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The Process of Cornification in the Horny Teeth of the Lamprey Involves Proteins in the Keratin Range and Other Keratin-Associated Proteins

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Lorenzo Alibardi and Anna Segalla (2011) The process of cornification in the horny teeth of the lamprey involves proteins in the keratin range and other keratin-associated proteins. *Zoological Studies* **50**(4): 416-425. A microscopic and electrophoretic study was conducted on the horny teeth of the lamprey to increase our knowledge of the process of cornification. Electron-dense bundles of keratin but no keratohyaline-like granules accumulated in the cytoplasm of transitional cells that were incorporated in the dense stratum corneum of the tooth. Mature corneocytes were delimited by a cell corneous envelope and formed corneous microridges on the tooth surface. Although the increase in the electron density of the corneous layer suggested the presence of sulfur, the low to absent reaction for sulfhydryl groups indicated that cysteine was largely oxidized to form disulphide bonds in the corneous material of the teeth. A 2-dimensional electrophoretic analysis of the corneous material from the horny teeth showed the presence of acidic proteins, most likely keratins of 45-66 kDa. Keratin 10 immunoreactivity was present in the teeth. Based on the size, it is likely that acidic and basic non-keratin proteins of 16-20 kDa were also present in the oral mucosa, generally in higher amounts than keratins. This suggests that the low-molecular-weight basic proteins are likely associated with acidic keratins to produce the dense corneous material of the tooth, a process that also occurs in hard skin derivatives of other vertebrates. http://zoolstud.sinica.edu.tw/Journals/50.4/416.pdf

Key words: Lamprey, Horny teeth, Cornification, Keratins, Keratin-associated proteins.

The intense process of keratinization in the vertebrate epidermis, referred to as cornification, takes place by the formation of a fibrous framework of keratin filaments bound to a matrix material formed by keratin-associated proteins (KAPs, Resing and Dale 1991, Rogers 2004, Alibardi 2006). Keratins of 40-67 kDa are now relatively well known in various vertebrates, including fish (Schaffeld and Markl 2004, Schaffeld and Schultess 2006). In contrast, the presence and molecular structure of proteins associated with intermediate-filament keratins remain poorly known in horny structures of non-mammalian vertebrates. KAPs contribute to the hardening of skin derivatives such as scales, claws, hair, feathers, and horns in amniotes, and are also likely present, although in low amounts, in skin derivatives of anamniotes (fish and amphibians). In some fish, known skin derivatives are represented by breeding tubercles, adhesive organs, and horny teeth (Mittal and Banerjee 1979, Mittal and Whitear 1979, Zaccone et al. 1995, Das and Nag 2005). In amphibians, known skin derivatives include nuptial pads, claws, and beaks (Luckenbill 1965, Forbes et al. 1975, Maddin et al. 2009).

Recent studies in amphibians (anamniotes) indicated that the process of cornification of skin derivatives, such as claws and beaks, requires basic matrix proteins of 20-30 kDa in conjunction with keratins (Alibardi 2010a b). Therefore, the presence of KAPs in the epidermis and skin derivatives of fish still remains hypothetical.

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The process of keratinization in the epidermis of fish is limited in its extent and to certain body areas, and no corneous layer is generally formed in the epidermis. Only in specialized regions of the epidermis as mentioned above do fish keratinocytes undergo a true process of cornification. In such cells, the cytoplasm rapidly fills up with numerous bundles of filaments that transform the cells into corneocytes capable of high resistance to friction and mechanical wear (Mittal and Banerjee 1979, Mittal and Whitear 1979, Zaccone et al. 1995, Das and Nag 2005).

Among piscine vertebrates, cyclostomes represent an independent and ancient evolutionary line of eel-shaped vertebrates (agnathans or jawless fishes) separated from the true fish (cartilaginous and bony fishes). Cyclostomes are a specialized group of mostly parasitic forms that usually attach onto fish. The epidermis of cyclostomes consists of a multi-stratified epithelium containing 3 main types of cells: mucous, granular, and skein cells (Spitzer et al. 1979, Alarcon et al. 1993, Zaccone et al. 1995). The main type of cell present in the epidermis, mucous cells, also contains keratin filaments, but the production of keratin in the epidermis is limited, and no corneous layer is formed. Only in some regions of the oral epithelium, epidermal cells (keratinocytes) form pointed cones of hard corneous material, the teeth, which are utilized to attach to fish hosts. Tooth differentiation involves a true process of cornification that shows similar cytological characteristics to those observed in the process present in skin derivatives of terrestrial vertebrates (Uheara et al. 1983). Teeth are cyclically replaced by the formation of new teeth from the germinal epithelium, so that in a random sampling, 1 or 2 generations of teeth may be observed.

The present study therefore utilized the oral mucosa with the resident teeth of a freshwater cyclostome as a tissue potentially richer in both keratins and associated proteins than in the normal epidermis. The research was carried out to determine whether the process of cornification in living agnathans (cyclostomes) requires the presence of KAPs in conjunction with keratins, as in skin derivatives of other vertebrates. The present study employed immunocytochemical, ultrastructural, and 2-dimensional (2D) electrophoretic separation of corneous proteins extracted from the horny teeth of a freshwater lamprey.

MATERIALS AND METHODS

Microscopic methods

Fourteen young freshwater lampreys (*Lenthenteron zanandreai*) of 20-30 cm in length were collected from streams around Padova, Italy, and kept for 2-3 d in oxygenated water tanks. Specimens were sacrificed by decapitation, and the oral disk containing numerous teeth was fixed for the histological and ultrastructural examinations, or stored in a freezer at -40°C for 1-2 mo for subsequent biochemical analyses. For the electron microscopic study, pieces (2 × 2 mm) of the oral disk were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 5 h, post-fixed for 2 h in 2% osmium tetroxide, dehydrated in a graded ethanol solution, infiltrated with propylene oxide, and embedded in Durcupan resin (Sigma-Aldrich, Milan, Italy). Some pieces of the tail epidermis were also fixed with the same method for comparison purposes (keratinized vs. non-keratinized epidermis).

Tissues were sectioned with an ultramicrotome, and sections 1-2 μm thick were collected on glass slides, stained with 0.5% toluidine blue, and examined under a light microscope. Some sections of 40-90 nm thick were collected with an ultramicrotome (LK-Nova, LKB, Bromma, Sweden) on copper grids, stained with uranyl acetate and lead citrate according to routine methods, and observed under a Philips CM-100 electron microscope operating at 80 kV (Eindhoven, The Netherlands).

Other pieces of the oral disk were fixed for 3-5 h in Carnoy fluid (1 part acetic acid and 9 parts 80° ethanol), dehydrated in 90° ethanol, and embedded in Lowcryl resin (vendor, location) under ultraviolet (UV) irradiation at 0-4°C. After sectioning with the ultramicrotome, 2-4 μm thick sections were collected on chromoallume-albumin-coated slides and immunostained for keratins. Immunocytochemistry was performed using the K10 monoclonal anti-cytokeratin antibody (Chemicon, Tamecula, CA, USA). Sections were preincubated for 30 min at room temperature with 1% bovine serum albumin (BSA) in 0.05 M Tris/HCl buffer at pH 7.6 containing 5% normal goat serum. Sections were then incubated overnight at 4°C in the primary antibody diluted in Tris buffer (1: 200). In the controls, the primary antibody was omitted. Sections were rinsed in buffer and incubated with a secondary anti-mouse FITC-conjugated antibody (Sigma, St. Louis, MO, USA, at a 1: 70

dilution) and rinsed in buffer, and sections were observed under a fluorescence microscope using a fluorescein filter. Images were collected using a Euromex CX-5000 digital camera (Euromex, Amsterdam, The Netherlands) connected to a personal computer.

Some sections were also reacted to detect sulfhydryl groups (-SH) using the ferric ferricyanide reaction of Frederich-Chevremont (reported in Troyer 1980).

Electrophoretic analysis

Working under a stereomicroscope, the oral disk was cut into smaller pieces containing numerous teeth using a dissecting scalpel and sharp tweezers. The fragments were immediately immersed in extracting solution, consisting of a low-salt, reducing solution (see details in Alibardi 2010a b). Briefly, tissues were homogenized in the extracting solution containing 8 M urea/50 mM Tris-HCl (pH 7.6), 0.1 M 2-mercaptoethanol, 1 mM dithiothreithol, and 1 mM proteinase inhibitor (Sigma). After 6-8 h of extraction, the particulate matter was removed by centrifugation at 14,000 g for 30 min to obtain the protein solution.

Part of the supernatant solution was directly utilized for 2D electrophoretic separation. Another aliquot of the supernatant solution was further filtered in order to select only proteins within a specific MW range (of < 100 kDa). In this case, the initial protein solution was centrifuged for 1 h at 15,000 g to concentrate the proteins using a MW cut-off filter of 100 kDa (Vivaspin, Sigma-Sartorium, St Louis, MI, USA). The filtered protein solution was used for the following 1-dimensional (1D) and 2D separation. Protein concentrations in the unfiltered and filtered solutions were determined by the BCA method according to the manufacturer's instructions (Sigma).

For sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation (1D separation), the loaded samples contained about 30 μg of protein. The sample was solubilized for 15 min in 10% glycerol, 3% SDS, and 30 mM DTT, in 40 mM Tris at pH 6.8. Electrophoretic separation (Minigel, GE Healthcare Bio-Science AB, Sweden) utilized 12% acrylamide and 6 M urea in a cooled apparatus for 3 h with a current at 25 mA. The gel was stained with a standard silver staining method (Heukeshoven and Dernick 1988).

For the 2D electrophoresis analysis, 45-50 μg of protein samples were loaded in the electrophoretic cell apparatus. The sample was further re-suspended in rehydration buffer (2% CHAPs, 8 M urea, 0.5% Immobil precast gel (IPG) buffer (pH 3-10), and 100 mM DTT9) and loaded in the gel. For the 1st-dimension run, an Isoelectric Phocus System apparatus was utilized (IPGphor™, GE Healthcare, Chicago, IL, USA). Isoelectric focusing (including the rehydration step) was carried out for 23 h using Immobilon 13-cm-long strips with a linear pH gradient of 3 to 10. For the 2nd dimension, proteins were separated with an overnight run at room temperature and 12 mA, using SDS-PAGE 12% acrylamide gels with the addition of 6 M urea. Finally, the gel was stained with a standard silver-staining method (Heukeshoven and Dernick 1988). An MW standard (14.4-97 kDa) included in the GE Healthcare Low Molecular Weight calibration kit was utilized.

RESULTS

Light microscopy and immunocytochemistry

The epidermis of *L. zanandreai* had a variable thickness and cell morphology. In the tail, a minor number of cell layers was seen, while the thickness increased in the oral epithelium and in the epithelium at the base of horny teeth. In the tail, 3 or 4 irregular cell layers (tiers) were seen above the basal layer, and the more-apical 2 layers were composed of large dark and pale cells (Fig. 1A). Some sparse granular and pigmented cells were seen among the keratinocytes (data not shown). The basal layer rested over a dense dermis made of oriented collagen fibrils.

The epithelium of the oral disk showed some variations in thickness in different areas of the mouth and even in different areas of the same section. In general, the epithelium consisted of 10-12 irregular layers (tiers) of fusiform cells (basal tiers) to more-polygonal cells in the upper 3 or 4 tiers (Fig. 1B). External cells were often fusiform to flat, but no corneous layer was observed. The external layer was stained for sulfhydryl (SH) groups using the ferric ferricyanide reaction of Frederich-Chevremont (data not shown).

The teeth appeared as conical or rounded elevations of the epidermis made of a folded epithelium covered with a conical-shaped corneous layer (Fig. 1C). Often both the outer tooth and the forming inner tooth which would eventually replace the outer tooth were present in the same section (Fig. 1C). The 2 corneous teeth were thicker at

the apex, but were terminated at the base (here indicated as the root) by a thinner corneous layer (double arrows and double arrowheads in Fig. 1C). Between the 2 corneous layers, a thick (outer) epithelium was present, while a thick inner epithelium was seen beneath the inner horny tooth. The Frederich-Chevremont reaction for SH groups showed a weak positive reaction in the most-superficial keratinocytes of the epidermis, but the horny tooth appeared mainly negative (Fig. 1D).

The only immunofluorescent structure of the epidermis, which was positive for K10-immunoreaction, was the horny layer, while some positive cells were sparsely seen in the remaining epithelium (Figs. 1E, F). Higher magnification showed that fusiform corneocytes of the horny layer were intensely immunolabeled for K10. In the controls, immunolabeling was lower or absent (Fig. 1G).

TEM observations

The surface of the oral epithelium was mainly occupied by 0.5-1.5 μm long microvilli with associated vesicles which represented cross-sectioned microridges with their cytoplasmic folds located on the surface of the epithelium (Fig. 2A). In the cytoplasm, sparse keratin bundles or individual keratin filaments of low electron-density were seen, often converging to the desmosomes joining these cells into an epithelium. Numerous,

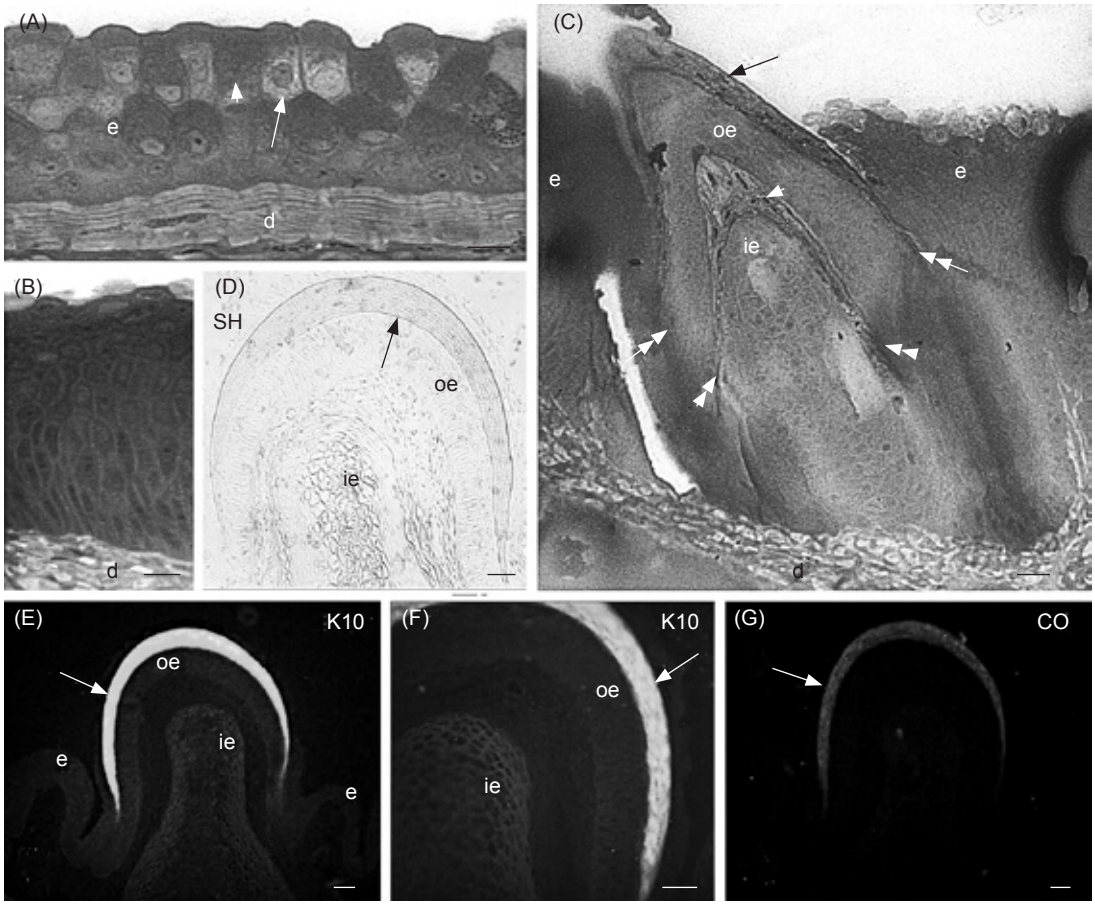

Fig. 1. Light microscopy using toluidine blue staining (A-C) and immunofluorescence for K10 (D-F) of epithelia. (A) Tail epidermis showing large pale keratinocytes (arrow) and darker keratinocytes (arrowhead). Scale bar = 10 μm. (B) Thick region of the oral epithelium with superficial, flat desquamating cells (arrow). Scale bar = 10 μm. (C) Tooth showing the outer horny layer (outer tooth, arrow) and inner horny layer formed underneath (inner tooth, arrowhead). Double arrows indicate the inner limit (roots) of the outer tooth. Double arrowheads indicate the roots of the inner tooth. Scale bar = 20 μm. (D) Intense immunolabeling for K1 of the unique horny layer (arrow) of a tooth. Scale bar = 25 μm. (E) Detail of the horny layer (arrow) formed by compacted fusiform keratinocytes. Scale bar = 25 μm. (F) Control section with an immunonegative horny layer (arrow). Scale bar = 25 μm. (G) Negative (control, CO) section. The arrow indicates the horny layer. Scale bar = 25 μm. d, dermis; e, epidermis; ie, inner epidermis of the oral epithelium; K10, immunoreaction for K10; oe, outer epidermis of the tooth; SH, sulfydril groups reaction.

dense, irregular mucous granules (0.05-0.1 μm in size) were present in external and intermediate cells of the epithelium, and were especially localized in the external cytoplasm (Fig. 2B). In more-superficial cells, small mucous granules mainly faced the external surface at the base of the microridges, and some granules had discharged their contents into the extracellular space (data not shown).

Nuclei were mainly euchromatic, and few heterochromatin clumps were present in epithelial cells. In both the oral epithelium and epithelium of the teeth, lower tiers of epithelial cells were joined by numerous desmosomes, and they frequently showed 0.2-0.3 μm vesicles of intermediate or low electron-density, probably containing mucus or glycoproteins (Fig. 2C).

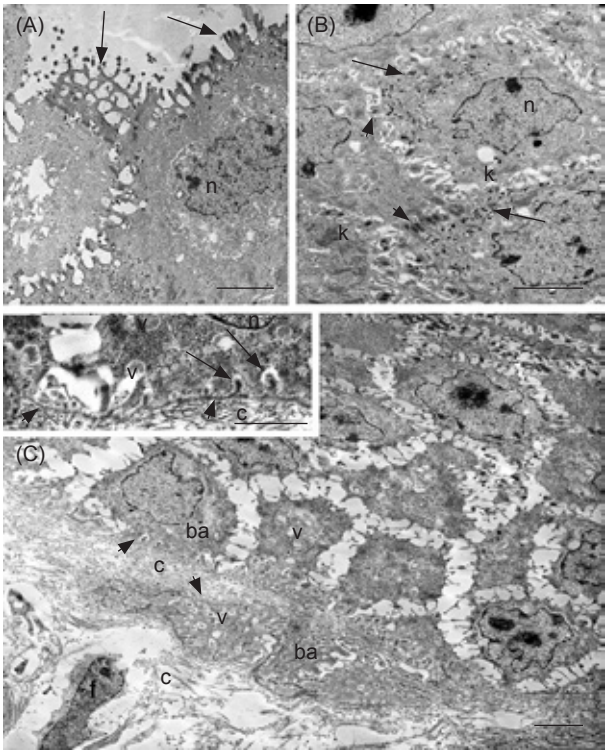

Fig. 2. Ultrastructural features of the oral epidermis (A, B) and basal layers of the inner horny epidermis (C). (A) External layer showing superficial microridges (arrows). Scale bar = 2 μm. (B) Spinosus-like cells joined by desmosomes (arrowheads) and containing small mucus granules (arrows). Scale bar = 2 μm. (C) Irregularly folded basal epidermis (arrowheads indicate the basal membrane). The keratinocytes contain pale vesicles often storing amorphous material of medium electron-density (see inset). Scale bar = 2.5 μm. The inset (Scale bar = 1 μm) details the basal membrane with some dense components (pedicels) that penetrate into the keratinocyte cytoplasm (arrows). ba, basal layer/cells; c, collagen fibrils; n, nucleus; v, vesicles (mucus).

In the basal-most cells of the epithelium and in basal cells of tooth roots (indicated by double arrows and double arrowheads in Fig. 1C), a basal membrane was surrounded by a very irregular surface. The cytoplasm of some basal cells of this epithelium deeply penetrated the dermis but was also recognizable in tangentially sectioned regions, since the perimeter of the epidermal cells was surrounded by a basement membrane (Fig. 2C). The dense lamella of the basement membrane formed micro-folds at the base of epidermal cells (inset of Fig. 2C). Numerous banded and non-banded collagen fibrils were in contact with the basement membrane.

Living, non-cornified cells of the inner and outer teeth appeared similar, although the outer epithelium contained flatter and spinosus keratinocytes (Fig. 3A). The roots of both the inner and outer teeth were surrounded by spinosus-like keratinocytes containing sparse and low electron-dense keratin bundles (Figs. 3A, B). Cells forming the corneous layer in the root region appeared electron-dense and were connected to non-corneous keratinocytes through numerous corneo-desmosomes that formed small electron-dense folds. The surface of the external corneous layer, which was oriented toward the epithelial surface, showed the presence of more or less regularly spaced folds forming the corneous microridges (inset in Fig. 3A and arrowhead in Fig. 3C). The plasma membrane of corneous cells, especially those forming the microridges, appeared thickened by the deposition of a 100-200 nm-thick layer of an electron-dense material (the "cell corneous envelope", see inset in Fig. 3A). The external surfaces of both the inner and outer teeth were in contact with living, spinosus cells of the epithelium containing sparse glycogen particles and keratin bundles, and featuring largely euchromatic nuclei.

The corneous folds observed in the roots and on the outer surface of the corneous layer largely disappeared or showed loss of their serrated shape in the transitional and pre-corneous layer of the upper, non-root area of the tooth (Figs. 3C, 4). The corneous folds disappeared at 10-20 μm from the roots moving toward the tip of the tooth. The living epithelium of both the inner and outer teeth was formed by 4-6 layers of spinosus keratinocytes that became flat in the transitional area with the stratum corneum (SC). Pre-corneous cells of both the inner and outer teeth were electron-pale and contained sparse ribosomes and numerous dark keratin bundles and vesicles (Figs. 3A, C, 4A). The vesicles contained an amorphous, low-

electron-dense material or appeared empty. In upper keratinocytes forming the transitional layer before cells entered the corneous layer, keratin bundles tended to be concentrated along the cell periphery. This was further observed in dark cells compacted into the SC. These keratin bundles appeared as different shapes according to the sectioned plane. The bundles varied from very irregular belts to round organelles resembling granules, and were often surrounded by ribosomes (Fig. 4B, lower inset of Fig. 4C). Despite the shape of the bundles being variable, their texture and electron density clearly indicated that they were only formed by condensing keratin bundles, while no other type of organelles, in particular

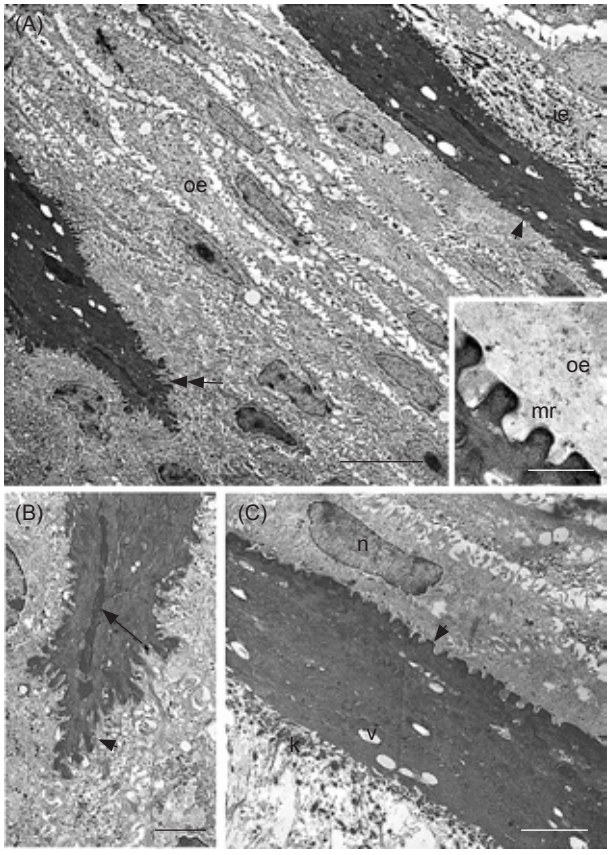

Fig. 3. Ultrastructural features of inner and outer horny teeth. (A) Detail of an area including the outer root (double arrow), the interposed spinosus epithelium, and part of the inner horny layer (arrowhead). Scale bar = 5 μm. The inset (Scale bar = 0.5 μm) shows the corneous microridges of the external part of the inner horny layer. (B) Detail of the numerous corneous folds (arrowhead) of the inner root. The arrow indicates a flat pyknotic nucleus of a corneocyte in this area. Scale bar = 1 μm. (C) Detail of the transitional layer rich in keratin bundles underneath the 1st corneous cell of the outer tooth. The arrowhead points to cornified microridges facing the external living cells of the oral epithelium. Scale bar = 2 μm. k, condensing keratin bundles; ie, epithelium of the inner tooth; mr, microridges; n, nucleus; oe, epithelium of the outer tooth; v, vesicles.

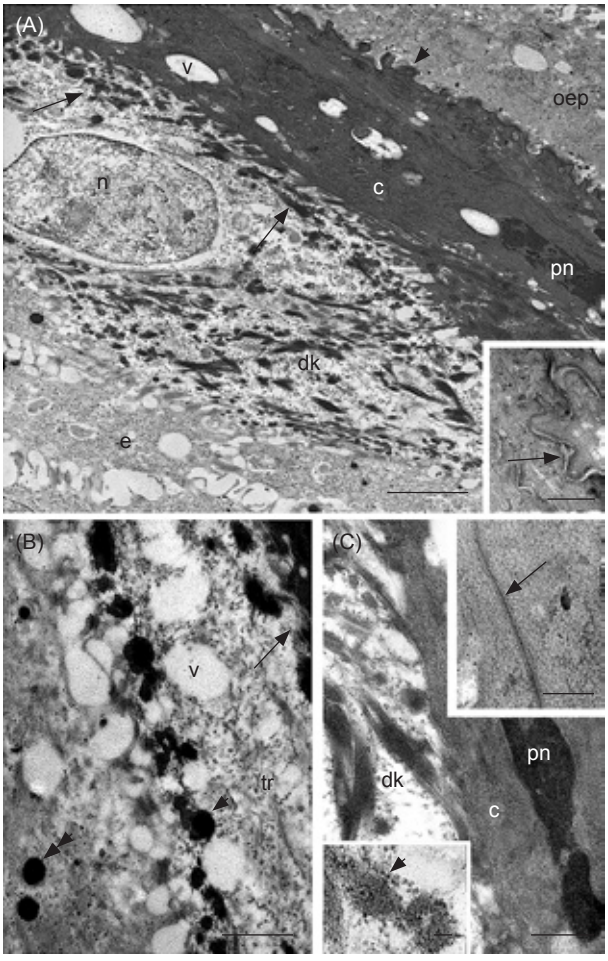

Fig. 4. Ultrastructural features of a cornifying outer tooth. (A) Detail of the packing of dense keratin bundles (arrows) in transitional cells. Scale bar = 2 μm. The inset (Scale bar = 0.5 μm) shows details of the interdigitations among corneocytes delimited by the electron-dense thickening of desmosomal remnants (arrow). (B) Detail of organelles present in a transitional cell such as round dark keratin (arrowhead) and irregular keratin bundles (arrow). The double arrowhead points to 2 rare rounded keratin bundles contained in a spinosus cell. Scale bar = 0.5 μm. (C) Long, dense bundles of dark keratin packed against a corneocyte containing a pyknotic nucleus. Scale bar = 0.5 μm. The upper inset (Scale bar = 200 nm) shows a dense plasma membrane (arrow) between mature corneocytes. The lower inset (Scale bar = 100 nm) shows the presence of ribosomes (arrowhead) around keratin bundles of a transitional cell. c, corneous layer; dk, dense keratin bundle; e, epithelial cell; n, nucleus; oep, oral epithelium; pn, pyknotic nucleus; tr, transitional cell; v, vesicle.

no distinctive types of other granules were seen. Nuclei of pre-corneous cells contained increased amounts of heterochromatin, and they became pyknotic (made of condensed chromatin) in the SC (Figs. 4A, C). The undulating plasma membrane of corneocytes gave rise to small inter-digitations on the external endings of these cells that connected with other corneocytes (inset of Fig. 4A).

The corneous layer was formed by 2 or 3 cells in the roots of the teeth that increased to 4-8 cells by the tip of the tooth. Dense corneocytes showed an irregularly thickened plasma membrane due to deposition of electron-dense material. Specialized junctions or corneo-desmosomes were occasionally seen between the corneocytes. Corneocytes were therefore mainly connected through a thin, electron-dense material deposited between the remnants of the plasma membranes (arrow in the upper inset of Fig. 4C). The mechanical connectivity among cells of the SC was also strengthened by the numerous inter-digitations present at the extremities of the corneocytes. In these areas, a dense line of corneo-desmosome remnants was often visible (arrow in the inset of Fig. 4A).

Electrophoretic separation

After 1D or 2D electrophoresis, several protein bands or spots were isolated, as indicated by the silver-staining in both the alpha-keratin range (40-66 kDa) and in lower molecular range (Fig. 5). Separation by 1D electrophoresis of the filtered solution showed a main band at 18-20 kDa, other minor bands at 68-70, 47-50, and 35-37 kDa, and 3 bands at 15-16 kDa (Fig 5A).

In the 2D separation of the filtered solution, proteins in the keratin range (40-66 kDa) showed a main spot at 62-64 kDa with a pI of 4.8, together with other minor spots of lower MW (40-50 kDa) but similar pI values (4.5-5.1, see Fig. 5B). The 2 lines of protein material seen at 47-48 and 30 kDa represent an artifact of protein migration, probably contamination from the protein standard.

The most-intense protein spots in the filtered solution were seen in the non-keratin range of the gels, both in the acid and basic ranges. In the former group, the main spots were found at 25 kDa with a pI of 5.1, at 18 kDa with a pI of 4.8, and at 16 kDa with pI values of 4.2 and 4.4, and a weaker spot around a pI of 5. In the basic range, spots at 8-10 kDa with a pI of 7.1 and another weaker spot with a pI of 8.2 were seen. Larger spots were observed at 16-17 kDa with pI values of 8.2, 8.5, 9.0, and 9.5. A spot at 22 kDa with a pI of 9.7 and another protein spot at 26 kDa with a pI of 8.4 were also seen (basic range, non-keratin protein spots).

The unfiltered solution, here presented for comparison, showed a slightly different spot pattern, but the prevalent protein spots observed in the filtered solution were still present (Fig. 5C).

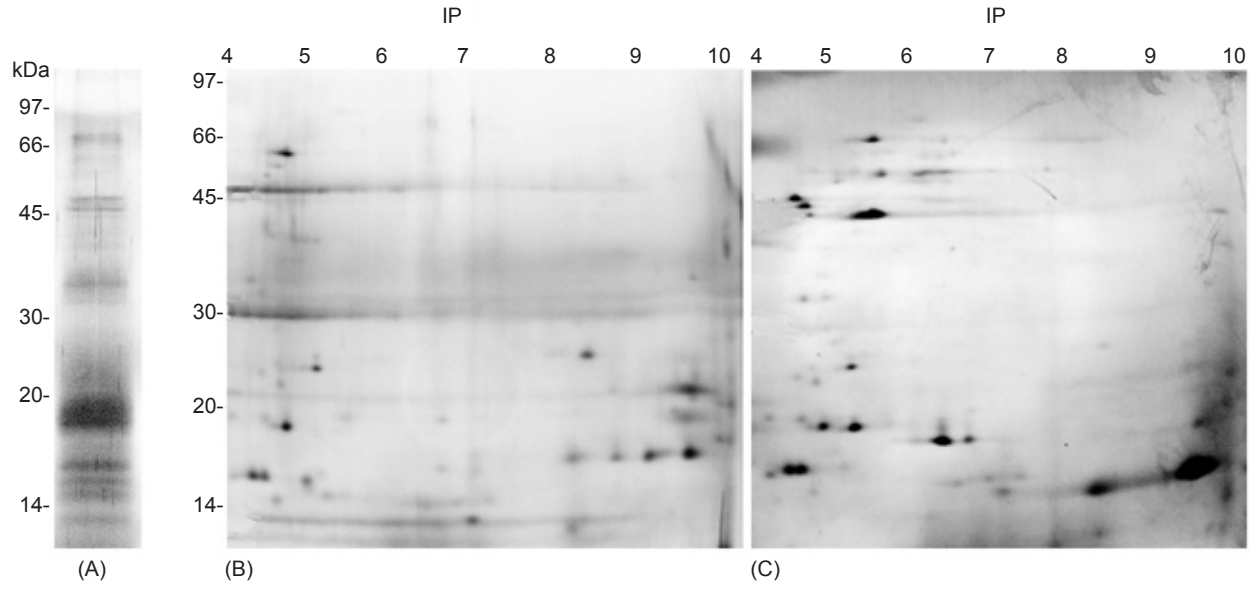

Fig. 5. Electrophoretic separation of tooth epidermal proteins with molecular weights (arrows). Protein bands (A) or spots (B, C) were stained with silver stain (see "MATERIALS AND METHODS" and "RESULTS"). (A) Mono-dimensional gel of filtered solution (with a cutoff at 100 kDa). (B) Case of a 2-dimensional gel of the filtered solution. (C) Another case of the non-filtered solution.

In the keratin region (40-66 kDa), more spots were seen than in the previously described filtered solution. In particular, 1 spot at 66 kDa showed a pI of 5.6, another at 57-60 kDa had pI values of 5.7 and 6.2-6.5, and 2 close spots at 47-50 kDa had pI values of 4.7-4.8. In the non-keratin range, a spot at 25-27 kDa had a pI of 5.2, 2 spots at 18 kDa had pI values of 5.0 and 5.5, and another 2 spots at 17 kDa had pI values of 6.5 and 6.7. A double spot present at 15-16 kDa had pI values of 4.3-4.5. In the basic pH range, a diffuse and large spot was seen at 18 kDa with a pI of 9.5. Finally, 2 main spots were seen at 15-16 kDa with pI values of 8.2 and 9.5, but other proteins were present within this basic range that formed a band (Fig. 5C, low right corner).

In an attempt to test whether some of the protein spots reacted to mammalian keratins, some gels, after blotting, were immunoreacted with keratin antibodies using the general pancytokeratin antibody from Sigma. However, the protein spots produced no cross-reactivity with this antibody, and no labeling was seen in either the keratin or non-keratin protein range.

DISCUSSION

The present study on a freshwater lamprey (*L. zanandreai*) indicated that in the horny teeth of primitive aquatic vertebrates such as cyclostomes, intense cornification of the superficial layers of the teeth takes place (Mittal and Whitear 1979, Uehara et al. 1983). The process of the addition of new keratinocytes to the roots of the inner and outer teeth, both in terms of the time of migration from the basal layer and of the direction of migration, was not the focus of the present study and therefore remains to be specifically analyzed.

The ultrastructural features of the horny teeth of *L. zanandreai* recall similar aspects present in the lamprey *Entosphenus japonicus* (Uehara et al. 1983), in breeding tubercles of the catfish *Bagarius bagarius* (Mittal and Whietar 1977), and in the adhesive organ of the catfish *Pseudochenensis sulcatus* (Das and Nag 2005). This process also resembles that observed in claws and beaks of amphibians (Maddin et al. 2009, Alibardi 2010a b). Previous descriptions of other species of lampreys are fully confirmed, with the exception of the presence of keratohyaline granules. In fact, the granules and bundles observed in pre-corneous cells were here identified as keratin bundles sectioned in a transverse or irregular plane. Keratohyaline granules are a unique characteristic of mammalian epidermis and are related to the accumulation of filaggrin and loricrine proteins (Resing and Dale 1991, Alibardi 2006).

Mucus and glycoproteins are non-specific inter-keratin matrix molecules in non-terrestrial vertebrates, and are largely secreted, while true matrix molecules remain intracellular (Mittal and Banerjee 1979). The numerous mucous granules and scarce tonofilaments typical of normal epidermis in cyclostomes and in other fishes, are replaced by numerous tonofilaments and scarce mucous granules in cells forming the corneous derivatives, including those of the oral teeth (Uehara et al. 1983).

The ultrastructural analysis indicated that the synthesis of keratin filaments and of KAPs in ribosomes of keratinocytes occurs rapidly, and these proteins are immediately assembled into bundles of variable shapes and textures. Prevalent keratin filaments are formed in spinosus cells, including those of the normal epidermis that contain 54-, 43-, and 40-kDa keratins corresponding to mammalian K7, K18, and K19 (Alarcon et al. 1994). From the teeth, different from the epidermis, 60-66 kDa proteins in the keratin-range were also extracted, perhaps more specialized for the formation of the hard SC. The specific analysis of proteins in the keratin range was not in the scope of the present research. It is however known that fish keratins generally have molecular weights ranging 40-62 kDa (keratin range, see Schaffeld and Markl 2004, Schaffeld and Schultess 2006). The intense immunoreactivity to K10 indicates that this or a cross-reactive protein, typical of corneous layers of mammalian epidermis (O'Guin et al. 1987), was also present in the horny teeth of the lamprey, but was absent from the remaining, non-keratinized epidermis.

The present study confirms previous analyses of the positivity of K10-like keratins in lamprey teeth (Zaccone et al. 1995). In the corneous material, some degradation of keratin-intermediate filaments can occur, but most of the darkening is probably derived from incorporation of a certain amount of non-keratin proteins, here indicated as fish (f)KAPs, around the initial keratin tonofilaments.

In pre-corneous (transitional) keratinocytes, tonofilaments suddenly turn electron-dense, and some ribosomes still remain associated with this corneous material. This cytological aspect indicates that proteins synthesized by these

ribosomes are immediately aggregated to the initial core made of keratins. Similar processes were seen in amphibian hard structures (Maddin et al 2009, Alibardi 2010a b), and in scales, hairs, and feathers of corneocytes in reptiles and mammals (Resing and Dale 1991, Rogers 2004, Alibardi 2006). The low to absent reactivity for SH groups in pre-corneous and corneous cells of the horny teeth suggests that sulfur-rich proteins are completely oxidized, which may explain the increased electron-density, as sulfur bonds to uranyl acetate and lead citrate (Fraser et al. 1972, Jessen 1973). Activation of specific genes for putative fKAPs probably occurs in transitional keratinocytes before they are incorporated in the SC of teeth. As indicated from electrophoretic separation, proteins in the keratin range of 40-68 kDa present in the teeth and oral epithelium of the lamprey are essentially acidic, like keratins in other fishes analyzed so far, including cyclostomes (Schaffeld and Markl 2004, Schaffeld and Schultess 2006). The present study on the oral epithelium containing teeth showed that the extracted fraction of proteins outside the keratin range, both acidic and basic, is often prevalent over proteins in the keratin range.

It is likely that some of these basic proteins are associated with keratins or other proteins of the oral epithelium, especially proteins in the basic range (with pI values of 7.1-9.5), and can interact with acidic proteins including keratins. These basic KAPs substantially contribute to the hardness of the horny teeth, like other KAPs that determine the hardness of corneous tissues in mammalian hair, nails, claws, and horns. In the latter, different types of KAPs are present, such as high-glycine tyrosine, high-sulfur, and ultrahigh-sulfur proteins (Rogers 2004). Non-keratin proteins in the acidic range from the lamprey oral mucosa may represent, aside from other types of components, post-translational or even degradative products from basic proteins of similar MWs, but further study is required on this point.

In hard derivatives of mammals such as hair, claws, and horns, sulfur-rich and ultra-sulfur-rich KAPs are believed to be responsible for the electron-density of corneous tissues, as cysteine and cystine react with osmium to form an electron-dense, inter-keratin (matrix) material. Also, the lead citrate that is utilized to stain sections for ultrastructural observations is mainly adsorbed by the matrix components of the corneous material (Fraser et al. 1972, Jessen 1973). It is also likely that in hard derivatives of fish and cyclostomes, the increase in electron-density is derived from deposition of cysteine-rich KAPs among keratins and on cell membranes of keratinocytes. This was also indicated in previous studies on amphibian corneous structures, such as claws and beaks (Maddin et al. 2009, Alibardi 2010a b). The specific nature and amino acid sequences of these proteins deserve further study utilizing molecular biological tools.

In conclusion, the present study indicates that a large proportion of proteins present in the oral mucosa containing the horny teeth of the lamprey are not keratins. Non-keratin proteins include basic proteins of 16-20 kDa that are likely associated with acidic proteins in the keratin range of the teeth. Future proteomic studies and determination of the amino acid sequences of these basic proteins will clarify their molecular characteristics, organization, and interactions with keratins responsible for the production of the hard corneous material of teeth and other skin derivatives in fish and amphibians.

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