

Chapter 18

Immunolocalization of Enamel Matrix Protein-Like Proteins in the Tooth Enameloid of Actinopterygian Bony Fish



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Abstract Tooth enameloid in bony fish is a well-mineralized tissue resembling enamel in mammals. It was assumed that the dental epithelial cells are deeply involved in the formation of enameloid. However, unlike enamel matrix which fully consists of several ectodermal enamel matrix proteins (EMPs), whether enameloid matrix contains ectodermal EMPs has been debated for a long time. In the present study, transmission electron microscopy-based immunohistochemical examinations, using the protein A-gold method with antibodies and antiserum against mammalian amelogenin, were performed in order to search for EMP-like proteins in the cap enameloid of basic actinopterygians, *Polypterus* and gar. Positive immunoreactivity was detected in the cap enameloid matrix just before the appearance of many crystallites along collagen fibrils, indicating that the cap enameloid contains EMP-like proteins. Immunolabelling was usually found along the collagen fibrils but was not seen on the electron-dense fibrous structures. Therefore, it is conceivable that the ectodermal EMP-like proteins in cap enameloid are involved in crystallite formation along collagen fibrils.

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18.1 Introduction

Cap enameloid is a well-mineralized tissue that occupies the tooth tip of actinopterygians (ray-finned bony fish) and corresponds to mammalian tooth enamel. Enameloid is an attractive tissue for elucidating biomineralization in fish and evolution of dental hard tissues in early vertebrates because its process of formation is different from that of mammalian enamel. Unlike amelogenesis in mammals, enameloid is formed by both odontoblasts and dental epithelial cells. Most of the organic matrix, including abundant collagen fibrils, is provided by odontoblasts during the matrix formation stage of enameloid, and many odontoblast processes are present in the matrix. Therefore, enameloid is homologous to the outermost layer of dentin. Dental epithelial cells are mainly engaged in the degeneration and removal of organic matrix and in the formation of large crystals during the maturation stage of enameloid formation (Sasagawa and Ishiyama 2005a, b).

The pattern of mineralization in enameloid is accordingly different from that in enamel. The mineralization process in cap enameloid is summarized as follows. During the matrix formation stage, many matrix vesicles (MVs) and electron-dense fibrous structures (EDFSs) that are probably derived from MVs (Sasagawa and Ishiyama 2003) are observed in the collagen-rich enameloid matrix. MVs are often the first sites at which crystallites appear. Then, many slender crystallites are deposited along the collagen fibrils in the enameloid, in a manner similar to the dentin and bone, during the mineralization stage. During the next maturation stage, the dental epithelial cells remove the degenerated organic matrix, including collagen fibrils, from enameloid and supply inorganic ions to boost crystal growth, like the maturation stage of amelogenesis in mammals. In matured enameloid, bundles are formed from thick elongated crystals that become twisted with each other, which are thought to keep the structure of degenerated collagen fibrils. However, the presence of ectodermal EMPs in enameloid is an unsolved question, in spite of expected active participation of the inner dental epithelial (IDE) cells.

Polypterus and gar possess both collar enamel in their teeth and ganoine in the scales, other than cap enameloid (Fig. 18.1a). EMP-like proteins were detected in collar enamel and the ganoine layer by means of immunohistochemistry (Sasagawa et al. 2012, 2014, 2016). It was assumed that the collar enamel and the ganoine are an ectodermal element, and EMP-like proteins play an important role in biomineralization in these tissues. Therefore, those species might be a suitable model for examining the localization of EMP-like proteins in cap enameloid. However, available data are limited concerning the ectodermal EMP-like protein in cap enameloid of *Polypterus* and gar. Concerning gar, transmission electron microscopy (TEM)-based immunohistochemistry recently detected EMP-like proteins in enameloid matrix (Sasagawa et al. in prep). In the present preliminary study, we report the fine structure of the initial mineralization and immunolocalization of EMP-like proteins in the enameloid matrix of *Polypterus*, in addition to gar.

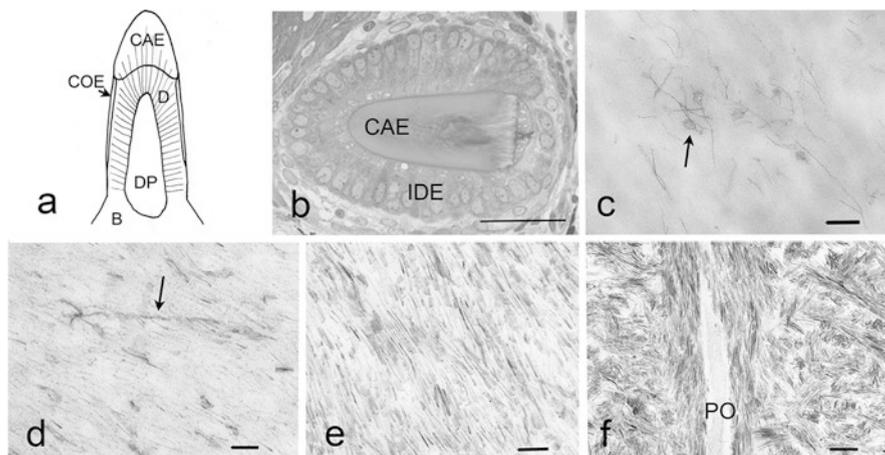


Fig. 18.1 (a) Schematic sketch of the structure of erupted teeth in *Polypterus* (Modified after Sasagawa et al. 2013). (b–f) Light (b) and transmission electron (c–f) micrographs showing enameloid formation during the early stage of mineralization (b, c) and the late stage of mineralization (d–f), in *Polypterus*. (b) Histological section of a tooth germ stained with TB. Mineralization has not started yet. (c) EDFSs that may originate from MV (arrow). (d) Initial fine crystallites found on EDFSs (arrow) and collagen fibrils. (e) Crystallites oriented along the collagen fibrils. (f) Slender crystallites form bundles and seem to align in the direction of collagen fibrils. *B* bone, *CAE* cap enameloid, *COE* collar enamel, *D* dentin, *DP* dental pulp, *IDE* inner dental epithelium, *PO* a process of odontoblast. Bar = 50 μm (b), 200 nm (c, f), 100 nm (d, e)

18.2 Materials and Methods

The actinopterygian fish *Polypterus*, *Polypterus senegalus* (three specimens, total length 9.5–19 cm), and gars, *Lepisosteus oculatus* (three specimens, total length 16–55 cm), were used in the present study. Local university animal care committees approved the euthanasia and all other animal procedures.

We subjected the tooth germs during the stages of enameloid formation to TEM observations and TEM-based immunohistochemical examinations using antibodies against mammalian amelogenins (AMEL). An affinity-purified, polyclonal anti-27 kDa bovine AMEL antibody (BAA) (Shimokawa et al. 1984) and anti-25 kDa porcine AMEL antiserum (PAA) (Uchida et al. 1989, 1991) were used in the present study. In previous studies, positive immunoreactivity with the anti-BAA and anti-PAA has been detected in the collar enamel of teeth and ganoine of ganoid scales in gar and the collar enamel of teeth in *Polypterus*, respectively (Sasagawa et al. 2012, 2014, 2016). For the immunohistochemical analyses, jaws containing tooth germs were placed in 4% paraformaldehyde-0.2% glutaraldehyde fixative (0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4) for 3 h at 4 °C. The specimens were then dehydrated with N, N-dimethylformamide and embedded in LR-White resin (LR-W, London Resin, Reading, UK) at –20 °C. The protein A-gold (PAG) method was employed for immunohistochemical analyses.

For TEM immunohistochemistry, ultrathin sections of the tooth germs obtained from LR-W resin blocks were mounted on nickel grids. The specimens were floated on a drop of 1% NGS for 15 min and then incubated overnight on a drop of the relevant antibody or antiserum diluted 1:200–1:400 with BSA-PBS. The sections were then washed and transferred onto a drop of PAG conjugate (5 nm gold, EMPAG5, BBI) diluted 1:10 with BSA-PBS for 30 min after being treated with 1% NGS for 10 min. These sections were stained with platinum blue (TI blue, Nissin EM, Tokyo, Japan) and lead citrate (TI-Pb) and often stained with phosphotungstic acid (PTA) in addition to TI-Pb (TI-Pb-PTA) and then examined in a TEM (JEM-1010, JEOL). Negative controls were performed by incubating sections in PBS lacking antibody or antiserum, or in normal rabbit serum (NRS) instead of antibody or antiserum. For TEM studies, ultrathin sections were mainly stained with lead citrate alone and examined using a TEM. Semi-thin sections that had been stained with 0.1% toluidine blue (TB) were also prepared for light microscopy. Details of the methods were described in previous reports (Sasagawa et al. 2012, 2014, 2016).

18.3 Results

Formation of cap enameloid is divided into three stages, namely, matrix formation, mineralization and maturation, according to morphological studies. In the present study, we mainly examined the stage of mineralization (Fig. 18.1b).

18.3.1 Initial Mineralization of Enameloid in *Polypterus*

In the TEM observation, abundant collagen fibrils were visible in the enameloid matrix during the early stage of enameloid mineralization. Many EDFSs and a few MVs were also observed in the enameloid matrix. There were no crystallites in the EDFSs, MVs and collagen fibrils, during the early stage (Fig. 18.1c). During the late stage of mineralization, fine, slender crystallites were found in both the EDFSs and collagen fibrils (Fig. 18.1d). In area where mineralization process is more advanced, many marked slender crystallites accumulated along the collagen fibrils (Fig. 18.1e) and formed bundles that aligned in the direction of the collagen fibrils (Fig. 18.1f). Afterwards, the crystallites increased in size, associated with the degeneration and removal of collagen fibrils.

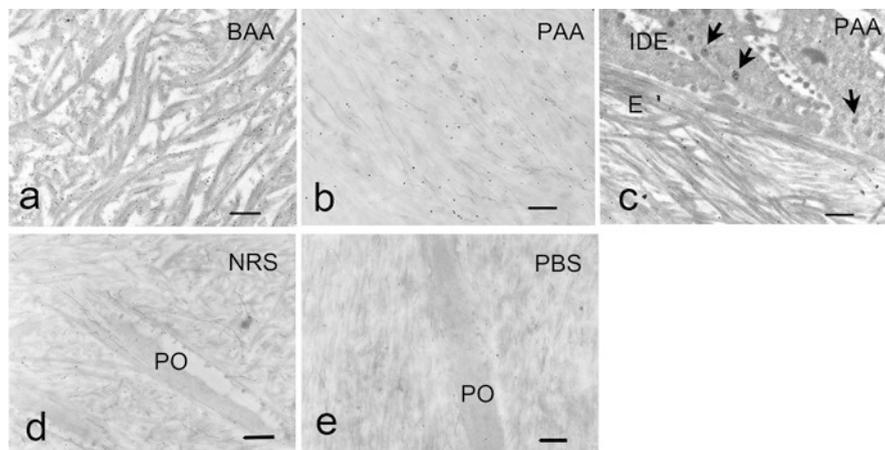


Fig. 18.2 Transmission electron micrographs of immunohistochemistry in gar, PAG method using an anti-bovine AMEL antibody (BAA) and an anti-porcine AMEL antiserum (PAA). **(a)** Many PAG particles are found on the collagen fibrils, BAA, stained with Ti-Pb-PTA. **(b)** Few PAG particles are seen on the electron-dense fibrous substances, PAA, Ti-Pb. **(c)** PAG particles are found in the granules (arrows) in the IDE cells, PAA, Ti-Pb-PTA. **(d, e)** Control sections incubated in NRS instead of antibodies **(d)** or in PBS lacking antibodies **(e)**, Ti-Pb. *E* enameloid, *PO* a process of odontoblast. Bar = 500 nm **(a–e)**

18.3.2 Immunohistochemical Localization of EMP-Like Proteins in Enameloid Matrix

18.3.2.1 Gar

During the early stage of mineralization, a number of PAG particles were observed in the enameloid matrix, in which crystallites did not appear yet. Most of the PAG particles were observed on the collagen fibrils (Figs. 18.2a, c). The EDFSSs showed no immunoreactivity (Fig. 18.2b). Only a few PAG particles were seen in pre dentin. In the IDE cells, electron-dense granules in the distal cytoplasm often contained PAG particles (Fig. 18.2c). Only a few PAG particles were seen in the negative control sections (Fig. 18.2d, e). During the late stage of mineralization, when many fine crystallites were visible along collagen fibrils, only a few PAG particles were found in the enameloid. During the former stage of matrix formation and the subsequent stage of maturation, little immunolabelling by the antibodies was found in the tooth germs (data not shown).

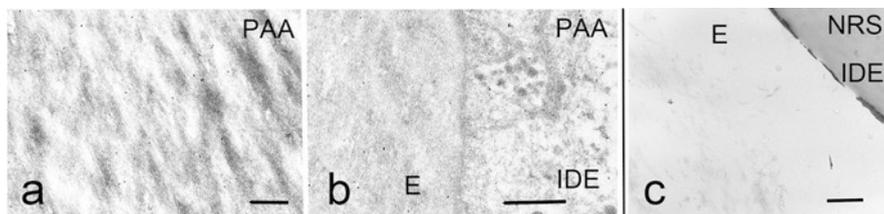


Fig. 18.3 Transmission electron micrographs of immunohistochemistry in *Polypterus*, PAG method using an anti-porcine AMEL antiserum (PAA), stained with Ti-Pb-PTA. (a) PAG particles are found on the collagen fibrils in the enameloid. (b) PAG particles are also visible in the granules of the IDE cells (IDE). (c) Control sections incubated in NRS instead of antibodies, in the early stage of enameloid maturation, Ti-Pb. Bar = 500 nm (a-c)

18.3.2.2 *Polypterus*

In the enameloid matrix during the early stage of mineralization, many PAG particles were found along the collagen fibrils (Fig. 18.3a). The EDFs exhibited no immunoreactivity. Immunoreactivity was also detected in the granules of IDE cells (Fig. 18.3b). During the late stage of mineralization, in which many slender crystallites accumulated along the collagen fibrils, immunolabelling was scarcely found in the enameloid (data not shown). Only weak labelling was visible in the negative control sections by NRS (Fig. 18.3c, Sasagawa et al. 2012)

18.4 Discussion

Positive immunolabelling by the antibodies against mammalian AMEL was detected in the enameloid matrix during the early stage of enameloid mineralization. The results indicated that EMP-like proteins are present in the matrix of enameloid in *Polypterus* and gar. Immunoreactivity was also found in the granules of IDE cells, indicating that EMP-like proteins were secreted from IDE cells. During the stage of enameloid matrix formation, immunolabelling was hardly found in enameloid matrix. EMP-like proteins might exist in the matrix just before the appearance of crystallites along collagen fibrils in the enameloid mineralization stage. Immunoreactivity was scarcely detected in the mineralizing enameloid matrix, in which fine crystallites were deposited along collagen fibrils, during the late stage of mineralization, suggesting that the EMP-like proteins had degenerated or had been removed. EMP-like proteins are probably present for a short period during the stage of mineralization (Fig. 18.4).

PAG particles were mainly observed on collagen fibrils in the enameloid. It is possible that the EMP-like proteins are involved in mineralization along the collagen fibrils in enameloid, because the initial crystallites appeared in collagen fibrils in the subsequent substage. On the other hand, EDFs exhibited no immunolabelling. It is likely that EDFs are not the structure that contains EMP-like proteins. In

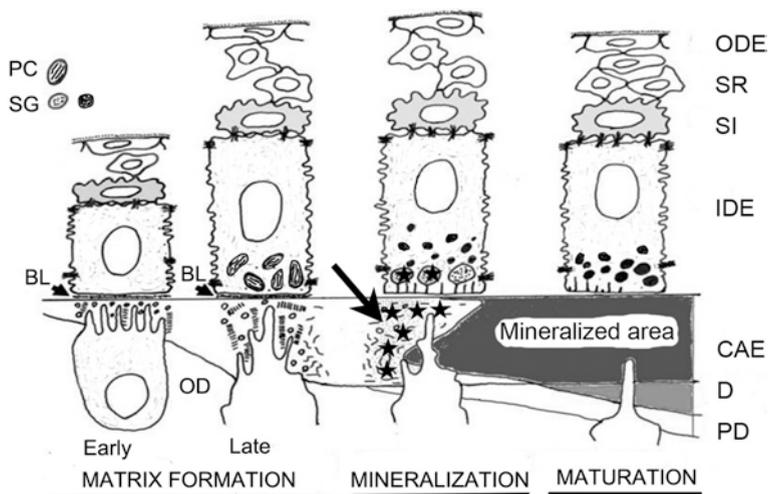


Fig. 18.4 Schematic drawings showing IDE cells during three stages of enameloid formation in gar (Modified from Sasagawa and Ishiyama 2005a). The long arrow and solid stars indicate positions of positive immunoreactivity for anti-mammalian AMEL antibodies. *BL* basal lamina, *CAE* cap enameloid, *D* dentin, *IDE* inner dental epithelial cells, *OD* odontoblasts, *ODE* outer dental epithelial cells, *PC* procollagen granule, *PD* predentin, *SG* secretory granule, *SI* stratum intermedium, *SR* stellate reticulum

a previous morphological study using TEM (Sasagawa and Ishiyama 2003), it was assumed that the EDFs were the site of initial mineralization in cap enameloid. However, in the present TEM study, fine crystallites seemed to appear at both the EDFs and collagen fibrils simultaneously. Because this phenomenon was observed in the enameloid matrix of gar (Sasagawa et al. [in prep](#)), this initial mineralization process in the enameloid seems to be very similar in *Polypterus* and gar. Even if the EDFs are one of the sites of initial mineralization, the EDFs might not be related to crystallite formation along the collagen fibrils in enameloid.

In mammals, AMEL occupies approximately 90% of EMPs. It is assumed that AMEL plays an important part in producing the structure of the enamel layer and to encourage crystal growth during amelogenesis. In *Polypterus* and gar, EMP-like proteins have been detected in the collar enamel and ganoine by several immunohistochemical studies using anti-mammalian AMEL antibodies (Ishiyama et al. 1999; Sasagawa et al. 2012, 2014, 2016; Zylberberg et al. 1997). According to recent genetic analyses, however, gars do not have an AMEL gene, but ameloblastin, enamelin, and many other secretory calcium-binding phosphoprotein (SCPP) genes are present and are expressed in both teeth and scales (Qu et al. 2015; Braasch et al. 2016). It is likely that the EMP-like proteins in actinopterygian fish have epitopes similar to mammalian AMEL, and the anti-mammalian AMEL antibodies may have cross-reacted with these proteins. It is probable that the EMP-like proteins detected in enameloid matrix are SCPPs (Kawasaki et al. 2017).

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Conflict of Interest The author declares no conflicts of interest associated with this manuscript.

References

- Braasch I, Gehrke AR, Smith JJ, Kawasaki K, Manousaki T, Pasquier J, Amores A, Desvignes T, Batzel P, Catchen J, Berlin AM, Campbell MS, Barrell D, Martin KJ, Mulley JF, Ravi V, Lee AP, Nakamura T, Chalopin D, Fan S, Weisel D, Canestro C, Sydes J, Beaudry FEG, Sun Y, Hertel J, Beam MJ, Fasold M, Ishiyama M, Johnson J, Kehr S, Lara M, Letaw JH, Litman GW, Litman RT, Mikami M, Ota T, Saha NR, Williams L, Stadler PF, Wang H, Taylor JS, Fontenot Q, Ferrara A, Searle SMJ, Aken B, Yandel M, Schneider I, Yoder JA, Volf J-N, Meyer A, Amemiya CT, Venkatesh B, Holland PWH, Guiguen Y, Bobe J, Shubin NH, Palma FD, Alfoldi J, Lindblad-Toh K, Postlethwait JH (2016) The spotted gar genome illuminates vertebrate evolution and facilitates human-teleost comparisons. *Nat Genet* 48:427–437
- Ishiyama M, Inage T, Shimokawa H (1999) An immunocytochemical study of amelogenin proteins in the developing tooth enamel of the gar-pike, *Lepisosteus oculatus* (Holostei, Actinopterygii). *Arch Histol Cytol* 62:191–197
- Kawasaki K, Mikami M, Nakatomi M, Braasch I, Batzel P, Postlethwait JH, Sato A, Sasagawa I, Ishiyama M (2017) SCPP genes and their ancestors in gar: rapid expansion of mineralization genes in osteichthyans. *J Exp Zool (Mol Dev Evol)* 328B:645–665
- Qu Q, Haitina T, Zue M, Ahlberg PE (2015) New genomic and fossil data illuminate the origin of enamel. *Nature* 526:108–111
- Sasagawa I, Ishiyama M (2003) Fine structural observations of the initial mineralization during enameloid formation in gar-pikes, *Lepisosteus oculatus*, and polypterid, *Polypterus senegalus*, bony fish. In: Kobayashi I, Ozawa H (eds) *Biomineralization (BIOM2001)*; formation, diversity, evolution and application. Proceedings of the 8th international symposium on biomineralizations. Tokai University Press, Kanagawa, pp 381–385
- Sasagawa I, Ishiyama M (2005a) Fine structural and cytochemical mapping of enamel organ during the enameloid formation stages in gars, *Lepisosteus oculatus*, Actinopterygii. *Archs Oral Biol* 50:373–391
- Sasagawa I, Ishiyama M (2005b) Fine structural and cytochemical observations on the dental epithelial cells during cap enameloid formation stages in *Polypterus senegalus*, a bony fish (Actinopterygii). *Connect Tissue Res* 46:33–52
- Sasagawa I, Yokosuka H, Ishiyama M, Mikami M, Shimokawa H, Uchida T (2012) Fine structural and immunohistochemical detection of collar enamel in the teeth of *Polypterus senegalus*, an actinopterygian fish. *Cell Tissue Res* 347:369–381
- Sasagawa I, Ishiyama M, Yokosuka H, Mikami M (2013) Teeth and ganoid scales in *Polypterus* and *Lepisosteus*, the basic actinopterygian fish: an approach to understand the origin of the tooth enamel. *J Oral Biosci* 55:76–84
- Sasagawa I, Ishiyama M, Yokosuka H, Mikami M, Shimokawa H, Uchida T (2014) Immunohistochemical and western blot analyses of collar enamel in the jaw teeth of gars, *Lepisosteus oculatus*, an actinopterygian fish. *Connect Tissue Res* 55:225–233
- Sasagawa I, Oka S, Mikami M, Yokosuka H, Ishiyama M, Imai A, Shimokawa H, Uchida T (2016) Immunohistochemical and western blot analyses of ganoine in the ganoid scales of *Lepisosteus oculatus*, an actinopterygian fish. *J Exp Zool (Mol Dev Evol)* 326B:193–209

- Sasagawa I, Ishiyama M, Yokosuka H, Mikami M, Oka S, Shimokawa H, Uchida T (in prep) Immunolocalization of enamel matrix protein-like proteins in the tooth enameloid of spotted gar, *Lepisosteus oculatus*, an actinopterygian bony fish
- Shimokawa H, Wassmer P, Sobel ME, Termine JD (1984) Characterization of cell-free translation products of mRNA from bovine ameloblasts by monoclonal and polyclonal antibodies. In: Fearhead RW, Suga S (eds) Tooth enamel IV. Elsevier, Amsterdam, pp 161–166
- Uchida T, Tanabe T, Fukae M (1989) Immunocytochemical localization of amelogenins in the deciduous tooth germs of the human fetus. *Arch Histol Cytol* 52:543–552
- Uchida T, Tanabe T, Fukae M, Shimizu M, Yamada M, Miake K, Kobayashi S (1991) Immunochemical and immunohistochemical studies, using antisera against porcine 25 kDa amelogenin, 89 kDa enamelin and the 13-17 kDa nonamelogenins, on immature enamel of the pig and rat. *Histochemistry* 96:129–138
- Zylberberg L, Sire J-Y, Nanci A (1997) Immunodetection of amelogenin-like proteins in the ganine of experimentally regenerating scales of *Calamoichys calabaricus*, a primitive actinopterygian fish. *Anat Rec* 249:86–95

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