A BIOPHYSICAL STUDY ON CHARACTERIZATION OF FISH TEETH BY RAMAN-MICROSPECTROMETRY AND MICROFLUOROMETRY

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To make a biophysical standard for histological identification of fish teeth, fluorescence and Raman-spectra are employed for typical teeth of various fish, such as the sea-bream, shark, trout, and hagfish in comparison with human teeth. The tooth tissues of sea-bream, mainly containing hydroxy-apatite, resemble those of human teeth. In the teeth of the trout and hagfish, no difference can be found between the surface and deep layers and they appear like decalcified human cement. Shark teeth, rich in fluoroapatite, are much different from both human enamel and dentin. According to the similarity of Raman-band patterns, it is possible to arrange them in order from trout to sea-bream through the human enamel and finally to the shark. These suggest that the structural characteristics of teeth in the fish kingdom do not coincide with differentiation of human tooth tissues.

Various crystalline structures of vertebrate teeth range from an undifferentiated type to such a differentiated type identified with three hard tissues as the human tooth. This variety was evident in various types of teeth in the fish kingdom. The most undifferentiated type has been noted in the teeth of lower vertebrate species such as eel, while multiple compositions of differentiated hard tissues were found in the sea-bream tooth. According to the morphological basis of these fish teeth, hard tissue composites have been classified empirically into the enameloid, dentin, vasodentin etc. as well as those in higher vertebrates.

On the other hand, some biological natures of mammalian teeth have been reported by using modern optical techniques. Biophysical studies on lower vertebrate teeth are still lacking. We attempt here to compare componental tissues of various fish teeth and to know how to classify those hard tissues in contrast with human tooth tissues by the use of microfluorometry and Raman-microspectrometry.

MATERIALS AND METHODS

Fresh teeth obtained from the sea-bream (*Pagrosomus major*, Temminck et Schlegel, *Sparus swinhonis*, Günter), shark (*Carcharvinus* sp.), rainbow trout (*Salmo irideus*, Gibbons) and Pacific hagfish (*Eptatretus burgeri*, Girard) were compared with human teeth (Figs. 1a–d). The teeth were stored in cold ethanol and cut with a dentistry engine and the cut face was polished on a rough whetstone and then on five agate-whetstones by a dropwise supply of water until the surface became completely smooth. Some specimens were polished on both sides of the surface for micro-fluorometry. The tooth was ground sagittally in the bucco-lingual direction without thermal decomposition. Thus, tooth slices were mounted on the slide glass with non-fluorescent glycerol and air bubbles were removed from the slide *in vacuo*. The coverslip was fixed with manicure fluid and the specimen was prepared for measurement at indicated areas under a microfluorometer devised by us. As previously reported by Horibe (5) and Katsura (6), the absorption was measured at 280 nm (A280) under a microspectrophotometer (Zeiss, UMSP-1) and the relative fluorescence intensity (RF) was obtained in reference to A280. To facilitate the RF, a ratio of the emission to A280 per unit round area of 20 μm of diameter at the microscopical image field was measured in different component tissues of fish teeth. Raman-microspectrometry was made on a round area of 50 μm in diameter projected at 5145 Å of argon ion laser (Spectra Physics Model 164) with a spectrophotometer (JASCO Model R-800, Japan Spectroscopic Co.). Thus, spectra were measured on the cut face of tooth specimens at 80–100 mW in combination with interference filter. The scattering angle of the projection laser beam on the plane was kept at an angle of 15° in all the specimens.

RESULTS

I. Relative fluorescence (RF) of fish teeth

The tooth is a complex organ of various tissue composites. There are many varieties in the mode of tooth construction of the fish species such as the vasodentin, osteodentin and orthodentin (16). On the cut surface of the trout tooth, the surface layer (S) is defined from the deep layer (denticle) which is an incomplete dentin as described as an example of vasodentin (Fig. 1d). The development of dentin has been described elsewhere (7). In the osteodentin found in shark species, the surface layer is often multiple in the edge shape and a trabecular bone develops in the deep layer. On the other hand, in the teleost as in the sea-bream, the

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- FIG. 1. a: Cut-face of the sea-bream tooth. $\times 100$
Ca: cap (enameloid), S: surface layer, D: deep layer.
b: Cut-face of the human tooth. $\times 4$
E: enamel, De: dentin, C: cement.
c: Cut-face of the shark tooth. $\times 8$
S: surface layer with serratus edge
D: deep layer.
d: Cut-face of the trout tooth. $\times 20$
S: surface layer.

enameloid (Ca) is identified in the outer top of the surface layer and the travecular dentin in the deep layer resembles a mode of orthodentin. This structure appears to be similar to that of human tooth consisting of the enamel, dentin etc. These layers can be identified on the cut face of tooth slices under a microscope. Ac-

FIG. 1.

FIG. 2. Relative fluorescence (RF: F_{440}/A_{280}) of fish teeth with reference to that of human teeth.

E: enamel, De: dentin, C: cement, Ca: cap,

S: surface layer, D: deep layer, bar: standard deviation of 10 samples, circles without bar: single samples.

cording to the preliminary studies (5), the fluorescence of tooth tissues has been measured in reference to the absorption at 280 nm (A_{280}). As the measuring site varies in thickness, the fluorescence intensity can be measured as an RF at 440 nm (F_{440}) to A_{280} per unit area by the measuring spot of 20 μm in diameter. Generally on emission spectra of teeth, a maximum fluorescence was observed at 440 nm,

FIG. 3. Phosphate bands of hydroxyapatite in Raman-spectrum.

when the human tooth was excited with 366 nm. The relative fluorescence of the human enamel was less than that of the dentin or cement (Fig. 2). When it was compared with the fluorescence of fish teeth, most of the RF was similar to the level of human enamel. This was one fifth of the fluorescence intensity of human dentin. The RF was lowest especially in the surface layer (S) and cap (Ca) of the sea-bream. In the other fish species, even in the hagfish, the RF of the surface and deep layers was as low as that of human enamel.

II. Raman-spectra of tooth composites

Teeth are rich in calcium, magnesium, fluoride, and phosphorus which compose hydroxyapatite, $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_9]$; HOAP. The HOAP represents four vibration modes of PO_4^{3-} in Raman-spectra. The bands at 432, 595, 965 and 1045 cm^{-1} correspond to ν_2 , ν_4 , ν_1 , and ν_3 modes (Fig. 3).

The ν_1 was compared between purified HOAP and fluoroapatite (FAP). The ν_1 band of FAP shifted a bit to the higher side than 960 cm^{-1} of HOAP (Fig. 4a). This transition of the band was clearly shown in the difference spectrum subtracting FAP from HOAP (HOAP-FAP). No shift of ν_1 was observed among the human enamel, dentin, and cement, revealing constancy at about 965 cm^{-1} .

As in Figs. 3 and 4, the ν_3 bands were subdivided into b at 1030 cm^{-1} , d at 1045 cm^{-1} , f at 1075 cm^{-1} in HOAP, and c at 1040 cm^{-1} in FAP in which band d appeared to be lower than that of HOAP (Fig. 4b). In the human enamel, the ν_3 -b

FIG. 4. Comparison between bands of hydroxyapatite (HOAP) and fluoroapatite (FAP).

a: Shift of ν_1 band of FAP.

b: Difference between ν_3 -c (FAP) and ν_3 -d (HOAP).

FIG. 5. Bands in human tooth tissues.

a: Enamel with or without EDTA treatment.

b: Decrease of $\nu_3\text{-c}$ in the cement.

and -d were identified as in the HOAP (Fig. 5a), and the $\nu_3\text{-e}$ was changed a little from the $\nu_3\text{-f}$ of HOAP. After treatment with EDTA to remove calcium from the human enamel surface, the $\nu_3\text{-e}$ and -d became sharper, as the spectrum of HOAP (Fig. 4b).

In the human dentin (Fig. 5b), the $\nu_3\text{-e}$ band of the enamel was unchanged and the $\nu_3\text{-c}$ and -a bands were added instead of the $\nu_3\text{-b}$ and -d bands of the enamel. In contrast with the dentin bands, they were not distinct in the human cement.

III. Raman-spectra of fish teeth

The structure of teeth is most organized in the sea-bream in which the outer

FIG. 6. Comparison of bandings between the surface layer (S) and deep layer (D) of sea-bream teeth.

From a (ν_1 band) and b (ν_3 bands), the S resembles FAP nature more than the D.

cap (enameloid, Ca), surface layer (S) and deep layer (orthodontin, D) are identified (Fig. 1a). The ν_1 of S was a band at 965 cm^{-1} which nearly corresponded to that of the human enamel. But, the ν_1 band of D shifted a bit to the lower side (Fig. 6a). The ν_3 -a, -c, -d, and -f bands were observed on S (Fig. 6b), which most closely resembled the modes of FAP (Fig. 4b). However, the spectrum of D (Fig. 6b) looked like that of human enamel. This suggests that D contains HOAP as well as human enamel.

The spectra were compared between the deep layers of the shark and sea-bream. There was a difference in ν_1 bands. The ν_1 of the shark shifted a bit to the higher side (Fig. 7a).

In the ν_3 bands, the ν_3 -a, -c, -d, and -e were observed on the deep layer of the shark tooth (Fig. 7b). This spectral profile looks that of the S of sea-bream, resembling the presence of FAP.

In the tooth of the hagfish, an incomplete tooth, there were only two signals of the ν_3 -a and -e (Fig. 8a). The spectral profile from 800 to 1800 cm^{-1} was similar to that of the mouse masseter muscle (Fig. 8b), so that it is unlikely that HOAP or FAP is incorporated in the tooth. However, the maximal band in the surface of trout tooth was much broader than ν_1 of human enamel (Fig. 9a). The ν_3 -a, -b and -e bands were found in the S (Fig. 9b) which resembled the D of the sea-bream (Fig. 6b). From these, the tooth of the hagfish seems to be a papillary prominence

FIG. 7. Comparison of bandings of the deep layer (D) of teeth between the shark and sea-bream. ν_3 -c and -d bands are much more distinct in the shark.

FIG. 8. Raman-spectra of the hagfish tooth.

a: ν_3 -a and -e bands.

b: General spectral profile looks like the mouse masseter muscle.

which differs from the trout tooth capped with the HOAP layer.

DISCUSSION

From previous studies (3-5, 8, 9), autofluorescence was known to distribute generally in human teeth. Fluorescence can be expected to be found in a wide range of vertebrates. On the evolutionary basis of teeth, fish teeth may range in wide varieties of histogenesis from the teleost to the hagfish. By using these varieties of fish teeth, fluorescence-and Raman-spectra can be compared in the present study.

In such lower vertebrates as fish, autofluorescence was mostly similar in RF without any tissue varieties. The RF did not vary between the surface layer and deep layer of teeth, and was mostly maintained at a low level as in human enamel (Fig. 2). In the human tooth, however, the RF varied tissue by tissue, as the RF of dentin was several times higher than that of enamel. On the contrary, fluorescence variety was minimal in the fish teeth. The reason why a higher fluorescence exists in the human tooth is not known, but it may depend on the accumulation of fluorescent substance in such bony tissue as human dentin and cement which seem to be lacking in the lower vertebrate teeth.

O'Shea (11) reported that the Raman-spectra of apatites reflected the crystalline structure and its decalcification. In addition, Raman-spectra were changed in relation to reconstruction of recrystallized hydroxyapatite (9, 10, 14, 15). Thus, Raman-spectrometry is useful to show phosphate vibration modes, as the tooth contains hydroxyapatite. The ν_1 and ν_3 bands of phosphate vibration were confirmed as suitable markers of apatite conformation. From this consideration,

FIG. 9. A sequential change of spectra in various dental surfaces (S) of fish with reference to the human enamel.

a: Band shifts to the higher side in the Ca of sea-bream and a little bit in the shark.

b: An order of the similarity of ν_3 bands of various teeth. It is reasonable to arrange in order from trout, human, sea-bream to shark.

Raman-spectra were observed on various tooth tissues of different fish species. Fish teeth have been regarded as the product of evolutionary mystery in the vertebrate phylogeny. There has been a discontinuous line of evolution in morphology and function. To look at some physical nature of fish teeth, comparative studies on Raman-spectra have been attempted by the authors (9). To this extent, spectral varieties were found in various typical teeth of fishes, such as sea-bream (teleost), shark, trout, and hagfish. The cap of the teleost tooth indicates the presence of FAP, while the deep layer contains such HOAP as in the human enamel (Fig. 9a). The relation of fluoride to enamloid was suggested by Suga (12, 13). It is highly possible to incorporate FAP to the cap and surface layers of teleost teeth.

From our data, the shark tooth, known as an osteodentin, seems to be rich in FAP which agrees with the reports by Fosse *et al.* (1) and Goto (2). The tooth of the trout, consisting of the vasodentin, is not clearly defined between the surface and deep layers. But, the spectrum resembles that of human cement. Furthermore, the tooth of the hagfish, a trabecular denticle, looks like a papillary prominence without any HOAP signal. On various dental surfaces which may represent more crystalline structures of various fish species, there is a sequential change of Raman-

spectra of ν_3 bands (Fig. 9b). According to the similarity of the band patterns, it is possible to arrange the trout to the sea-bream in order through the human enamel and finally to the shark. If it was reasonable to arrange this way, the dental surface of the hagfish remains primitive and that of the trout is secondary in developing crystalline apatite. The signal of FAP is developed in the sea-bream tooth and most developed in the shark tooth. Human enamel is, however, situated between the trout and sea-bream teeth on the spectral basis. This hypothetical concept may need further phylogenetic studies on the crystalline structure of lower vertebrates.

Thus, Raman-spectra suggest that the teeth of fish kingdom are not simply classified along their morphology. They are multiplied in chemical and morphological manifestations. In comparison with the orthodontin of sea-bream the physical nature of the human tooth is far different in the lack of FAP and in the presence of high fluorescence of the dentin. The teeth of the other lower vertebrates are very different from the human tooth. The changes in such physicochemical natures as Raman- and fluorescence-spectra may take part in future studies on the phylogeny of hard tissues.

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