Journal of Research in Medical and Dental Sciences
Volume 5, Issue 2, Page No: 80-90

Available Online at: www.jrmds.in
eISSN No. 2347-2367: pISSN No. 2347-2545

Hydrogen Peroxide Tooth Whitening Agent Alters the Protein Content of Enamel

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DOI: 10.5455/jrmds.20175212

ABSTRACT

Tooth whitening is a relatively quick, inexpensive and conservative treatment options for managing discoloured teeth and can be applied easily by dentists and patients. Bleaching agents at various concentrations have been marketed for dental office and home application. The aim of this study was to assess the effects of 30% hydrogen peroxide on the protein content of enamel. The effect of bleaching for 30 minutes with 30% hydrogen peroxide on the protein content of sound enamel was also investigated. Protein was extracted from the enamel by precipitation with trichoracetic acid after dissolution of the inorganic phase and the amount of protein was quantified using the Lowry and the Bradford protein assays. From 0.01g of sound enamel, the mean amount of protein detected using the Lowry, and the Bradford assays were50.56µg ± 0.06, and 3.08µg ± 0.01 respectively. After surface treatment of the enamel with 30% hydrogen peroxide, the mean protein values using same protein assays were 9.2 µg ± 0.04, and 1.2 µg ± 0.004 respectively. These results indicate that bleaching treatment with 30% hydrogen peroxide resulted in a significant reduction in protein content. The contribution of the protein modified or extracted from mature, sound enamel by bleaching with 30% hydrogen peroxide to the mechanical properties of enamel should be further investigated.

Key words: Tooth Whitening – Hydrogen Peroxide – Enamel Proteins– Protein assay – Mechanical properties.

HOW TO CITE THIS ARTICLE: Reza Shah Mansouri, Nabil Khzam, Hydrogen Peroxide Tooth Whitening Agent Alters the Protein Content of Enamel, J Res Med Dent Sci, 2017, 5 (2): 80-90, DOI: 10.5455/jrmds.20175212

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Received: 12/01/2017
Accepted: 10/06/2017

INTRODUCTION

Hydrogen peroxide (HP) is one of the most popular agents used for either professional or self-administered bleaching. It dissociates into free radicals which oxidize complex chromogens such as pigment molecules [1]. *In vivo* studies of teeth suggest that these modifications are unlikely to be permanent [2-4]. The dissociative decomposition of the hydrogen peroxide-urea conjugate carbamide peroxide also can be used to produce HP. High concentrations of bleaching agents such as 35-37% carbamide peroxide (CP) and 30-35% HP have been developed for in-office use by the dental practitioner [5], while several-fold lower concentrations of these agents are used in products designed for home use.

The adverse effects of different bleaching agents are thought to occur through modification of hard tooth structures, including enamel, dentine and cementum. The most important adverse effect due to teeth whitening is thought to be a reduction of enamel microhardness that may be attributed to changes in enamel proteins [6]. It was reported that bleaching agents adversely affect the surface morphology of dental hard tissue and that cementum was affected by bleaching even more than either enamel or dentine [7].

Numerous studies have described the mechanical properties of mature enamel, and tried to explain the unique mechanical characteristics of enamel from perspectives that include anisotropy, hierarchy, fracture characteristics and composition [8-13]. Several investigators have suggested that peroxides modify the chemistry of dental hard tissues [14-18], by changing the ratio between organic and inorganic components. Although water and

organic components such as protein comprise a minor part of mature enamel, they are very important for developing the structural organisation that determines its physical properties [19].

The critical importance of enamel function is indicated by the finding that enamel is about 3-fold tougher than crystalline hydroxyapatite [20]. After eruption of tooth and enamel maturation, it has been suggested that the fragmented enamel proteins such as enamelin, tuftelin and amelogenin proteins remaining inside the enamel act as "glue" and "cushion" between crystallites in the prisms structure that extends from the dentinoenamel junction (DEJ) to the enamel surface area. This arrangement is thought to influence enamel properties including compressibility, permeability and ionic conductivity [21, 22]. The elastic modulus and hardness of enamel may therefore be strongly influenced by its organic content [23]. There is some controversy about the effect of bleaching agent on the demineralisation of teeth. There was no evidence of deleterious effects on enamel or dentine exposed to 35% HP [24]. On other hand, it was reported there is a significant reduction in Knoop hardness of bovine crown enamel after application of 35% CP [25].

The aim of this study was to identify assays that can be used to determine the protein content of enamel samples after treatment with HP and to determine the effects of treatments with 30% HP on the protein content of surface-treated enamel, powdered enamel and extracted enamel polypeptides.

MATERIALS AND METHODS

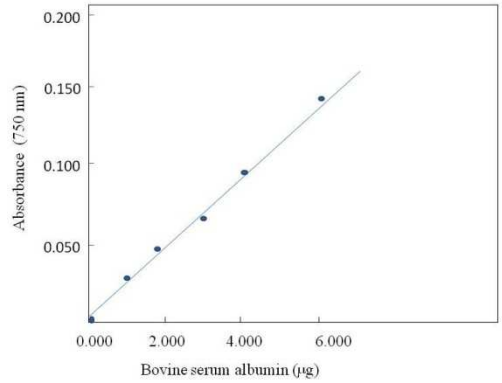
The study sample consisted of eight permanent molars and four permanent premolars that were extracted because they were either impacted, or required as part of an orthodontic treatment plan. The selected teeth were caries-free and had no defect or crack on the enamel layer. Teeth with a history of bleaching treatment were excluded from the study.

After collection of teeth, attached soft tissues were removed using scissors. The teeth were then washed and cleaned under running water with a soft toothbrush. Specimens were placed in containers encoded with the patient's identification number. Sterile Hank's balanced saline solution (HBSS, Invitrogen™), as recommended by Habelitz *et al*., 2002 was added to cover the teeth during storage [26]. The protein content of enamel samples obtained from these teeth was determined after solubilisation of the inorganic phase (demineralization) and protein precipitation using 20% Trichloroacetic Acid (TCA) [27]. The Lowry and Bradford assays were used to measure the protein content of samples by using bovine serum albumin (BSA) as protein standard.

Lowry protein assay

The Lowry method (1951) relies on two different reactions [28]. The first reaction is the Biuret reaction. This involves the reduction of copper ions (Cu^{2+}) in alkaline solution by peptide bonds. The second reaction uses the Folin reagent (a mixture of sodium tungstates, molydbates and phosphate) which reacts with the sidechains of tyrosine and/or tryptophan. The overall reaction gives a reduced product with a characteristic blue-purple colour. The colour depends mainly on the tyrosine and tryptophan content of the protein sample and can be quantified by measuring maximum absorbance of the reaction product at 750 nm. Although the intensity of colour can be related to the amount of protein in solution, strong dependence on tyrosine and tryptophan content for the reaction can skew the response of individual proteins or protein mixtures. A further limitation of the Lowry assay is interference caused by a range of substances including detergents, carbohydrates, glycerol, Tricine, EDTA, Tris (a buffer component), potassium compounds, sulfhydryl compounds, disulfide compounds, magnesium and calcium. Many of the interfering compounds are commonly used in the preparation of protein samples [29]. For example, precipitates can be formed because of the presence of detergents, lipids, potassium ions, and sodium phosphate [30]. The Lowry method is very sensitive. It can detect as little as ~ 0.01 mg protein/mL and is best used on solutions with protein concentrations in the range 0.01–1.0 mg/mL [31].

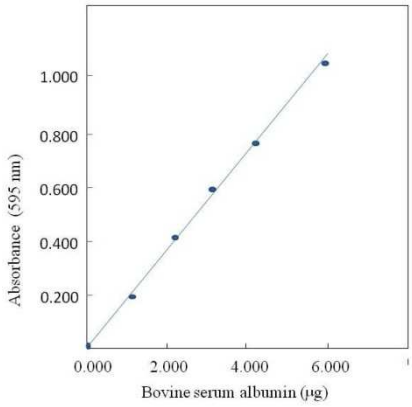
The Standard curve for BSA in the Lowry assay is shown in Figure 1. The correlation coefficients of the BSA standard curve was 0.999 for the Lowry assay. The Lowry assay was used in the present study to quantitate protein was the Bio-Rad *DC* protein assay applied in a 96 well microtitre plate format (Bio-Rad, Hercules, CA, USA) [28, 29, 32, 33].



Curve Name	Curve Formula	A	B	C	R^2
Curve	Y=C*X^2+B*X+A	0.0709	0.0245	0.00038	0.999

Y Axis, Absorbance at 750 nm, X Axis, Bovine serum albumin (µg)

Figure 1: Showing Standard curve for BSA in the Lowry Assay.



Curve Name	Curve Formula	A	B	C	R^2
Curve	Y=C*X^2+B*X+A	0.321	0.236	0.0115	0.999

Y Axis, Absorbance at 595 nm, X Axis, Bovine serum albumin (µg)

Figure 2: Showing Standard curve for BSA in the Bradford assay.

Bradford protein assay

The Bradford assay (1976) relies on the binding of dye Coomassie Brilliant Blue G-250 to protein at an acidic pH [34]. At low pH the free dye has absorption maxima at 470 and 650nm, but when bound to protein it has an absorption maximum at 595nm. The formation of protein-dye complex stabilises the anionic form of the dye producing a blue colour, even under acidic conditions when most of the molecules in solution are in the cationic form. The amount of dye binding appears to vary appreciably depending on the content of the basic amino acids arginine and lysine in the protein [29]. Hence, it may be difficult to choose a relevant standard. Bovine serum albumin, which is widely used as a convenient protein standard but naturally binds a wide variety of drugs and several dyes, gives absorbance values per µg of protein that are 50% higher than for the soluble protein bovine gamma globulin. This can lead to lower estimates for the protein content of some samples. Also, an incomplete reaction may result as many proteins are not fully dissolved in the acidic reaction medium [29, 34]. The Bradford method offers advantages that include simplicity in preparing the reagent, a rapid colorimetric response to the reaction between protein and dye, and relative stability of resultant colour [34].

Like the Lowry assay, the Bradford assay measures the amount of protein in solution by comparison of absorbance values given by a standard curve of known protein amounts. The Standard curve for BSA in the Bradford assay is shown in Figure 2. The correlation coefficients of the BSA standard curve was 0.999 for the Bradford assay. Unlike the Lowry assay that preferentially detects aromatic amino acids, the Bradford assay detects predominantly more general binding of the commassie dye to protein.

The assay system used in the present study was the Bio-Rad DC protein assay kit adapted to a 96 well microtitre plate format (Bio-Rad, Hercules, CA, USA).

All teeth were decoronated at the cementoenamel junction using a handpiece and diamond bur with distilled water irrigation. The decoronated teeth were embedded in clear epoxy resin for ease of handling during sectioning. One experimental group of molars was surface treated with 30% HP as described below before decoronation and embedding for sectioning. All teeth were machine-sectioned using an Accutom 50 (Struers A/S, Ballerupt, Denmark). The machine was programmed to cut each crown into 1 mm slices across its entire thickness using a diamond-impregnated disc under Double Distilled(DD) water irrigation. A pilot study was used to establish parameters for sectioning the coronal tooth structure. Medium force, a wheel speed of 2000 rpm, and a low feed-speed of 0.05 mm/second were used to generate sections with flat cut surfaces. These guidelines were adapted from the protocol developed by Farah *et al*. (2009) [35].

The thin sections obtained allowed ready identification of the DEJ and enabled precise separation of enamel from the dentine with sterile nail clippers. Extreme care was taken to avoid contamination of the cut enamel samples from dentine. Pure enamel samples were cut from 1mm sections, with the operator using a Heine 2.5 x magnifying head loupe (Heine Optotechnik, Herrsching, Germany) and wearing a mask and double gloves to minimise contamination with keratin. The adherent smear layer resulting from sectioning was removed from all cut pieces of enamel by immersion in 0.5ml of 100% ethanol in an Eppendorf tube. The tube was placed in an ultrasonic bath for 1 minute, the enamel blotted dry using clean tissue paper and allowed to air dry.

Teeth samples were divided into four groups and treated as follows:
Group 1 Untreated teeth (control).
Group 2 The teeth were surface treated with 30% HPby applying The BEYOND™ Whitening Accelerator (BEYOND Dental and Health Inc. Houston, Texas USA), (3x10 min) and washing with DD water between treatments, and at the completion of the treatment.
Group 3 Powdered enamel obtained from the teeth was treated with 30% HPfor 30 min and washed briefly with 1.5 ml 20% TCA.

Enamel obtained from the teeth in groups 1, 2 and 3 were demineralised using 20% TCA and the TCA precipitated protein recovered as a pellet by centrifuging at 13,200 rpm for 10 min at 4^{o}C.

Group 4 Protein extracted from sound enamel demineralized using 20% TCA was treated with 100 µl of 30% HP for 30 minutes. The enamel protein was recovered as a pellet by treating with 20% TCA and centrifuging at 13,200 rpm for 10 min at 4^{o}C.

Enamel samples were crushed into a coarse powder using a sterile amalgam plugger, and the combined weight of each group measured. Individual crushed enamel samples were separately resuspended in 20% TCA until the mineral phase of enamel was dissolved and the protein content precipitated. Samples containing ~0.01g of enamel were extracted by resuspension in 1.5 ml of 20% TCA containing 15 µl of a proteinase inhibitor cocktail (Roche). The cocktail inhibits a broad spectrum of serine and cysteine proteases. The proteinase inhibitor cocktail was prepared as a stock solution by dissolving 1 pill of Roche proteinase inhibitors (complete minus EDTA) in 20 ml DD water. TCA precipitated enamel protein was recovered as a pellet by centrifuging at 13,200 rpm for 10 min at 4^{o}C.

TCA remaining with the extracted protein of the group 4 sample was removed prior to treatment with 30% HPby washing the sample with 300 µl of 80% acetone (stored at -20ºC) for 20 minutes on ice and centrifugation (13,200 rpm, 10 min, 4ºC). The supernatant was completely removed and the sample allowed to air dry. The dried sample was resuspended in 100 µl 30% HP and incubated for 30 minutes at room temperature. After centrifugation at 13,200 rpm for 10 min at 4°C the supernatant containing the 30% H_2O_2 treatment was discarded and the pellet retained for further processing. Unless otherwise specified, the samples were then washed twice with 1 ml 20% TCA to remove any remaining mineral phase and eliminate residual HP.

TCA remaining in the pellets was removed by washing all the samples with 300 µl 80% acetone (at -20ºC) for 20 min on ice and centrifugation at 13,200 rpm for 10 min at 4ºC. The supernatant was removed completely, the samples allowed to air dry and each protein pellet dissolved in 400 µl 0.1 M sodium hydroxide (NaOH).

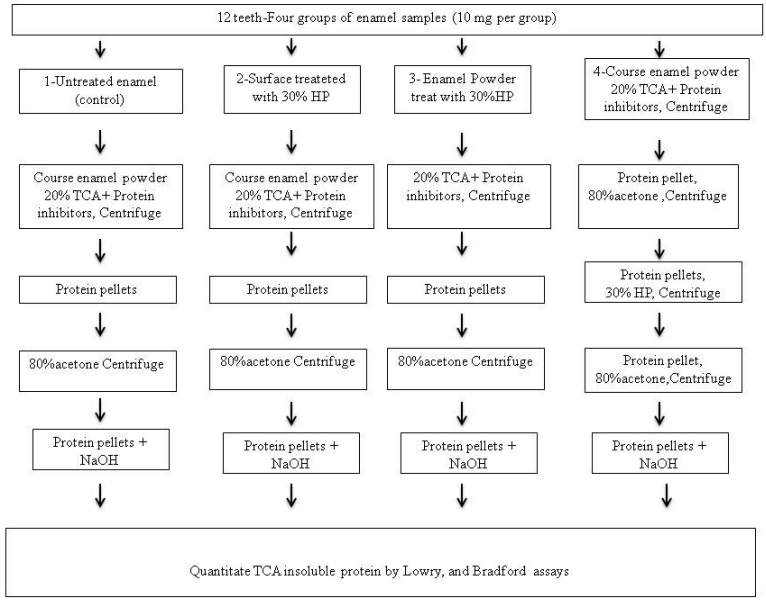


Figure 3: Showing a flowchart of the experimental design for all protein assays.

Table Error! No text of specified style in document.**: Recovery from 1 mg of input BSA detected using the Lowry and Bradford assays.**

Protein assay	Lowry	Bradford
	Protein Mean ± SD (µg)	Protein Mean ± SD (µg)
Control BSA	704 ± 14	608 ± 16
Test BSA 30% HP for 30 min	640 ± 17 (91% recovery)	515 ± 5 (85% recovery)

SD = Standard deviation, BSA = Bovine serum albumin , HP = Hydrogen peroxide

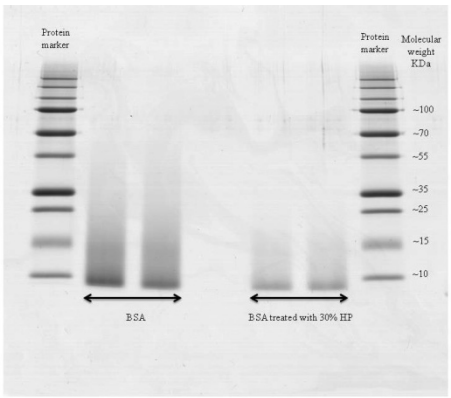


Figure 4: Showing the BSA samples separated by SDS-PAGE and stained with Coomassie Blue R250. (Invitrogen Life technologies, Ohio, USA)

The samples from each group were then divided into 200µl aliquots that were frozen at -20˚C. One 200 µl sample was assayed for protein using the Bradford and Lowry methods. The other 200 µl samples are stored at -20˚C for analysis by mass spectrometry. The overall experimental procedure is summarised In Figure 3.

RESULTS

Oxidation of bovine serum albumin with 30% HP: Effects on detection using the Lowry and Bradford assays:

The detection of BSA using either the Lowry or Bradford method was modestly affected by treating the BSA with HP, i.e. the Lowry and Bradford assays gave 9 and 15% reductions in protein detected after treatment with HP compared with an untreated control sample, respectively. Although, 30% HP may denature,

modify or disrupt polypeptides, it only slightly affected the ability of either assay to detect BSA after TCA precipitation and dissolution in 0.1 M NaOH. However, it should be noted that the Bradford assay recorded a significantly lower level of protein than the Lowry assay (Table 1).

Samples of TCA-precipitated BSA (20 µl in 0.1M NaOH) were neutralised with 4 µl 0.5 M HCl and treated with 6 µl 6x SDS-lysis buffer. The samples were then separated by SDS-PAGE and stained with Coomassie Blue R250 (Figure 4). Both the control and the 30% HP-treated protein samples were found to be degraded, with most coomassie-stained material running at or near the dye front. A band at 68 kDa expected for intact BSA was not found. The BSA had been readily dissolved in 0.1 M NaOH to facilitate reproducible determination of protein content. Exposure to 0.1 M NaOH was not expected to lead to protein degradation. The reason for the unexpected degradation of BSA may have been due to hydrolysis caused by use of HCl to neutralize the protein sample prior to electrophoresis.

The protein content of enamel samples

Surface-treatment of teeth with 30% HP resulted in ~50% reduction in the amount of TCA –precipitated protein detected by either the Lowry or Bradford assay compared with otherwise identically-treated control samples (Table 2). The 50% loss may have been due to oxidative damage causing increased solubility or a loss of reactivity with the Lowry or Bradford reagents. The much higher percentage loss of detectable protein incurred with the HP-treatment of enamel samples than in the BSA-based control experiment may be consequences of:

a) Extensively damaged/degraded products were a higher proportion in enamel samples than the BSA because the enamel samples contained ~200-fold less protein than the BSA samples (10mg of BSA compared with <50 µg of protein in the 10 mg enamel samples).

b) Less efficient TCA precipitation of HP-modified enamel peptide products than for BSA because the input enamel peptides had much lower molecular weights than BSA (68 kDa). The HP-treatment of enamel polypeptides would be expected to yield a group of much smaller peptide products than HP-treated BSA.

There was a major reduction in the amount of protein detected in surface-treated enamel samples washed with water (instead of TCA) to remove the residual HP i.e. the Lowry and Bradford assays (Table 2) detected 15% and none of the input enamel protein in the water-washed sample, respectively. This result indicates that HP treatment rendered water-soluble 35% (85-50%) of the TCA-precipitated peptides in surface-treated mature enamel.

Table 2: Showing the mean amount of protein recovered from 10 mg of sound enamel when teeth were surface treated then washed with TCA or water to remove excess HP.

Protein assay	Lowry Protein Mean ± SD (µg)	Bradford Protein Mean ± SD (µg)
Untreated teeth (control) (TCA washed)	5.2 ± 1.2	2 ± 0.6
Surface treated with 30% HP (TCA washed)	2.8 ± 1.0	1.1 ± 0.4
Untreated enamel (control) (water washed)	49.3 ± 3.2	10.2 ± 2.1
Surface treated with 30% HP (water washed)	8.3 ± 2.8	0

HP = Hydrogen peroxide ; SD = Standard deviation ; TCA = Trichloracetic acid.

Table 1: Showing the mean amount of protein recovered when crushed enamel or enamel protein from 10 mg of sound enamel was treated with 30% HP and washed with TCA or water to remove excess HP

Protein assay	**Lowry** Protein Mean ± SD (µg)	**Bradford** Protein Mean ± SD (µg)
Powdered enamel treated with 30% HP (TCA washed)	66.4 ± 2.4	20 ± 1
Extracted enamel protein treated with 30% HP (TCA washed)	46.8 ± 4.4	8.4 ± 2.4
Powdered enameltreated with 30% HP (water washed)	6.6 ± 2.0	0.28 ± 0
Extracted enamel protein treated with 30% HP (water washed)	37.4 ± 8.7	1.68 ± 0.8

SD = Standard deviation; HP = Hydrogen peroxide

The remaining 15% of the protein detected due to retention of aromatic groups sensed by the Lowry assay can only be explained as enamel-associated peptides that were oxidatively cleaved to an extent that they are unable to bind the Bradford.

The results obtained after treating protein in powdered enamel samples with 30% HP showed that substantial amounts of protein were recovered (detected by both the Lowry and Bradford assays) when TCA (Row 1) was used instead of water (Row 3) to wash treated samples free of HP. Washing HP-treated samples with water severely diminished the quantity of protein detected by both assays i.e. 10% of the Lowry protein detected after TCA washing was recovered after washing with water while only 1% of Braford protein was detected. This result indicated even greater losses of protein in the powdered samples compared with teeth samples were surface-treated with HP and washed with water prior to enamel dissolution with TCA. In contrast, when TCA-purified enamel protein was treated with 30% HP, the amount of protein recovered was comparable after the TCA or water wash for the Lowry assay but not for the Bradford assay (Table 3).

DISCUSSION

HP and the by-product urea from CP are both low molecular weight compounds. This allows them to diffuse into enamel [36, 37]. The mechanism of action of HP-based bleaching agents is thought to be due to the ability of HP to form oxygen free radicals that interact with adsorbed coloured organic molecules. These macromolecules and pigment stains are oxidised giving rise to breakdown products that are smaller and less able to absorb light [38-41]. The formation of colourless or less pigmented compounds give the colour changes seen as a whitening action [42].

HP, which is unlikely to modify the inorganic content of the enamel, can oxidise proteins in ways that might modify the protein content of the enamel [43]. One way to investigate the effects of HP-mediated oxidation of enamel is to use proteomic techniques to determine the protein content and protein composition of enamel in response to this bleaching agent. To the best of our knowledge, this has yet to be done by others. The present study provides a preliminary test of the notion that the residual protein content of enamel contributes to its mechanical properties.

There are few reports on the protein content of sound enamel [27. 44, 45, 46]. The main protein in mature enamel matrix formed during amelogenesis is proteolysedamelogenin. This protein and its proteolytic products are important for enamel formation mediated by ameloblasts[47]. Enamel proteinases, which are also secreted by the ameloblasts, have two important roles during enamel formation [48. 49]. First, during the secretory stage, they process enamel matrix proteins, including amelogenin, into a large number of stable cleavage products. Both the intact enamel proteins and their proteolytic cleavage products are believed to play active roles during amelogenesis. A second function of enamel proteinases is to degrade the enamel matrix during the transition to maturation stages, which allows the enamel layer to attain its high degree of mineralization by the time of tooth eruption or shortly thereafter.

The residual protein in mature enamel is primarily composed of polypeptides derived from the enamelin and amelogenin classes of protein [27, 50]. HP is expected to penetrate regions rich in enamel polypeptides rather than the much more compact inorganic phase. It has been suggested that the urea associated with CP might dissociate H-bonds, causing conformational change and denaturation of proteins such as enamelin and amelogenin, thereby increasing the permeability of enamel to peroxides and free radicals [51]. This is unlikely as the concentration of urea in CP does not approach those needed to denature proteins e.g. 6-8 M urea.

In the proteomic studies, a test with the model protein BSA using two different protein assays (Lowry and Bradford) before and after treatment with 30% HP for 30 min followed by precipitation with TCA and dissolution in 0.1 M NaOH, showed only a 9-15% reduction in the amount of control protein in BSA after bleaching treatment. Although HP may oxidise, denature or cleave protein, the ability of either protein assay to detect protein appeared only modestly affected. It was also specifically noted that treatment with 30% HP gave a significantly greater reduction in the amount of protein detected in the Bradford assay than the Lowry assay.

When 10 mg samples of enamel obtained from teeth were surface-treated with 30% HP and washed with water to eliminate residual HP, there was a considerable reduction in protein content. The Lowry assay detected 49.3 ± 3.2 µg

protein in an untreated enamel sample and 8.32 ± 2.76 μg in a sample surface-treated with 30% HP. This amounts to a statistically significant (~ 83%) difference in the amount of protein recovered from the control and HP-treated samples. When the Bradford assay was applied to the same sample, no protein was detected in the enamel. When the same experiment was carried out but the samples were washed with 20% TCA, the Lowry assay detected 5.2 ± 1.2 μg of protein in the control sample and 2.8 ± 1.0 μg and in the sample surface-treated enamel with 30% HP. This amounted to a ~ 50 % decline in the amount of protein. The Bradford assay gave a comparable result. These results lead us to conclude that treatment with 30% HP causes about 50% of enamel peptides to become either soluble in TCA and lost from enamel, or unresponsive to the Bradford and Lowry assays due to protein oxidation. Washing HP-treated enamel with water leads to solubilisation of another 35% of the Lowry positive protein and all the Bradford positive protein. The remnant (15%) protein of the enamel protein remains associated with the enamel and is responsive to the Lowry but not the Bradford assays. This interpretation, which suggests the maintenance of a sub-population of Lowry-positive, Bradford-negative polypeptides in HP-treated teeth, is supported by experiments where ground enamel and enamel polypeptides were purified using TCA extraction, with the purification exposed to 30% HP, and purified. In the absence of proteomic analysis by mass spectrometry of this sub-population, we hypothesise that the assay tests mentioned includes enamel peptides that are significantly lower in molecular weight than the parent untreated polypeptides due to cleavage of the polypeptide backbone.

The TCA precipitation used to purify mature enamel protein should have denatured enamel polypeptides and rendered them insoluble in water. Since the Lowry assay can detect the amino acids tryptophan and tyrosine independent of the polypeptide backbone, while the Bradford assay requires polypeptide backbones of a size sufficient for dye binding, it is suggested that the HP treatment caused considerable damage to enamel polypeptides backbones and much less to their aromatic sidechains. This would have rendered the HP-treated enamel peptides less susceptible to the binding of Coomassie blue 250G without dramatically affecting the Lowry reaction. These results suggest that surface-treatment of teeth with 30% HP is likely to extensively cleave the polypeptide backbone of enamel peptides, rendering the bulk of this material soluble and leading to substantial loss from the preparation during water washes. However, this loss is unlikely to have been complete because a detectable sub-population of enamel protein (~20% of input Lowry-detectable polypeptide but no Bradford-detectable polypeptide) remained associated with the enamel obtained from HP-treated teeth. Whether Lowry positive and Bradford negative polypeptide sub-population is important for the nano-mechanical properties of enamel have yet to be established. Mass spectrometry studies designed to identify this sub-population of polypeptides are in progress.

The 30% HP treatment used to remove stains from teeth appears likely to remove significant amounts of organic material from enamel structure. The present research suggests that this loss is not associated with structural weakness, as indicated by the H and E values determined by nano-indentation the enamel i.e. the polypeptide components of mature enamel may not contribute significantly to the hardness and stiffness properties of the enamel. This interpretation may be premature. Despite the likelihood of considerable oxidative damage to remnant of enamel polypeptides caused by 30% HP, we have been unable to exclude the possibility that populations of peptides that are differentially unresponsive to the Lowry and Bradford assays remain in the HP-treated enamel and confer important biomechanical properties.

CONCLUSION

Surface-treatment of teeth with 30% HP modified the reactivity, solubility and polypeptide backbone of enamel polypeptides, altering the amount of polypeptide that can be recovered by demineralisation and protein precipitation with 20% TCA and assayed using the Lowry and Bradford assays. Treatment of samples with water to remove residual HP removed additional reactive material in a manner most consistent with cleavage of the polypeptide backbone increasing polypeptide solubility and leaving a sub-population of small peptides that were still reactive with the Lowry reagent but not the Bradford regent. The identification of this sub-population of peptides and their contribution to the nano-mechanical properties of enamel has yet to be determined.

Future research and recommendations:

To the best of our knowledge, a proteomic study (mass spectrometry) has yet to be carried out to test the oxidizing agent HP's ability to modify

the protein content of the enamel. Such a study would further investigate the idea that the residual protein content of enamel may contribute to its biomechanical properties. Moreover, such study should be carried out soon after extraction of protein in appropriate medium without any delay or freezing the extracted protein to achieve better retrieval of the protein content.

Acknowledgements
Ethical approval (category A, approval number 10/112) from the University of Otago Ethics Committee have been granted. Informed consent was obtained from participants who voluntarily donated their extracted teeth.

Conflicts of interest:
The authors declare that there is no conflict of interest regarding the publication of this article.

Author's Contributions:
Reza Shah Mansouri: Performed all the procedures, conducted a literature review of similar articles and contribute significantly to the discussion.
Nabil Khzam: Contributed significantly to the drafting and revision of the manuscript.

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