Carbonated Soft Drink-Soaking Change the Crystallographic Properties of Human Tooth Enamel -A Micro-XRD Study

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Abstract: To elucidate the crystallo-chemical reaction between the enamel and carbonated soft drink, a micro-X-ray diffraction analysis (micro-XRD) was conducted using the human tooth sections soaked in the drink (Sprite®) for 1 and 7 days. In this study, 4 typically and heavily dissolved teeth were selected from 20 teeth, and the divergence of the crystallographic properties of the enamel was analyzed.

All of the untreated human tooth enamels exhibited only the apatitic XRD patterns. We confirmed that the carbonated soft drink changed the macro- and microscopic morphological features of individual tooth enamel. The following 4 results were obtained from our study: i) the unit cell dimensions and crystallinity of the untreated enamel apatite varied between the layers and the individual teeth; ii) after the soaking experiment, the crystallographic properties of the enamels changed remarkably and displayed non-uniformity; iii) no relationship was discerned between the enamel’s crystallographic properties and the sensitivity of the carious attack; and iv) the intermediate reaction product of soaking was uncovered; however, it was not identified at this time. The presence or absence of occurrence and variations in the amount of intermediate product reflected the complex chemical and crystallo-chemical reactions between the decalcification solution and the human tooth enamel crystallites.

We clearly established that the untreated human tooth enamels differed in their crystallographic properties and did not react in the same manner, which resulted in varied apatitic structures after soaking. These results necessitate reconsideration of the generally accepted caries protection methods that are applied as common standards for all individuals and tooth enamels.

Key words: Human tooth enamel apatite, Carbonated beverages, Caries, Unidentified materials, Micro XRD

Introduction

From studies on human tooth enamel caries, it could be inferred that the appearances of native enamel caries and artificial carious lesions are determined by their diversities in original structural and physico-chemical properties1,2). Although the enamel crystallites revealed similarities to hydroxyapatite in electron diffraction and X-ray diffraction (XRD), our studies also revealed significant differences between the enamel mineral and pure hydroxyapatite3,4,5). The chemical composition of the tooth enamel varied not only with the tooth type but also with the site of the enamel6,7,8). Owing to the dietary changes, dental caries caused by carbonated beverages has been reported frequently in the recent decades9-11). The biological apatites comprising of the human tooth enamel depicted remarkable deviations in their crystallographic characteristics12,13). Nonetheless, detailed formation and progression mechanisms remain to be clarified.

These conflicts may arise from the fact that the characteristics of tooth enamel are not macro- and micro-structurally similar among individuals and sections of the enamel14). The biological apatites comprising of the human tooth enamel depicted remarkable deviations in their crystallographic characteristics15,16). Therefore, the formation mechanism of dental caries should be studied from the microscopic and not from the macroscopic perspective.

This study aimed to clarify the variable erosive potency of carbonated soft drinks toward individual human teeth enamel from a microscopic physico-chemical perspective using micro-XRD technique.

Materials and Methods

Human Tooth Samples

Artificial caries production was performed using the carbonated soft drink Sprite® (Coca-Cola [Japan] Co., Ltd., Tokyo, Japan), and the durations of control, 1 day, and 7 days were fixed, as suggested by previous studies1,2). Twenty human third molar teeth used in this study were extracted for clinical reasons and pooled in purified water. The teeth se-
Figure 1. Photographs of the human tooth sections.
Photographs of the 4 selected human tooth samples, HE1, HE2, HE3, and HE4.
Cont: untreated, D1: after the soaking duration of 1 day, and D7: after the soaking duration of 7 days. Inset photographs are the two-times enlarged view of the analyzed areas. ※ indicated dissolved enamel, and only the waterproofing bond remained. The arrows and suffix after the sample name represent the micro-XRD analysis region; for instance, HE1-2 is the inner layer of the sample HE1.
lected for this experiment did not have carious lesions. Two longitudinal sections, approximately 0.5 mm thick, were cut from the middle portion of each tooth using a low-speed diamond saw (IsoMet, Buehler Co. Ltd., Lake Bluff, IL, USA). These sections were adhered onto the microscopic cover glass using a waterproofing bond (TAISUI-BOND-SOKKAN, Bijutsu Shuppan Ed-

Figure 2. Micro-XRD patterns of the human tooth enamel samples of untreated and soaked samples. The sample notations are the same as in Figure 1. The principal diffraction peaks are identified with those of the apatitic structure, indicated as bars. Note: additional broaden peaks can be observed (arrowheads).
Artificial caries were produced using a carbonated soft drink. Each bonded section was placed into a separate plastic mesh bag and soaked in a 1.5 L carbonated soft drink bottle for the periods of control, 1 day and 7 days, with the durations being denoted as Cont, D1, and D7, respectively. After the soaking period, the samples were washed in distilled water and placed in a desiccator. The soaked teeth were dissolved with each passing day. The samples were screened on the basis of their macroscopic and microscopic features.
After the soaking experiment in accordance with the preceding experiment. Among the 20 samples examined, 4 typical heavily dissolved samples, denoted as HE1, HE2, HE3, and HE4, were selected and subjected to crystallo-chemical analysis.

The ethics committee of Nihon University School of Dentistry at Matsudo approved this study (approval number: 17EC-015).

Microscopic Observation

A stereomicroscope (Leica M60® and Leica DFC295®, Wetzlar, Germany) was used to observe and photograph all the samples.

Micro-XRD with PSPC (PSPC-micro-XRD)

The crystallographic properties of the outer and inner layers of the samples were analyzed using a micro-XRD instrument (RINT-2500, RIGAKU, Co. Ltd., Tokyo, Japan) equipped with a curved position sensitive proportional counter (PSPC). The curved PSPC captured the XRDs from 0 to 160 degree (2θ) simultaneously. Resolution in practice: the measuring range was 3–160 degree (2θ) to prevent the direct beam.

X-ray was generated as follows: X-ray rotary target: Cu, accelerating voltage: 50 kV, current: 300 mA, instrumental resolution: 0.2 degree (2θ), monochromator: graphite, incident X-ray beam diameter: 100 μm, sample rotation axes: χ, ω, and θ, counting duration: 0.5 h. The obtained XRD data were analyzed with JADE (MDI, Materials Data Inc., Liver-

Table 1. The crystallographic properties of the human tooth enamel before and after soaking treatment in carbonated soft drink.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Duration Day (identifier)</th>
<th>Cont (No.1)</th>
<th>D1 (No.3)</th>
<th>D7 (No.5)</th>
<th>Sample</th>
<th>Duration Day (identifier)</th>
<th>Cont (No.2)</th>
<th>D1 (No.4)</th>
<th>D7 (No.6)</th>
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<tr>
<td></td>
<td>FWHM (300) n.d</td>
<td>0.410</td>
<td>0.439</td>
<td></td>
<td></td>
<td>FWHM (300) 0.367</td>
<td>0.393</td>
<td>0.393</td>
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<tr>
<td></td>
<td>crystallite size 113</td>
<td>208</td>
<td>194</td>
<td></td>
<td></td>
<td>crystallite size 211</td>
<td>218</td>
<td>203</td>
<td></td>
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<tr>
<td></td>
<td>FWHM (300) 0.364</td>
<td>0.401</td>
<td>0.443</td>
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<td>FWHM (300) 0.367</td>
<td>0.423</td>
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<td>202</td>
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<td></td>
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<td>0.386</td>
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<tr>
<td></td>
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<td>0.410</td>
<td></td>
<td></td>
<td>FWHM (300) 0.457</td>
<td>0.406</td>
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<tr>
<td></td>
<td>crystallite size 190</td>
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<td>208</td>
<td></td>
<td></td>
<td>crystallite size 186</td>
<td>210</td>
<td>218</td>
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</tbody>
</table>

The unit for the a- and c-axis length and the crystallite size is Å and for FWHM 2θ degree.
The calculated estimated standard deviation (e.s.d) values for the a- and c-axis length were within 0.005.
The full-width-half-maximum (FWHM) values were for (300), and the sizes were along the c-axis.

cf. HE4-1; HE4, Cont, Outer
more, CA, USA). For comparison, the powder diffraction file (ICDD, International Centre for Diffraction Data) for hydroxyapatite (PDF#09-0432) was used. The unit cell dimensions reflected the ionic substitutions and the crystallinity as the peak broadening. The crystallite size along the a-axis derived from the FWHM(300) value of the human enamel was calculated by JADE.

Results

Among the 20 human tooth samples displaying heavy dissolving reactions macro- and microscopically, 4 are described here to demonstrate the unique reactions occurring in the enamel.

Fig. 1 presents the photographs of the selected human tooth samples HE1, HE2, HE3, and HE4 before the soaking experiment (untreated) of Cont and after the soaking experiment of D1 and D7 durations. The insets photographs are the two-fold magnifications of the analyzed areas. The arrows and the numbers indicate the micro-XRD analyzed regions.

It can be observed from the figure that, as the soaking duration increased from D1 to D7, noticeable morphological changes occurred in comparison with Cont. In the areas marked with ※ in Fig. 1, the enamel had completely dissolved and only the waterproofing bond material remained. In Fig. 1-A, the enamel of Cont is translucent, and the ultrastructure is not visible. The Hunter–Schreger bands can be clearly visualized when compared with Cont and D1. Furthermore, the enamel uncertainty was higher than in Cont. The deficiency of the dental enamel configuration can be witnessed in D7. The dimple of the dissolved enamel can be seen near the dentinoenamel junction. The Hunter–Schlegel bands can be more clearly observed in D7 than in D1. In Fig. 1-B, the Hunter–Schreger bands could be confirmed in Cont. The appearance of the bands did not significantly change in D1. However, enamel uncertainty increased, and some of the enamel surface presented roughness. In D7, the surface properties of the enamel are rougher, and the Hunter–Schreger bands had almost resolved. In addition, the dimple caused by the dissolved enamel can be observed near the dentinoenamel junction. In Fig. 1-C, the Hunter–Schreger bands that were already confirmed in Cont are unclear. The cloudiness of the enamel surface is more prominent in D1 and D7 when compared with that in Cont. The enamel surface of D1 is comparatively smooth. The Hunter–Schreger bands can be seen clearly in D1. However, in D7, the surface of the enamel is slightly roughened, and the Hunter–Schreger bands are indistinct. In addition, a dimple can be noticed near the dentinoenamel junction by enamel elution. In Fig. 1-D, the Hunter–Schreger bands are not discerned in the enamel of Cont. As the soaking duration increased from D1 to D7, the Hunter–Schreger bands became clearer. The dimple near the dentinoenamel junction was not decipherable in D7 and a half or more of the enamel thickness was eluted as a whole.

Fig. 2 portrays the micro-XRD patterns of the outer and inner layers of the HE1, HE2, HE3, and HE4 samples of Cont, D1, and D7. The major intense diffraction peaks in these patterns were almost coincident with those of the apatitic structure, for example, hydroxyapatite (HA). The unit cell dimensions and FWHM (300) were calculated for these apatitic structures (Table 1). In some cases, the additional peaks other than those of the apatitic structures were observed at 4.31 Å (20.6°, 2θ), 3.91 Å (22.75°, 2θ), and 3.55 Å (25.1°, 2θ) as stronger and relatively broader peaks not only in the micro-XRD patterns of the D1 and D7 samples (the inner and outer layers of HE1, HE2, HE3, and HE4) but also in the Cont samples (the outer layers of HE2 and HE3) (arrowheads in Fig. 2). The peak at 3.55 Å may be overlapped with that of the apatitic structures; however, this peak was stronger and broader and always occurred together with the peaks at 4.31 Å and 3.91 Å. The peak at 4.31 Å could be assigned to brushite (DCPD), 4.237 Å, within the error range of the micro-XRD instrument used in this study, but the other strong peaks of brushite were not observed in this study. Therefore, the presence of brushite is questionable. Table 1 and Fig. 3 provide the calculated unit cell dimensions of the human enamel apatite. The a-axis length values of the Cont samples ranged from 9.396 Å to 9.459 Å and the c-axis length values from 6.846 Å to 6.881 Å. All the Cont samples demonstrated a declination of the a-axis length value from the outer to the inner layers (Fig. 3; HE1 Cont a-axis). While the c-axis length values of the HE1 (Cont), HE2 (Cont), and HE3 (Cont) did not show meaningful inclinations, HE4 (Cont) displayed the greatest inclination both in the a-axis and in the c-axis length values among the samples. Fig. 3 also showed changes in the unit cell dimensions during the soaking treatment. With increasing duration, the relationship between the outer and the inner layers became disordered both in the a-axis and the c-axis length values. In the c-axis length, the relationship between the outer and the inner layers seemed to be settled after the duration of 7 days (Fig. 3; D7 c-axis). Fig. 4 demonstrates the changes in the FWHM (300) values of the human enamel apatites during the soaking treatments. The crystallinity was represented as the crystallite size along the a-axis direction and calculated by JADE in this study (Table 1). These FWHM values were altered irregularly along the duration. The D0 samples revealed a decrease in crystallinity and an increase in the FWHM value from the outer to the inner layers (Fig. 4, D0). These associations become disordered with duration. After the duration of D7, the relationship seemed to be settled with larger values in the outer layer and smaller values in the inner layer.

Discussion

This study aimed to clarify whether the chemical processes of dental caries were common throughout the human teeth on the basis that the enamel displays wide diversities in its morphological and chemical properties[14]. The variations in the specific etching behaviors of tooth enamel were reported previously[5,19]. Using micro XRD, the crystallographic properties were analyzed for the outer and inner layers of the human teeth enamels subsequent to soaking in the carbonated soft drink (Sprite®) for Cont, D1, and D7.

This study reconfirmed that the carbonated soft drink Sprite® altered the morphological features of the individual tooth enamels, and gave the following 4 results: i) the unit cell dimensions and crystallinity of the native enamel varied with the layers and the individual teeth; ii) after the soaking experiment, these crystallographic properties of the tooth enamel changed remarkably and exhibited non-uniformity; iii) at the present time, no relationship was inferred between the enamel crystallographic properties and the sensitivity of the carious attack; and iv) the intermediate reaction product was found, although it was not identified in this study. Variations in the occurrence and the amounts of intermediate product reflected the complex chemical and crystallo-chemical reactions between the decalcification solution and the human tooth enamel crystallites.

The carbonated soft drink Sprite® changed the morphological features of individual tooth enamels

From the histopathological observations of the natural human tooth enamel caries, histological structures, such as Retzius lines and prism sheath, are the key structural factors in initiating and/or propagating the carious lesions[20,21]. These facts may be related to the fluctuating carbonate contents of these structures, with the carbonate-rich layers being selectively dissolved by the dilute acids[40]. Robinson et al.[22] and Anatomy
et al.\textsuperscript{29} noted that the chemical components of the normal and carious enamels in humans changed according to the sections and the layers.

Therefore, the noted morphological variations in this study were in accordance with the reports and, hence, should be noted carefully in the study of enamel properties.

\textit{i)} The unit cell dimensions and crystallinity of the untreated enamel varied with the layers and the individual teeth.

The unit cell dimensions of the untreated (D0) human enamel in this study varied as \(a = 9.396 - 9.459 \text{ Å} \) and \(c = 6.846 - 6.881 \text{ Å}\). The previously reported values were as follows: \(a = 9.4563 - 9.4900 \text{ Å}\) and \(c = 6.8982 - 6.930 \text{ Å}\), \(a = 9.45 - 9.62 \text{ Å} \) and \(c = 6.85 - 6.93 \text{ Å}\), \(a = 9.441 \text{ Å} \) and \(c = 6.878 \text{ Å}\), \(a = 9.4555 \text{ Å} \) and \(c = 6.8809 \text{ Å}\), and \(a = 9.441 \text{ Å} \) and \(c = 6.878 \text{ Å}\). At a glance, it is obvious that the values are inconsistent, with the existence of wide variations among them. Notably, each analytical method possesses unit-specific analytical limits, and the results are not directly comparable. However, these wide-ranging results revealed marked variations among the human tooth enamel crystallites.

The unit cell dimensions of apatite reflect its chemical composition\textsuperscript{29}. Therefore, these differing values indicate variations in the chemical composition of the human tooth enamel. The definite composition could not be analyzed in this study because of the limited XRD conditions.

All of the untreated samples signified a declination in the a-axis length value from the outer to the inner layers. In a previous study, a similar tendency of the a-axis dimensions of the enamel apatite was reported\textsuperscript{27}.

Discussions on the crystallite size of human tooth enamel continue. Elliott\textsuperscript{20} reported the crystallite sizes of tooth enamel to be 1600 Å (c-axis) x 410 Å (a-axis), 920 Å (c-axis) x 600 Å (width) as determined by XRD, and 70 x 700 x 263 Å (thickness) x 683 Å (width), at least 1000 Å (c-axis) by electron microscopy. In this study, no direct measurement of the crystallite sizes was performed, but the JADE analyzed results were in accordance with these values (Table 1).

Our results indicate that further accumulation of basic data on individual teeth is needed to elucidate variations in human tooth enamel. For future studies, a more precise analysis is warranted. We plan to examine the apatite crystal structure using crystal structure analysis software, such as by the Rietveld method. More detailed variations in the enamel apatite crystallites will then be clarified.

\textit{ii)} After the soaking experiment, the crystallographic properties of the tooth enamels changed remarkably and depicted non-uniformity.

This study also exposed that the unit cell dimensions of the human tooth enamel changed remarkably upon soaking. Using a synchrotron XRD \textit{in situ} analysis, Sui et al.\textsuperscript{31} reported a decrease in the diffraction intensities as well as changes in the preferred orientations and the crystalline length, that is, in the crystallite size of the human third molar enamel with a lactic acid attack of up to 42.5 h. Their results agreed with our findings. The a-axis length dimension relationship between the outer and inner layers of the human tooth enamel, which was seen in the Cont samples, was not maintained after the soaking treatment. However, the c-axis length dimensions indicated that the relationships were settled as higher in the outer layers than in the inner layers as a whole. A similar relationship was observed in the FWHM values as higher in the outer layers and lower in the inner layers after the period of D7.

These facts suggest that the reactions of the carbonated soft drink with the human enamel were activated in the first few days, which were not determined in this study; nonetheless, after 7 days, these reactions were completed at least from the crystallinity perspective.

\textit{iii)} Presently, no relationship was discerned between the enamel crystallographic properties and the sensitivity of caries attack.

Several investigators reported chemical changes in the mineral compositions of carious teeth or more specifically, carious lesions\textsuperscript{32,33}; a preferential loss of carbonate from the enamel was observed. Suga\textsuperscript{34} confirmed the favored loss of Mg from the lesions. Johansen (1965) reported the occurrence of central holes in the crystals, both in healthy and carious enamels. Vogel and Frank\textsuperscript{35} and Daculsi and Kerebel\textsuperscript{36} documented that these holes developed in the core of the crystals during demineralization. Driessens\textsuperscript{37} suspected the presence of SCOHA, slightly carbonated hydroxyapatite or NCCA, and Na- and CO\textsubscript{3}-containing apatite in the carious lesions, but did not conclusively find evidence for it.

Regrettably, there was no apparent relationship between the crystallographic properties and the sensitivity of the human tooth enamel in this study. Many investigators suspected that the caries tends to start from the core of the enamel crystallites\textsuperscript{37,39}. Gutierrez et al.\textsuperscript{39} tried to prove the correlation between caries risk and XRD results. No such correlation was observed, but the authors demonstrated a possible link between enamel micropore form and carious disposition by employing small-angle X-ray scattering. Using these results, it is expected that detailed basic data could be gathered for clarifying the carious mechanisms.

\textit{iv)} The intermediate reaction product of soaking was detected, but not identified.

The unidentified products were noticed not only in the soaked samples but also in the untreated samples. These additional peaks could not be attributed to any of the calcium phosphates that were reported in the biological systems\textsuperscript{24,32,33}. Several researchers have reviewed the chemistry of dental caries comprehensively. Among the listed references, there is no description of the crystalline forms in carious lesions other than the apatite-series crystals of brushite, monetite, whitlockite and octacalcium phosphate, calcite, and dolomite, including the suspected minerals. Ca\textsubscript{3}Mg(CO\textsubscript{3})\textsubscript{2} and Ca\textsubscript{9}Mg(PO\textsubscript{4})\textsubscript{6}(HPO\textsubscript{4}) have been proposed as the non-apatitic mineral phases in dental caries\textsuperscript{42}. The intermediate product was not identified to be any of the calcium carbonates, such as calcite, aragonite, or vaterite, which are the suspected precursors in the hard tissue formations of the human body\textsuperscript{42-45}. These unidentified peaks exhibited remarkable broadening, indicating extremely low-grade crystallinity. Presently, it was not concluded whether these peaks are from a single phase. Therefore, we called the substance(s) unidentified material(s). These intermediate products should be analyzed in detail. Recent progress in analytical techniques, such as those using synchrotron\textsuperscript{46}, may help in exploring these unidentified materials in the enamel and other hard tissues.

This study clearly asserted the variations in the crystallographic properties of the human tooth enamel and further revealed that the samples did not react in the same manner, resulting in the differing apatitic structures after soaking. These variations in the manner of the reaction and the presence of intermediate products require reconsideration of the generally accepted caries protection methods that serve as common standards for all individuals and tooth enamel types.

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Conflict of Interest
The authors have declared that no COI exists.

References


