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Effect of phenylmethylsulfonyl fluoride, a protease inhibitor, on enamel surface remineralization

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Phenylmethylsulfonyl fluoride (PMSF) is a protease inhibitor widely used in research, but fluoride is released during its action and this knowledge has been neglected in dental research. **Aim:** to evaluate if fluoride released by salivary protease action on PMSF affects enamel remineralization and fluoride uptake. **Methods:** Groups of 10 enamel slabs, with caries-like lesions and known surface hardness (SH), were subjected to one of the following treatment groups: Stimulated human saliva (SHS), negative control; SHS containing 1.0 µg F/mL (NaF), positive control; and SHS containing 10, 50 or 100 µM PMSF. The slabs were subjected to a pH-cycling regimen consisting of 22 h/day in each treatment solution and 2 h/day in a demineralizing solution. After 12 days, SH was again measured to calculate the percentage of surface hardness recovery (%SHR), followed by enamel fluoride uptake determination. The time-related fluoride release from 100.0 µM PMSF by SHS action was also determined. Data were analyzed by ANOVA followed by Newman-Keuls test. **Results:** The release of fluoride from PMSF by SHS was rapid, reaching a maximum value after 10 min. Fluoride released from PMSF was more effective in enhancing %SHR and increasing fluoride uptake in enamel compared with SHS alone ($p < 0.05$); furthermore, it was equivalent to the positive control ($p > 0.05$). **Conclusion:** In conclusion, fluoride released by saliva from PMSF is available to react with enamel and needs to be taken into account in research using this protease inhibitor.

Keywords: Protease inhibitor. Dental enamel. Fluorides. Tooth remineralization.

Introduction

Even before the salivaomics era in Dentistry[1], protease inhibitors had already been used to preserve the structure of proteins to be analyzed. Particularly, for human salivary proteome analysis, the subject is relevant because the proteins in saliva collected suffer rapid degradation[2], requiring the use of a cocktail of protease inhibitors during storage to stabilize the structure of the proteins[3].

Phenylmethylsulfonylfluoride (PMSF) has been used in research as a protease inhibitor to avoid the breakdown of proteins. It inhibits serine-proteases by a covalent linkage to the active site of the enzyme and, during the reaction, the serine hydroxyl links to sulfonyl group and fluoride is released into the media[4]. PMSF is an efficient inhibitor of salivary proteases[5] and has been used in dental research for a long time, e.g.: (1) to avoid proteolysis during saliva collection[6]; (2) in studies about adsorption of salivary proteins to enamel[7]; (3) in studies on salivary gland function[8]; (4) determination of GTF activity[9]; (5) inhibition of bacteria coaggregation in saliva[10], and (6) in isolation of salivary proteins[11] and proteomics analysis[12]. In these studies, PMSF has been used from 100 µM to 0.5 M. If it was totally hydrolyzed, the final F concentration into the batch media would range from 1.9 to 9,500 µg F/mL (ppm F).

This high F concentration released from PMSF could have an indirect effect depending on the research in question as fluoride at a concentration of only 1.0 µg F/mL can interfere with protein adsorption to and desorption from hydroxyapatite[13]. Likewise, the enzymes enolase and F_1F_oATPase of *Streptococci* species are inhibited by F at 20 to 45 µg /mL[14] and 10 ppm F prevents the enrichment of S. mutans in biofilms[15]. Furthermore, sub-ppm fluoride concentrations are sufficient to enhance enamel remineralization[16].

Although fluoride released from PMSF by salivary action can have an indirect effect in research and even producing an artifact, it could be a new approach in development of products for caries prevention. Therefore, the aims of this research were (1) to evaluate if fluoride is released by saliva from PMSF, and (2) to investigate its effect on fluoride uptake and remineralization of dental enamel.

Materials and Methods

Experimental design

Fifty bovine enamel blocks with caries-like lesions and of known surface hardness (SH) were randomly distributed into five groups of 10 each and allocated to one of the following treatments groups: (i) Simulated human saliva (SHS) as negative control; (ii) experimental groups containing 10, 50, and 100 mmol of PMSF/L of SHS; and (iii) SHS containing 1.0 µg F/mL (NaF) as a positive control. The blocks were placed for 22 h in the treatment solutions and 2 h in a demineralising solution, simulating a pH-cycling remineralizing regimen. After 12 days the enamel blocks were recovered, microhardness was again measured to calculate enamel SH recovery (%SHR), and enamel fluoride uptake was also determined.

Fluoride released from 100 µM PMSF by saliva action was assessed according to the time of incubation at 37 °C. The experiment was repeated 6 times and the increase of fluoride concentration in SHS was determined.

This study was conducted according to Resolution no. 196 from National Health Council, Health Ministry, Brasília, DF, Brazil.

Enamel blocks preparation and lesion creation

One hundred and nine enamel slabs (4 x 4 x 2 mm) of sound bovine incisors were prepared[17] and their baseline surface hardness (SH) measured using a 50 g load with a Shymadzu tester. Ninety-nine slabs, presenting indentations length from 40 to 46 mm, were selected for lesion creation. The slabs were painted with an acid-resistant varnish, except for a circular central area of 3.14 mm^2, in which caries-like lesions were induced[18]. The demineralising solution contained 0.05 M lactic acid, 0.2% Carbopol C907, and was 50% saturated with respect to hydroxyapatite at a pH of 5.0. Each specimen was placed in 6.3 mL of this solution for 16 h at 37 °C. The enamel SH was again determined and 50 slabs with indentations length from 110 to 150 mm were selected for the present study.

Treatments and pH-cycling remineralizing regimen

SHS was collected twice/day over ice in the morning and afternoon from seven (7) healthy adult volunteers by parafilm chewing and was then pooled. Sodium azide was added to the pooled saliva as a preservative (final concentration 0.02%). The pools were split into five fractions to prepare the treatment solutions. One fraction was separated to be used as a negative control treatment, and to three other, PMSF (Sigma) 10 µM (dissolved in isopropanol) was added to obtain final concentrations of PMSF at 10, 50 and 100 µM. A fluoride solution of 100 µg/mL (Orion) was added to the 5th fraction to obtain a final concentration of 1.0 µg F/mL (positive control treatment). Fluoride in these treatment solutions was determined daily with an ion-specific electrode, before the pH-cycling regimen.

Each enamel block was immersed individually in the SHS treatment solutions prepared each morning from 9:30 to12:30 h and from 14:30 to 17:00 h. Between these periods, the enamel blocks were immersed individually in the demineralising solution, which composition was identical to that described for the formation of early artificial caries. From 17:00 h until the next day, the enamel blocks were individually immersed in the SHS treatment solution prepared with SHS collected in the afternoon. Each slab was immersed in 4.0 mL of SHS solutions and in 12.0 mL of the demineralizing solution. The enamel blocks stayed in all solutions at 37 °C, and after each soaking they were washed with deionized water. The SHS solutions were changed twice a day and the demineralizing solution after the 6th day of cycling. This pH-cycling model used is similar to that used by White[18] to evaluate the ability of fluoride dentifrice to remineralize enamel.

Surface hardness analysis (SH)

After pH-cycling, the SH of the treated enamel blocks was measured again. Five indentations spaced 100 µm from each other, from the baseline and from those made

after the artificial caries development were made. A micro-hardness tester (Shimadzu HMV 2000) with a Knoop diamond indenter was used with a 50-g load for 15 seconds. The mean values of all five measurements at the three different times (baseline, after lesion creation and after pH cycling) were used to calculate the percentage surface microhardness recovery (%SHR) using the equation:

$$\%SHR = \frac{(\text{hardness after pH cycling} - \text{hardness after caries production}) \times 100}{\text{baseline hardness values} - \text{hardness after caries production}}$$

SH was evaluated because there is a good correlation (0.94) between remineralization of early carious lesions measured by this technique and by microradiography[18]. After surface microhardness analysis, all slabs were prepared for fluoride enamel analysis.

Analysis of Fluoride Concentration in Enamel

Five layers of enamel were sequentially removed from each dental slab under agitation in 0.5 ml of 0.5 M hydrochloric acid for 30, 30, 30, 60 and 60 s. An equal volume of TISAB II pH 5.0, modified with 20 g NaOH/L, was added to the acid extracts containing the dissolved enamel layer[19]. Fluoride was determined using an ion specific electrode (Orion 96-09) and an ion analyzer Orion E 940. The thickness of the enamel layer removed was calculated from the inorganic phosphorus concentration, determined by the Fiske and Subarrow method[20]. Phosphorus content of 17.4% and enamel density of 2.92 were assumed in order to calculate the amount of enamel removed and to estimate the depth of each enamel layer.

Fluoride release from PMSF by saliva

SHS was pre-incubated at 37 °C for 5 min. A volume of 0.51 mL of PMSF 5 µM, dissolved in isopropanol, was added to 25 mL of SHS. Aliquots of 1 mL of this saliva solution containing PMSF 100 µM were distributed in 18 assays tubes. After 5, 10, 20, 40, 60 and 120 min at 37 °C, three tubes were removed and 1 mL of TISAB (Acetate buffer 1.0 M, pH 5.0, containing 1.0 M NaCl and 0.4% 1,2-Cyclohexanediaminotetraacetic) was added to them. This procedure was repeated for six days. Fluoride released, and that in the SHS, was determined using an ion-selective electrode Orion 96-09 and an ion analyzer Orion EA-940.

Statistical analysis

The results were analyzed by analysis of variance (ANOVA) followed by Newman-Keuls test, with exception of comparison between fluoride concentration found in the solutions fresh and after the pH-cycling, which was evaluated by paired *t* test. For all statistical analysis, BioEstat 2.0 software[21] [Ayres et al., 2000] was used and the significance limit was set at 5%.

Results

The ANOVA showed statistically significant effects for fluoride release into saliva over time of incubation with PMSF ($p < 0.0001$), fluoride in the treatment solutions

used in pH-cycling, enamel fluoride uptake ($p < 0.0001$), and % of SH recovery ($p<0.0001$) after pH-cycling.

The effect of saliva on PMFS is shown in Figure 1. Fluoride release was very rapid and reached a plateau within 20 min. Fluoride concentration found at all times was statistically higher than that at time zero ($p < 0.05$), but after 10 min of incubation the concentrations were not statistically different ($p > 0.05$).

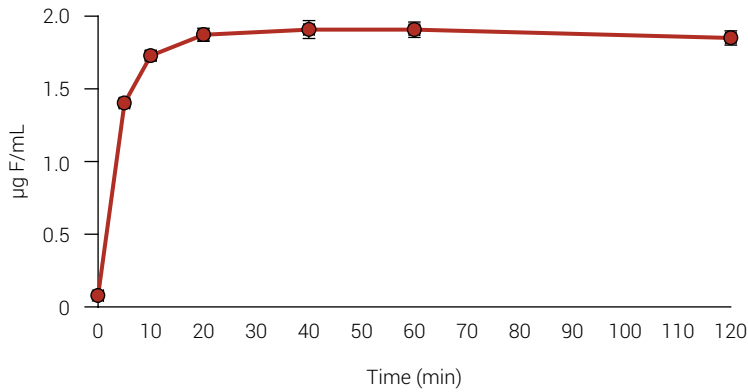

Figure 1. Means (n=6) of fluoride concentration (µg F/mL) in saliva over time of incubation with PMSF 100 µM. Bars denote SE; the statistical significance is described in Results section.

Table 1 shows that all treatments were more effective in increasing %SHR than the negative control ($p<0.05$). The %SHR of enamel blocks treated with SHS containing PMSF 50 and 100 µM was statistically higher than that treated with PMSF 10 µM ($p < 0.05$), but the difference between them was not significant ($p> 0.05$). All treatments with PMSF were equivalent to the positive control treatment ($p> 0.05$).

Table 1. Means (±SE) of enamel surface hardness recovery (%SHR, and fluoride concentration (µg F/mL) in the treatment solutions before and after the pH-cycling, according to the treatment groups.

Treatments groups	%SHR (n=10)	µg F/mL (n=24)	
		Before	After
SHS (negative control)	9.5 ± 1.8 [a]	[A] 0.076 ± 0.006 [a]	[B] 0.061±0.003[a]
SHS+PMSF 10 µM	32.9 ± 2.0 [b]	[A] 0.216±0.007 [b]	[B] 0.1308±0.010 [a]
SHS+PMSF 50 µM	47.9 ± 2.7 [c]	[A] 0.852±0.016 [c]	[B] 0.697±0.035 [b]
SHS+PMSF 100 µM	48.6 ± 3.1 [c]	[A] 1.888±0.032[d]	[B] 1.484±0.051 [c]
SHS+1.0 µg F/mL(Positive control)	43.2 ± 5.9 [b,c]	[A] 1.081±0.011 [e]	[B] 0.866±0.047 [d]

Means followed by different letters are statistically significant ($p<0.05$); lower case letters represent significant differences among treatments and capital letters represent significant differences between before and after pH-cycling for each treatment.

All treatment solutions containing PMSF presented greater ($p < 0.05$) fluoride concentrations than the negative control (Table 1). In addition, fluoride concentration in all treatment solutions decreased (before vs. after) significantly after the pH-cycling ($p < 0.05$). Also, all treatment solutions containing PMSF presented either before or after pH-cycling greater ($p < 0.05$) fluoride concentrations than the negative control (SHS). Before cycling, all treatment groups differed statistically in terms of fluoride concentration in the solutions ($p < 0.05$), but after the pH-cycling the difference between SHS containing 10 μM of PMSF and SHS (negative control) was no longer statistically significant ($p > 0.05$).

Figure 2 shows the enamel fluoride uptake data. All treatments were statistically more effective ($p < 0.05$) in incorporating fluoride into enamel than the negative control (SHS), up to approximately 30 mm from the dental surface (first three layers of enamel removed). The treatments with SHS containing PMSF 50 and 100 μM did not differ from the positive control treatment (1.0 μg F/mL) and between each other ($p > 0.05$). PMSF 10 μM formed lower fluoride concentration in enamel than the positive containing 1.0 μg F/mL ($p < 0.05$).

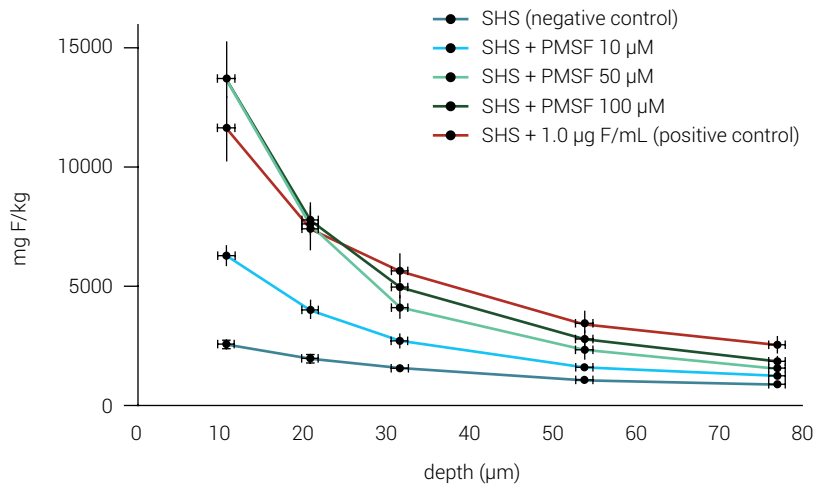

Figure 2. Means (n=10) of fluoride concentration (μg F/kg) in enamel according to the treatment groups and the distance from dental surface (μm). Bars denote SE; difference statistically significant ($p < 0.05$) between treatments are described in the text.

Discussion

The present findings showed that fluoride released from PMSF by saliva action has the same ability to enhance the remineralizing properties of human saliva as fluoride from the positive control (NaF) treatment. The release of fluoride by whole saliva is very fast (Figure 1) but the origin of the proteases is unknow, because they usually originate from oral mucosa tissue, salivary glands, or oral microorganisms[22]. The amount (mol) of fluoride released (Figure 1 and Table 1) is according to stoichiometry of the equation in figure 3:

Figure 3. Stoichiometric reaction between Serine-Enzyme and PMSF. The hydroxyl residue of Ser amino acid of the active site of enzyme links to sulfonyl group of PMSF and due to an electronic balance, free ion fluoride (F^-) is released to the media. The reaction is equimolecular, 1 mol of PMSF produces 1 mol of F^-.

Thus, the concentration of fluoride found after 10 min of incubation of PMSF 100 µM with SHS (Figure 1) was very close 100 µM of F (1.9 µg F/ml = 1.9 ppm F). In addition, the fluoride concentrations found in the treatment solutions of groups of SHS containing PMSF 10, 50 and 100 µM (Table 1) is according to the stoichiometry of the above chemical equation.

The results of enamel surface remineralization (Table 1) are supported by the fluoride concentrations present in the treatment solutions used. A positive correlation of 0.79 was found (data not shown) between %SHR and the concentrations of fluoride (µg F/mL) in the treatments used before the pH-cycling. After the pH-cycling, the fluoride concentrations in all groups decreased significantly (Table 1). This can be explained by the enamel fluoride uptake data (Figure 2), whereas the surface of enamel was remineralized with fluorapatite-like minerals. Indeed, a positive correlation of 0.97 was found (data not shown) between the mean of fluoride concentrations in the three first layers of enamel (~30 mm depth) and the %SHR (Table 1 and Figure 2).

Therefore, the robust findings of the present study show that during its action as protease inhibitor, PMSF releases fluoride into the batch media. Considering that PMSF has been used in research at concentration up to 0.5 M, it might result in up to 9,500 ppm F in the treatment solution. This unexpected very high fluoride concentration is not only a concern for research conducted in dentistry, because protease inhibitors are used for other research and therapeutic applications. Thus, the findings are an alert because artefacts in research may occur.

On the other side, although fluoride released from PMSF by salivary action can have indirect effect on research in progress, it could be a new approach in development of new products for caries prevention. In fact, fluoride has been used for a long time chemically bound to phosphate as monofluorophosphate (FPO_3^{2-}). In the past, it was believed that FPO_3^{2-} per se was the active moiety against caries. Nowadays it is accepted that its anticaries activity is due to the fluoride ion released by hydrolysis catalyzed by enzymes found in saliva and dental biofilm[23]. However, FPO_3^{2-} is hydrolyzed by salivary enzymes at a very slow rate[24], what could explain the relatively lower anticaries effect of dentifrice containing FPO_3^{2-} in comparison with NaF-based ones[25]. On the other hand, FPO_3^{2-} is indispensable as source of fluoride in a formulation containing Ca-based abrasives[26]. Thus, a molecule containing bound fluoride that was rapidly hydrolyzed by salivary action could have a better anticaries effect.

On the other hand, the present in vitro study presents some limitations. First, it was only evaluated the effect of fluoride released on surface enamel remineralization.

The effects on caries lesions remineralization and mainly the effect on reduction of enamel demineralization were not evaluated. Also, the effect on the enzymes inactivated by PMS was not evaluated in terms of reversibility.

In conclusion, fluoride released by saliva from PMSF is active to react with enamel and possibly may have other effects in research using this protease inhibitor.

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Data Availability

All data are available in UNICAMP repository.

Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

Conceived and designed the experiment: JAC. Performed the experiment: PECP. Analyzed the data: JAC, JF and DTZ. Wrote the paper: JAC. Reviewed the paper: PECP and DTZ.

Declaration of originality, interests, and financing:

This MS is original, and it was not supported by any manufacturer of oral hygiene products

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