respective colorimetric tests. Collagen alone yielded an average of 51.0%±5.6% of its true value in the Lowry assay while non-collagen alone was 40.0%±8.2% of its true value in the modified Lowry assay. When mixtures of collagen and non-collagen protein (albumin or hemoglobin) were measured in the Lowry and modified Lowry assay, there was positive interference. In mixtures of collagen and non-collagen, there was an average of 144±16 % recovery in the modified Lowry assay and 152%±28% in the Lowry assay. These results demonstrate the positive interference in both assays. To accurately measure the amounts of collagen and non-collagen protein released from teeth into the artificial saliva, it is necessary to separate them. Trichloroacetic acid (TCA) precipitation is a method to separate collagen from non-collagen. When the TCA precipitation method was performed on chicken egg white albumin and collagen individually, there were recoveries of 102±3 % and 15±1% respectively. These results demonstrate that

TCA is effective in precipitation of the non-collagen proteins while only a small amount of collagen is precipitated. When mixtures of non-collagen and collagen proteins were treated with TCA, there was an average recovery of 120%±14%, which is due to the non-collagen protein with a small contribution from the collagen.

The supernatant from the TCA precipitation contains the collagen. If a modified Lowry assay is performed on this fraction, the average percent recovery is 36%±3%, which is lower than expected. Collagen solutions are in acidic solutions and the modified Lowry requires alkaline conditions. If the pH of the collagen-containing supernatant is adjusted to alkaline conditions, the percent recovery is 74.5%±3.2%. This value is less than 100% but is consistent with the small amount of collagen precipitated by TCA. These results suggest that TCA precipitation is an effective way to separate collagen and non-collagen. Since a small amount of collagen is precipitated by TCA, the non-collagen value is likely a small overestimate of the true amount of non-collagen protein while the collagen value is likely a slight underestimate of the true value. Samples of Artificial Saliva (AS) were tested in both the TCA precipitation and modified Lowry assay: there were 0mg/ml values for both. The amount of protein in AS solutions surrounding the teeth was measured that received one whitening strip per day or none. The results are shown in Table 2. There was no protein released from untreated teeth: both the collagen and non-collagen results were 0 over the course of 20 days. When untreated samples were spiked with known amounts of gelatin in the modified Lowry assay, there was an average of 102.0±3.5% recovery, which suggests the samples themselves are not interfering in the assay. These results are in stark contrast to teeth treated with whitening strips. There was an average of 0.659mg collagen/g tooth released per day with a high of 1.11. When these treated samples were spiked with gelatin, the average percent recovery was 97.5±3.8%, which demonstrates the samples do not interfere with the assay. For non-collagen proteins, an average of 0.626mg protein/g tooth was released with a high of 0.896. It is likely to underestimate the true amount of collagen and overestimate the amount of non-collagen. This experiment was repeated with two whitening strips per day. The amount of non-collagen averaged 1.19±0.18mg/ml or 190% of the value for the once a day treatment. The value for collagen averaged 0.841 mg/ml±0.088 or 128% of the value for the once a day treatment. Hydroxyproline was detected in those treated with whitening strips but not for the untreated teeth (data not shown).

Table 2: Release of Non-Collagen and Collagen Proteins from Teeth with Whitening Strips.

Number of days in artificial saliva	Collagen protein released for untreated teeth(mg/ml)	Collagen protein released for treated teeth (mg/ml)	Non-collagen released from untreated teeth (mg/ml)	Non-collagen released from treated teeth (mg/ml)
1	0	0.912±0.167	0	0.604±0.151
2	0	0.741±0.143	0	0.635±0.184
3	0	0.708±0.152	0	0.728±0.080
4	0	0.398±0.092	0	0.521±0.079
5	0	0.452±0.120	0	0.667±0.083
6	0	0.887±0.258	0	0.896±0.235
7	0	0.606±0.189	0	0.792±0.216
8	0	0.540±0.091	0	0.562±0.125
9	0	0.563±0.096	0	0.500±0.068
10	0	0.516±0.102	0	0.563±0.104
11	0	0.628±0.180	0	0.375±0.120
12	0	0.762±0.151	0	0.650±0.091
13	0	1.108±0.256	0	0.444±0.107
14	0	0.586±0.107	0	0.722±0.126
15	0	0.797±0.244	0	0.722±0.171
16	0	0.842±0.211	0	0.729±0.079
17	0	0.551±0.060	0	0.583±0.108
18	0	0.478±0.102	0	0.542±0.089
19	0	0.363±0.090	0	0.542±0.110
20	0	0.746±0.211	0	0.750±0.136

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) of proteins from teeth: The non-collagen proteins extracted from teeth were separated using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE), which separates proteins by size or Molecular Weight (MW). As seen in this Figure 1A, there are prominent bands at MW of 70kDa and 11kDa. The MW of isolated collagen fraction is 130kDa as Figure 1B shows, which is the very similar to that observed for pure Type I human collagen

(data not shown). The MW of proteins released from teeth was also measured. There were no proteins visible for the untreated samples. This is consistent with the measurements of 0mg/ml non-collagen and collagen. For the samples treated with one round, there was a prominent band at 66kDa (data not shown). This value is similar but slightly less than the protein seen in the non-collagen fraction.

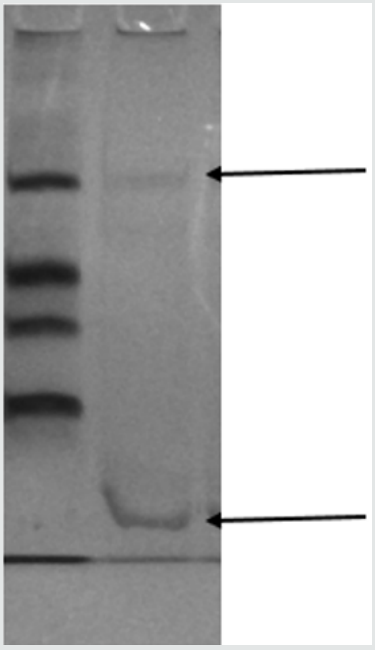

Figure 1: Effect of Hydrogen Peroxide and Proteases on Non-Collagen and Collagen Fractions Isolated from Teeth with SDS PAGE

Figure 1A: Markers dialysis fluid

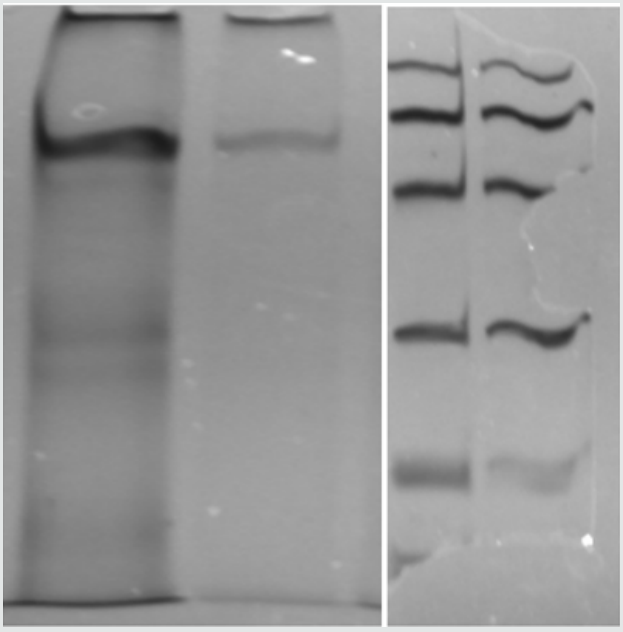

Figure 1B: Collagen markers untreated 1X treated.

SDS PAGE of Proteins Treated with Hydrogen Peroxide Or Proteases: SDS PAGE was used to characterize the effect of hydrogen peroxide on these teeth proteins. Non-collagen fraction was treated with either hydrogen peroxide or trypsin or both for 60 min and the protein(s) were separated using SDS PAGE. The untreated non-collagen fraction had a band at MW of 70kDa. As shown in Figure 1C, when the non-collagen fraction was treated with hydrogen peroxide, there was a decrease in the size of the higher MW protein. This was observed with both 6.7% as well as 3.3% hydrogen peroxide. The original size of 70kDa decreased to 64kDa. The 11kDa protein is not visible on this gel and this suggests the hydrogen peroxide produced protein fragments too small to be detected. This decrease in MW was not observed when trypsin alone was added. Trypsin is a protease that hydrolyzes at specific amino acids. It produces protein fragments that are smaller than the original MW. When the non-collagen protein fraction was treated with trypsin alone, a band appeared at 72kDa and at 28kDa which suggests the trypsin hydrolyzed some of the 72kDa protein. When both trypsin and hydrogen peroxide were mixed, there was a decrease in the size of the original protein (64kDa) as well as the fragment made by trypsin (24kDa). Both the size of the original protein and the trypsin-produced fragment are smaller when hydrogen peroxide is present. These results suggest that trypsin and hydrogen peroxide have a different effect on non-collagen fractions. Pure type I collagen showed a prominent band at the expected MW for its subunits (129kDa). As shown in Figure 1D, when type I collagen was treated with hydrogen peroxide, the original protein seen at 129kDa was not visible. There are no bands at the bottom, which suggests the size of fragments is too small to be detected in this method. When treated with collagenase, smaller proteins with a MW of 80, 66 and 47kDa were observed. When treated with both collagenase and hydrogen peroxide, the original protein band disappeared.

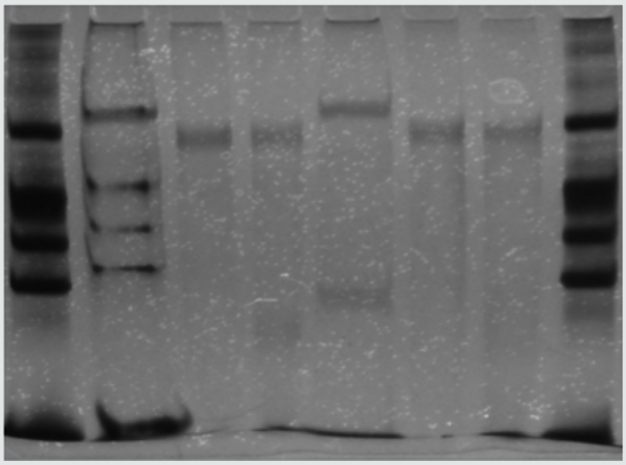

Figure 1C: Markers H_2O_2(%) 6.7 6.7 0% 3.3 3.3
Trypsin - + + - +

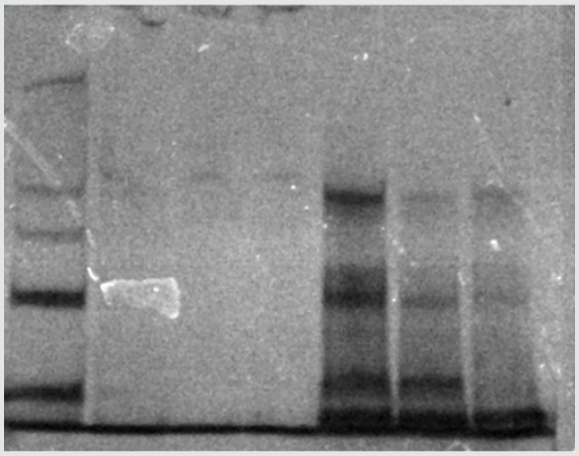

Figure 1D: Markers time(min) 10 20 30 10 20 30
H_2O_2(%) 6.7 6.7 6.7 0 0 0
Collagenase - - - + + +

Characterization of Size of Protein Fragments Released By Hydrogen Peroxide Treatment: In order to characterize the size of the fragments released from hydrogen peroxide treatment, proteins were mixed with varying concentrations of hydrogen peroxide, a protease, or both and placed in dialysis tubing with a molecular weight cut off (MWCO) of 3.5kDa. Only fragments smaller than this can leave the dialysis tubing. The amount of protein fragments released was measured using the Lowry or modified Lowry for non-collagen and collagen fractions respectively. The concentrations of hydrogen peroxide used are high enough to interfere in the modified Lowry assay. The amounts were shown to be the same inside and outside the dialysis tubing (data not shown). All released protein has a maximum size of 3.5kDa. The results are shown in Table 3. When either Type I collagen or collagen isolated from teeth treated with one or three rounds of whitening strips are placed in dialysis tubing, it is too large to leave the dialysis tubing and the amount retained is 100%. The same is observed if collagenase is added; this protease hydrolyzes the protein, but the resulting fragments are too large to leave the dialysis tubing. Similar results were obtained if the collagen was treated with pepsin (data not shown). Treatment of any type of collagen, either type I or that extracted from teeth with hydrogen peroxide, results in small protein fragments. After 30 minutes with 7.5% hydrogen peroxide, 65% of the total collagen had been converted to fragments 3.5kDa or smaller. The amounts were higher for the collagen from teeth treated with three rounds of whitening strips. The extra exposure to hydrogen peroxide with more whitening strips could have resulted in collagen that was already partially damaged by peroxide and produced the larger values. This amount of hydrogen peroxide is 1% higher than what is commonly used in over-the-counter whitening strips. When the amount of hydrogen peroxide was decreased, the amount of collagen fragments released also decreased but even 1.88% still produced greater than 50% collagen fragments.

Table 3: Effect of Hydrogen Peroxide on Formation of Peptides (MW<3.5 kDa) from Protein.

Protein	Treatment	Protein retained in dialysis tubing (% of total protein)	Protein released from dialysis tubing (% of total protein)
Type I collagen	None	100	0
	Pepsin	100	0
	7.5% H_2O_2	28.86±8.20	71.15±10.26
	3.75% H_2O_2	34.07±4.20	65.93±9.62
	1.88 % H_2O_2	48.52±10.28	51.48±9.02
Collagen from teeth treated with one round	None	100±0	0±0
	pepsin	100%	0±00
	7.5% H_2O_2	29.04±5.17	70.96±5.28
Collagen from teeth treated with three rounds	None	100±0	0±0
	pepsin	100±0	0±0
	7.5% H_2O_2	11.77±1.02	88.23±10.55
Non-collagen from untreated teeth	None	100±0	0±0
	Trypsin	100±0	0±0
	7.5% H_2O_2	13.51±4.78	86.43±11.54
Non-collagen from teeth treated with three rounds	none	100±0	0±0
	trypsin	100±0100	0±0
	7.5% H_2O_2	14.43±0.91	85.57±11.21
Chick egg white albumin	None	100	0±0
	Trypsin	100	0±0
	7.5 H_2O_2	39.03±8.25	60.96±8.26

The experiment was done on non-collagen fractions as well as chick egg white albumin treated with trypsin as the protease. Similar to collagen, no protein is released when trypsin is added. Hydrogen peroxide at 7.5% resulted in about 85% of the protein being released. These values were similar in non-collagen proteins from teeth either untreated with whitening strips or with three rounds. It has previously been observed that hydrogen peroxide enhances the action of various proteases on hemoglobin [18] but that was not true for these samples (data not shown). Two more experiments were performed to confirm these results. Various types of albumin solutions were mixed with hydrogen peroxide and precipitated with TCA. The Lowry assay was done on the supernatant which contains peptides too small to precipitate. The results, shown in Table 4, indicate that there were protein fragments released when albumin was treated with hydrogen peroxide but there were none released when the protein was treated with trypsin. This is consistent with previous results. The results in Table 4 also show that 71% of the protein is a size too small to be precipitated when the protein is treated with hydrogen peroxide. These values are consistent with the results of the dialysis experiment and demonstrate that hydrogen peroxide produces small protein fragments. The second method was to use gel filtration with a Sephadex G 10 column. Its exclusion limit is 10kDa and any protein that size or larger elutes immediately after the void volume. When non-collagen fraction was passed, it emerged in the first fraction. The same pattern was observed when treated with trypsin. When hydrogen peroxide was added, there were fragments in latter fractions. A similar pattern was observed for both Type I collagen and the collagen fraction (data not shown). All these results suggest that treatment of protein with hydrogen peroxide does produce small fragments.

Table 4: Release of Protein Fragments from Albumin Protein Treated with Hydrogen Peroxide or Trypsin.

Treatment of albumin with	Mg of protein fragment released
0% hydrogen peroxide	0
Trypsin	0
1.5% hydrogen peroxide	6.17±0.56
4.5% hydrogen peroxide	7.10±0.68
18.0 % hydrogen peroxide	7.88±090

Discussion

Teeth contain protein in both the enamel and dentin layer. Even though all proteins are made of the same components, the major protein in dentin, collagen, has different properties from other proteins. There is loss of both collagen and non-collagen protein from teeth treated with whitening strips. Beyond a certain threshold, hydrogen peroxide interferes with both measurements, but the levels were below this value. Another contaminant, EDTA, had to be removed from the non-collagen fraction. The loss of protein is consistent with a previous study that showed a loss of 71% of protein from enamel when 35% hydrogen peroxide was applied [7]. In another study, the amount of protein from extracted enamel showed an 82% loss when treated with 30% hydrogen peroxide [12]. Enamel protein is made by the ameloblasts and once damaged or lost, it cannot be replaced. This enamel protein represents non collagen protein. The loss of collagen is consistent with the observation that carbamide peroxide can damage the collagen. When demineralized dentin was exposed to carbamide peroxide, which degrades to hydrogen peroxide and urea, there was a visible change in the appearance and a decrease in the signal from Raman spectroscopy for the amide group [19]. Hydrogen peroxide has previously been shown to damage collagen. It can form a redox couple with either Fe (II) or either Cu (either II or I) and the site of attack varies with the metal [20]. In addition, it can cleave amide bonds under basic conditions [21]. The amount of hydroxyproline lost with one round in treatment is nearly identical to the amount of collagen lost. This result confirms that hydroxyproline is an accurate marker of collagen. The number of protein bands observed on SDS PAGE is lower than the expected number of proteins in teeth. Previous studies have shown that there is a low yield for dentin and enamel proteins when removed from teeth for numerous reasons including low solubility. In addition, certain proteins are only present during the formation of the tooth. There are proteins found in both the non-collagen and collagen fractions whose MW match those of known proteins in teeth. The MW of these proteins can be determined either by the protein itself or based on the predicted amino acid sequence of the gene. Another issue is the alternate splicing that produce proteins that differ slightly in size. Post-translational modifications can also produce aberrations in the MW based on the sequence of the gene that encodes the protein [22]. Bone acidic glycoprotein in rats has previously been shown to have a MW of 75 based on a Western blot [21]. Western blot yields the MW of the protein on SDS PAGE as opposed to a predicted MW based on amino acid sequence translated from the encoding gene. Dentin also contains dentin sialoprotein whose MW can vary based on the number of sialic residues attached. The range of this protein from 10.5 to 11.2kDa is based on the translated DNA sequence of the gene. While there are several types of collagen found in teeth, Type I collagen is the most common. It is made of two α-1 chains and one α-2 chain. The MW of these both subunits, once the signal peptide is removed is 129kDa based on the translated DNA sequence for the encoding gene. The MW of isolated collagen fraction is 130kDa as Figure 1B shows, which is the very similar to that observed for pure Type I human collagen (data not shown).

The results demonstrate that these observed decreases in protein are due to release of protein from the teeth. Instead of simply being degraded, the whitening strips cause a clear release of both collagen and non-collagen protein from teeth. Even small number of whitening strips, applied according to manufacturer's instructions, resulted in loss of protein from teeth. This amount increased if the number of strips per day increased. As noted

previously, collagen and the dentin itself are replenished in teeth providing the pulp is healthy. Since these are extracted teeth, that process could not occur. The released proteins have with slightly smaller MW to proteins shown to be in the teeth. This is consistent with results in Figure 1C where non-collagen proteins had slightly smaller MW when treated with hydrogen peroxide. When these samples were run on gels specifically for collagen (4%/6%), there were no bands. As shown in Figure 1D, collagen protein treated with hydrogen peroxide tends to disappear from the gel. When the concentrated samples from teeth that received two treatments a day were tested, the prominent band at 66kDa disappeared over time (data not shown). The decrease in MW of protein treated with hydrogen peroxide is consistent. It has previously been observed that treatment of bovine serum albumin or aldolase with 2.5% hydrogen peroxide can alter their MW on SDS PAGE [23]. The percent hydrogen peroxide used in those experiments was lower than the ones used here. The concentrations of hydrogen peroxide used are similar to those in over-the-counter whitening strips. The results from these experiments suggest that hydrogen peroxide changes proteins in different way than proteases do [24]. The latter hydrolyze at specific amino acid residues producing fragments that are reduced in size but still detectable on SDS PAGE. When hydrogen peroxide is used, the non-collagen proteins are reduced in size and the resulting fragments are too small to be detected [25]. While for collagen, the original protein bands disappear when hydrogen peroxide is added.

Conclusion

In conclusion, the results from these experiments demonstrate that hydrogen peroxide used in over-the- counter whitening strips can damage proteins in the teeth. There is a decrease in collagen and non-collagen proteins extracted from teeth that received whitening strips. The MW of proteins extracted are consistent with proteins known to be in teeth. In addition, treatment with whitening strips resulted in significant amounts of both collagen and non-collagen protein in the fluid outside teeth. The MW of the protein that left the tooth is slightly smaller than the protein in the tooth. This is consistent with the change in MW observed when proteins are treated with hydrogen peroxide. While this was true for non-collagen proteins, it was not possible to detect the effect of hydrogen peroxide on collagen using SDS PAGE. Other experiments demonstrate that treatment of collagen with hydrogen peroxide produces a large amount of small protein fragments. This result is very different than that which happens when treated with a protease.

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