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Ultrastructural visualization of cross-linked protein features in epidermal appendages

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SUMMARY

Upon vigorous extraction with ionic detergent under reducing conditions, the macroscopic structures of mammalian hair, bird feather and horny teeth of the hagfish became swollen and flexible but were substantially preserved. In each case, removal in this way of solubilizable constituents, such as disulfide-bonded keratins and associated matrix proteins, left a residue of ε-(γ-glutamyl)lysine cross-linked protein. Residual features in hair included cell envelopes in the cortex, cell envelopes and intracellular deposits in the medulla, and nearly the entire cuticle cells. In feather, extraction left largely intact the macroscopic barb structures but caused collapse of the rachis. In both rachis and barbs, considerable material resembling cell envelopes remained. In extracted hagfish teeth the cellular organization was clearly visible microscopically, including cell borders and remnant nuclei. Unlike the cornified envelopes of mature epidermal keratinocytes, which appear as doublets, reflecting their formation immediately beneath each plasma membrane of apposing cells, the borders of cells of hair cortex and hagfish teeth appeared single and continuous from one cell to the next. Thus the observed cross-linked features comprised four types: (i) condensation immediately beneath the plasma membrane (feather, hair medulla and cuticle) similar to cornified envelopes of epidermal keratinocytes; (ii) deposition between cells (hair cortex, horny teeth); (iii) cytoplasmic deposits (hair cuticle and medulla); and (iv) nuclear condensation (hair medulla, horny teeth). The results emphasize the importance of transglutaminases and their substrate proteins for the function of epidermal appendages and may provide a useful diagnostic test for perturbation of their normal structures.

Key words: feather, hair, isopeptide, hagfish teeth, transglutaminase

INTRODUCTION

A characteristic feature of mature epidermal cells of mammals, birds, reptiles and amphibians is the cornified (or cross-linked) envelope that forms at the cell periphery during the final stage of terminal differentiation (Matoltsy, 1977; Green, 1979). A number of distinct proteins are incorporated into this structure, which contains a high content of ε-(γ-glutamyl)lysine isopeptide bonds, through transglutaminase cross-linking activity (Hohl, 1990; Polakowska and Goldsmith, 1991; Reichert et al., 1993). Envelopes are also displayed by mature cells in epidermal appendages such as the nail plate (Hashimoto, 1971) and the hair shaft cuticle (Hashimoto, 1988), which also have high contents of isopeptide bonding (Zahn et al., 1980; Rice et al., 1993). Teleost fish exhibit protein cross-linking activity, apparently transglutaminase-mediated, during eggshell hardening (Oppen-Berntsen et al., 1990). The process of cornified envelope formation is not evident ordinarily in the epidermis or oral epithelium of such fish (Albright and Skobe, 1965; Henrikson and Matoltsy, 1968), but keratinization is observed in breeding tubercles and contact organs of the epidermis in some teleost species (Wiley and Collette, 1970). While little or no evidence suggests agnathan epidermis exhibits cornified envelopes, these ancient jawless fish (lamprey, hagfish) display a type of horny teeth, a noncalcified epidermal appendage whose properties resemble nail or claw in terrestrial animals (Dawson, 1963).

Cornified envelopes in epidermis were originally isolated by dissolving cellular contents with denaturants such as alkali or detergents under conditions that break disulfide bonds (Matoltsy, 1955; Sun and Green, 1976). Our initial observations that human hair, bird feather and hagfish teeth retain their integrity despite boiling in detergent and reducing agent were compatible with the cells in these structures having cornified envelopes. However, transglutaminase-mediated protein cross-linking in epidermal appendages is not limited to envelope formation. For example, a high degree of cross-linking in the hair shaft occurs in the medulla associated with the cytoplasmic protein trichohyalin (Rogers, 1988). The present investigation has been directed to localizing cross-linked structures accounting for the stability of hair, feather and horny teeth.

MATERIALS AND METHODS

Biochemical characterization

Before treatment, feather barbs were separated from the rachis (central shaft) with scissors. Human facial hair and white pelican (*Pelecanus erythrorhynchos*) flight feather samples (50-100 mg) were rinsed briefly at room temperature in 1% SDS to remove oils and debris, then heated in a boiling water bath for 1 hour in 10 ml of 1% SDS, 50 mM sodium phosphate buffer (pH 7.9). The SDS-insoluble residue was extracted 4-6 times, each for 2-6 hours with sonication inbetween, with fresh SDS-phosphate buffer to which was added ~5 mg of dithiothreitol (DTT). Hagfish (*Eptatretus stouti*) teeth were treated identically in parallel but usually in half the volumes. The proteins in soluble fractions were precipitated with 10% trichloroacetic acid and quantitated with ninhydrin after digestion for 6 hours with 10% sulfuric acid (Shiffman, 1966). The amount of lysine participating in cross-links was determined as previously described (Rice and Green, 1977) by exhaustive proteolytic digestion of the insoluble material, isolation of the ε-(γ-glutamyl)lysine isodipeptide fraction by ion-exchange chromatography and amino acid analysis of the desalted isopeptide fraction after acid hydrolysis. Amino acid analyses of isolated isodipeptide and SDS-DTT-insoluble material were performed after 24 hours hydrolysis in 6 M HCl by the Protein Structure Laboratory of the University of California, Davis.

Processing for ultramicroscopic examination

Except as noted, samples were routinely heated in a boiling water bath for 6 hours in 5-10 ml of 50 mM sodium phosphate or Tris-HCl buffer (pH 8), 2% SDS, 20 mM DTT, rinsed briefly in distilled water, transferred to a solution of 2% glutaraldehyde in 85 mM sodium cacodylate buffer (pH 7.4) and fixed for several days under refrigeration. The samples were washed twice in Zetterquist (veronal acetate) buffer (Pease, 1956) and post-fixed with 1% osmium tetroxide (EMS, Fort Washington, PA) in Zetterquist buffer for 2 hours at room temperature. After three 10-minute washes in 63 mM maleate buffer (pH 6.0), samples were stained for 1 hour at 4°C with 1% tannic acid, subsequently rinsed several times in maleate for a total of 1 hour, and stained overnight at 4°C with 0.5% uranyl acetate. The samples were dehydrated in a graded series of ethanol and propylene oxide and infiltrated with Araldite 502 overnight. Samples were embedded in fresh resin and cured at 60°C for several days. Sections (0.5 μm) were cut for each specimen using glass knives. Ultrathin (50-60 nm) sections, cut from blocks containing cross-sectional profiles, were placed on 200 mesh copper grids, stained with uranyl acetate and lead citrate and examined on a Zeiss 10A electron microscope. For measurements of envelope thickness, electron micrographs were scanned with an HP Scanjet IIc and imported into an NIH Image 1.49 program.

Table 1. Fractionation of human hair, pelican feather and hagfish teeth*

Property	Hair	Barbs	Rachis	Teeth
1. Extraction				
SDS soluble	1±1	10±4	1±1	2±1
SDS+DTT soluble	37±3	82±7	95±1	89±5
Insoluble	62±3	8±4	4±0.4	9±3
Total	100	100	100	100
2. Insoluble material				
Protease solubilized	66±14	62±9	90±7	75±11
Lys in cross-links	7.4±0.3	18.5±1.0	21.8±0.0	7.8±0.4

*Values tabulated (%) are the mean and range of two independent determinations.

RESULTS

Solubilization and cross-link analysis

Appendages extracted by heating with SDS were altered minimally in appearance, but upon inclusion of DTT they became markedly swollen. Hair, hagfish teeth and feather barbs became flexible but retained their original shape, while feather rachis collapsed into a formless mass. With the exception of feather barbs (10% extractable), only traces of protein were extracted from each sample by SDS in the absence of reducing agent (Table 1). Upon addition of DTT, the feather barbs and rachis were readily disrupted by sonication into small fragments, and extractable protein was removed by two further treatments. In contrast, hair and hagfish teeth samples, disrupted with difficulty and incompletely, required several more extractions. The remaining insoluble residues amounted to the majority of protein in the hair samples (62%) and a smaller proportion (4-9%) of the feather and hagfish teeth samples. Amino acid compositions of the insoluble material, including a substantial proline content (8-14%), were roughly similar to each other and to samples previously prepared from human foot callus (Table 2). Each material was largely solubilized by extensive proteolysis (62-90%) and contained a considerable proportion of constituent lysines (7-22%) participating in ε-(γ-glutamyl)lysine isopeptide bonds (Table 1).

Hair

Untreated hair sectioned with difficulty due to poor embedding, but treatment with SDS and DTT for 1 hour at room temperature led to marked improvement. As seen by comparison of Fig. 1A and B, the brief extraction permitted better visualization of the cellular structure of the cortex (and cuticle). White and brown hair were essentially identical except for the presence of scattered pigment granules in the brown hair. Sections of scalp and beard hair were comparable, although the medulla was more prominent in the latter.

Table 2. Amino acid compositions of cross-linked structures*

Amino Acid	Hair	Barbs	Rachis	Teeth	Callus†
Aspartic	4.9	8.6	8.1	8.2	5.3
Threonine	8.4	7.3	6.0	6.3	3.6
Serine	12.9	8.2	7.3	8.4	14.8
Glutamic	12.8	12.4	13.1	8.8	12.6
Proline	10.0	10.9	8.4	14.2	9.1
Glycine	7.8	7.9	8.6	9.9	18.8
Alanine	4.5	3.6	5.8	5.5	4.0
Valine	8.9	9.1	7.9	5.4	3.2
Methionine	0.4	1.9	1.6	1.4	1.1
Isoleucine	2.9	2.7	3.8	3.1	2.2
Leucine	5.8	4.1	6.7	6.4	4.4
Tyrosine	2.4	5.3	4.7	5.1	2.5
Phenylalanine	1.8	1.2	2.2	2.5	2.0
Histidine	0.8	1.2	1.3	2.4	1.9
Lysine	2.6	5.5	6.2	3.9	4.6
Arginine	7.0	3.5	5.1	6.1	4.0
Cysteic acid‡	6.1	6.6	3.2	2.4	5.9
	100.0	100.0	100.0	100.0	100.0

*Normalized values for each sample are means of two independent determinations.

†Unpublished data from Rice and Green (1979).

‡Determined after performic acid oxidation.

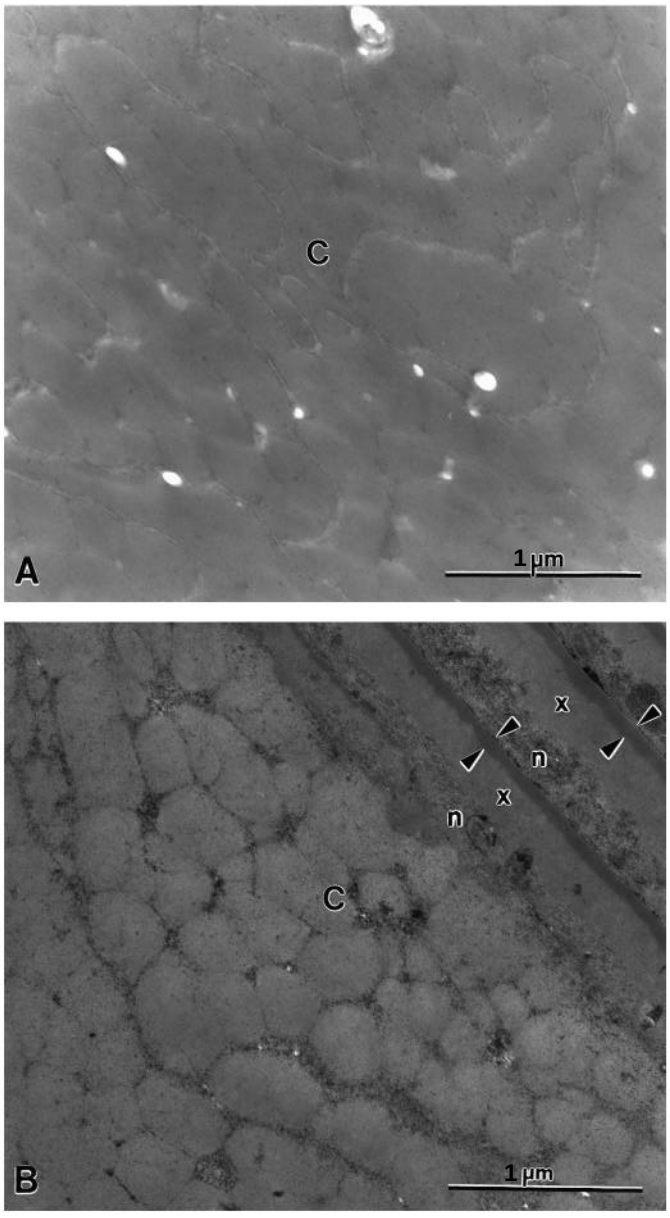

Fig. 1. Cross-sections of human hair without extraction (A) and after extraction for several hours at room temperature with SDS and DTT (B). The sections show primarily cortex (C). B includes cross-sections of two cuticle cells showing the endocuticle (n), exocuticle (x) and marginal band (between arrowheads).

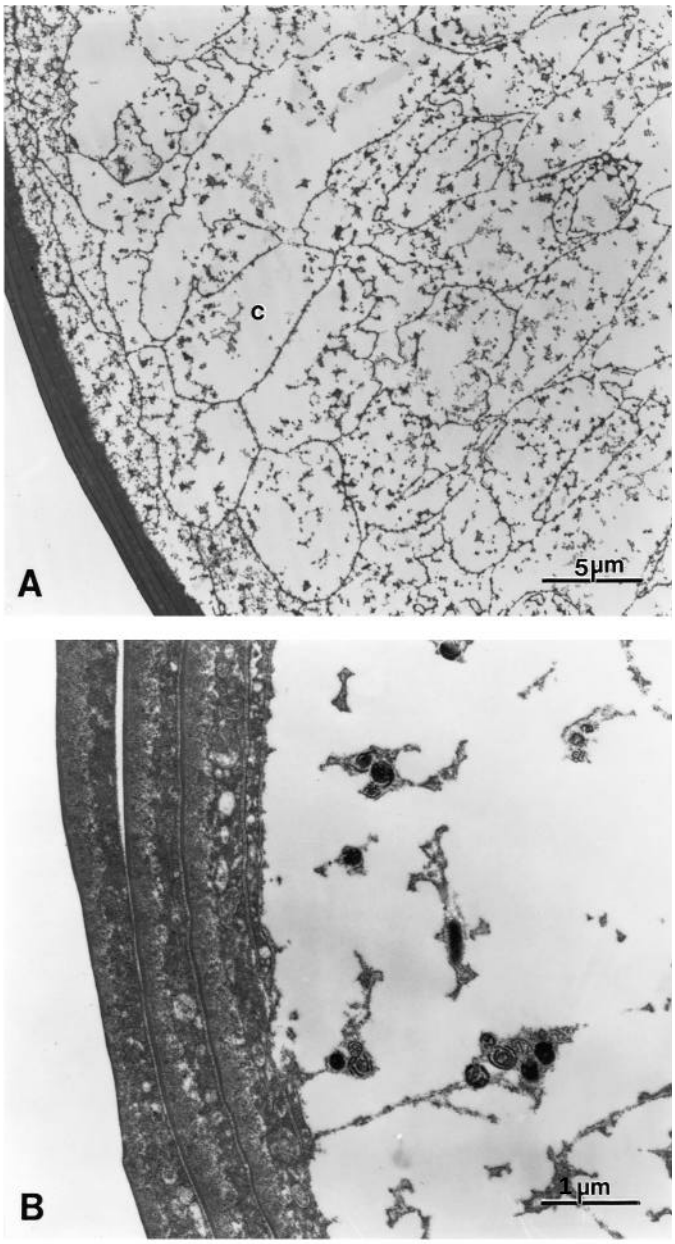

Fig. 2. Cross-sections of human hair extracted with SDS and DTT for 6 hours at 50°C (A) or 100°C (B). B emphasizes the contrast in degree of extraction in cortex and cuticle cells at 100°C.

As shown at higher magnification (Fig. 2B), cells of the cuticle boiled in detergent and reducing agent for 6 hours retained most of their internal structure, as judged by comparison with unextracted sections seen in this work and presented by others (Hashimoto, 1988). The solid marginal band at the outer periphery and the boundary between the smoother exocuticle and the more granular and heterogeneous endocuticle were clearly evident. Indeed, these features appeared hardly affected by the extraction. The marginal band was nearly 50 nm thick (Table 3), or 10% of the cell diameter, where the exocuticle and endocuticle were ~190 and ~310 nm, respectively. By contrast, cells of the cortex were nearly devoid of visible content following treatment (Fig. 2A). The only consistent feature surviving extraction was a honeycomb-like or sometimes filigree network of narrow deposits ~30 nm thick (Table 3) outlining the borders of the cells. Although aggregates of filament protein were evident in regions of some sections, treatment at 50°C instead of 100°C was often sufficient to remove cleanly the cell contents, leaving the extraction-resistant cell borders. Distinct in appearance, cells of the medulla were midway between the above extremes. They contained large deposits of amorphous material (Fig. 3A), often including nuclear remnants. In addition, distinct electron-dense borders ~30 nm thick were visible beneath the plasma membranes of apposing cells (Fig. 3B), resembling in appearance the cornified envelopes of epidermal keratinocytes.

Table 3. Thickness of envelope structures*

Appendage	Site	Species	Thickness (nm)
Hair	Cuticle	Human	46±5
		Mouse	56±9
	Cortex	Human	31±12
		Mouse	12±4
	Medulla	Human	31±12
		Mouse	16±4
Feather	Rachis (thin)	Pelican	24±5
	(thick)	Pelican	72±9
	Barb	Pelican	69±5
Horny teeth	Intercellular deposits	Hagfish	58±12

*For each sample, treated at 100°C for 6 hours, values are given for the means and s.d. of measurements at 6-10 sites.

The major features observed in detergent-treated human hair were also seen in mouse fur treated in the same way (Fig. 4). The most prominent feature was the extraction-resistant material filling cells of the medulla. This electron-dense material comprised rounded amorphous deposits coalesced near the cell periphery, similar in appearance to the nuclear contents. Along the axis of the hair, essentially as demonstrated with untreated hair (Hojiro, 1972), longitudinal sections showed the laddered appearance of medullary cells, each with indentations matching bulges in adjacent cortical cells. While the macroscopic hair morphology was preserved despite detergent treatment, structures in the cortex and cuticle in most sections appeared less resistent to extraction than those in the human hair. Cortical cell borders were much thinner, about half

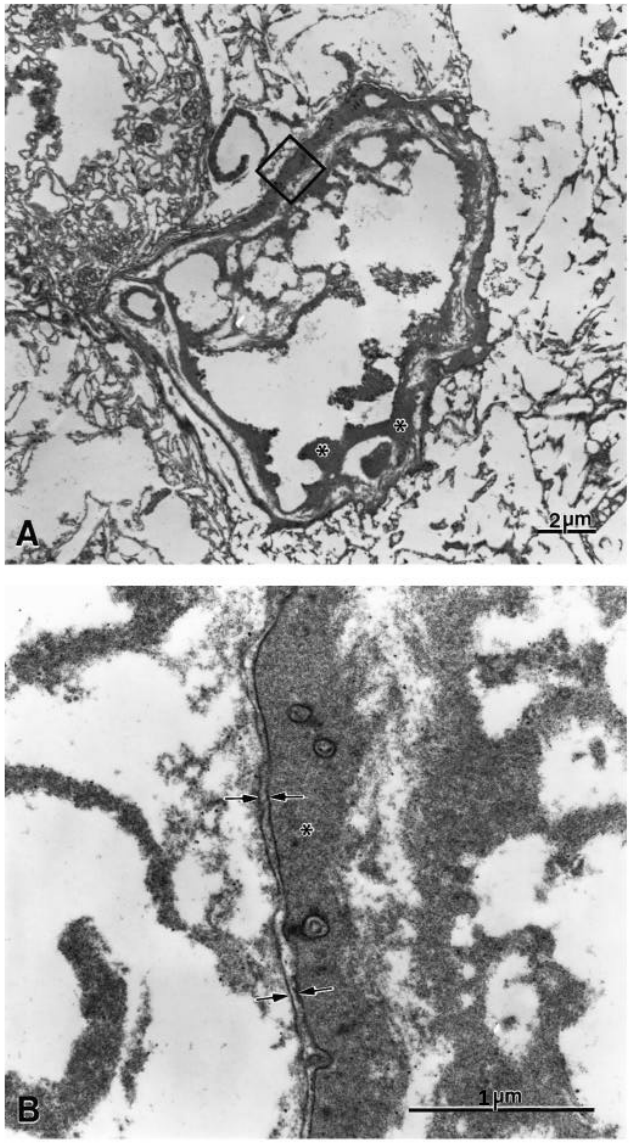

Fig. 3. Medulla cell of human hair extracted with SDS and DTT for 6 hours at 100°C. Inside the medulla cell in A, numerous amorphous deposits are evident (*). (B) Higher magnification of the cell periphery (boxed in A) showing that the cell is bounded by distinct electron dense thickened plasma membranes (arrows) resembling those in epidermal corneocytes.

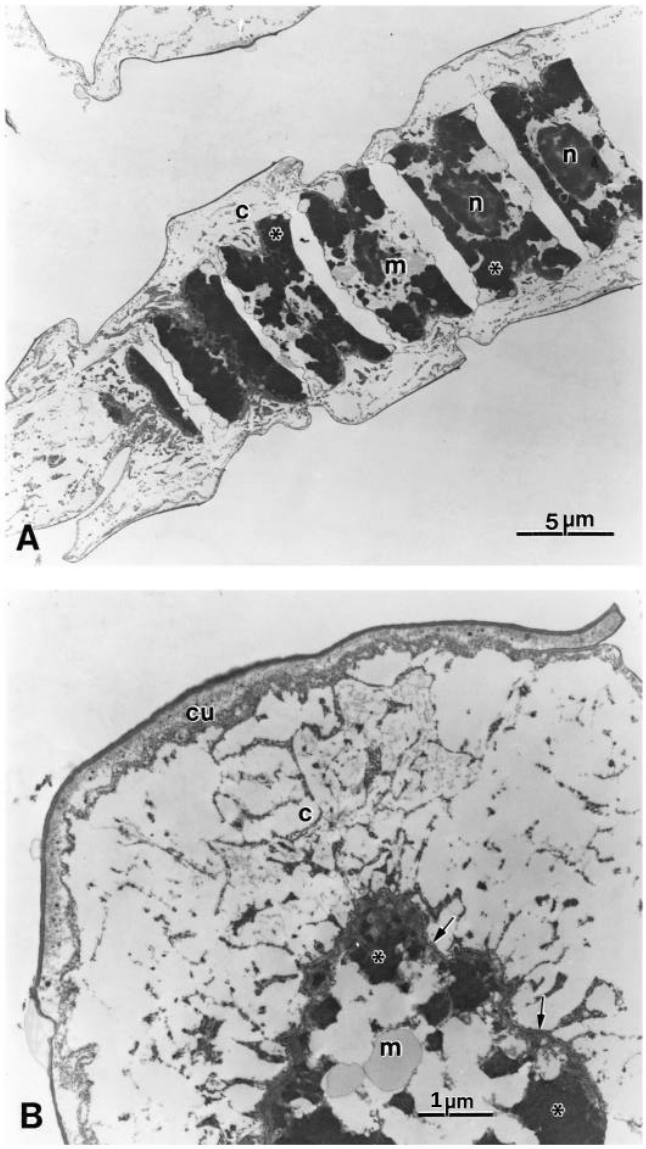

Fig. 4. Mouse hair extracted in SDS and DTT for 6 hours at 100°C. (A) Longitudinal section showing medulla cells with large deposits (*) and remnant nuclei (n) and a more completely extracted cortex (c). (B) Cross-section showing thickened plasma membranes (arrows) at the periphery of a medulla cell. The cortex (c) is much more completely extracted than the medulla (m) or cuticle (cu).

the thickness (Table 3), and seemed more fragmentary than in human hair, and the contents of cuticle cells appeared partially extracted.

Feather

Cross-sections of feather rachis treated with SDS but not DTT (Fig. 5A) resembled those observed by routine histological treatment (Spearman, 1966). The central medulla with large empty cells was surrounded by the cortex and cuticle of keratinized cells that varied in thickness and electron density. The barbs, radiating from opposite sides of the rachis shaft, also displayed a central medulla of empty cells surrounded by a cortex of electron-dense keratinized cells (Fig. 5B). The barbules protruding from them appeared resistant to SDS extraction, but addition of DTT produced a dramatic swelling of the barbs and barbules (Fig. 5C,D). Extracted barbs consisted primarily of structures resembling cornified epidermal cell envelopes. Some of these envelopes, especially at the outer edge of the barb, were thickened (Table 3) and displayed groups of small projections (Fig. 5D). Barbules extracted with DTT also consisted of envelopes, one edge of which was greatly thickened. In both cases, the thickened envelope resembled the marginal band of human hair cuticle in appearance.

Collapse of the rachis occurred upon extraction with SDS and DTT, even with treatment at room temperature. The collapse was faster and more complete with feathers from some species (pigeon) than others (white pelican, double crested cormorant, great egret). While this phenomenon prevented precise localization of the residual structures, detergent-resistant envelopes were evident even with pigeon feathers. In addition to scattered amorphous material, two types of envelope structures were observed in the pelican samples (Fig. 6A). One resembled in appearance and thickness (20-30 nm) the envelope structures at the borders of human hair medulla cells (Table 3). The other resembled in thickness (~70 nm) the asymmetric marginal band present on the outer side of the barb (Table 3), but it was clearly visible on the cytoplasmic sides of apposing cell borders at high magnification (Fig. 6B).

Horny Teeth

Hagfish teeth, which resemble human fingernail in hardness and cohesiveness, became swollen and flexible after treatment with detergent and reducing agent. Sections of the extracted material permitted ready visualization at low magnification of cell boundaries in a regular array (Fig. 7A inset). Intracellular proteins were largely removed by heating in SDS and DTT, leaving electron-dense material at the borders resistant to extraction. In certain regions, the cell boundaries were highly convoluted (Fig. 7A). A distinctive feature of the borders in most places (but not everywhere) was that only a single layer of material ~60 nm thick (Table 3) could be distinguished separating the cells (Fig. 7B), unlike the double layer present in

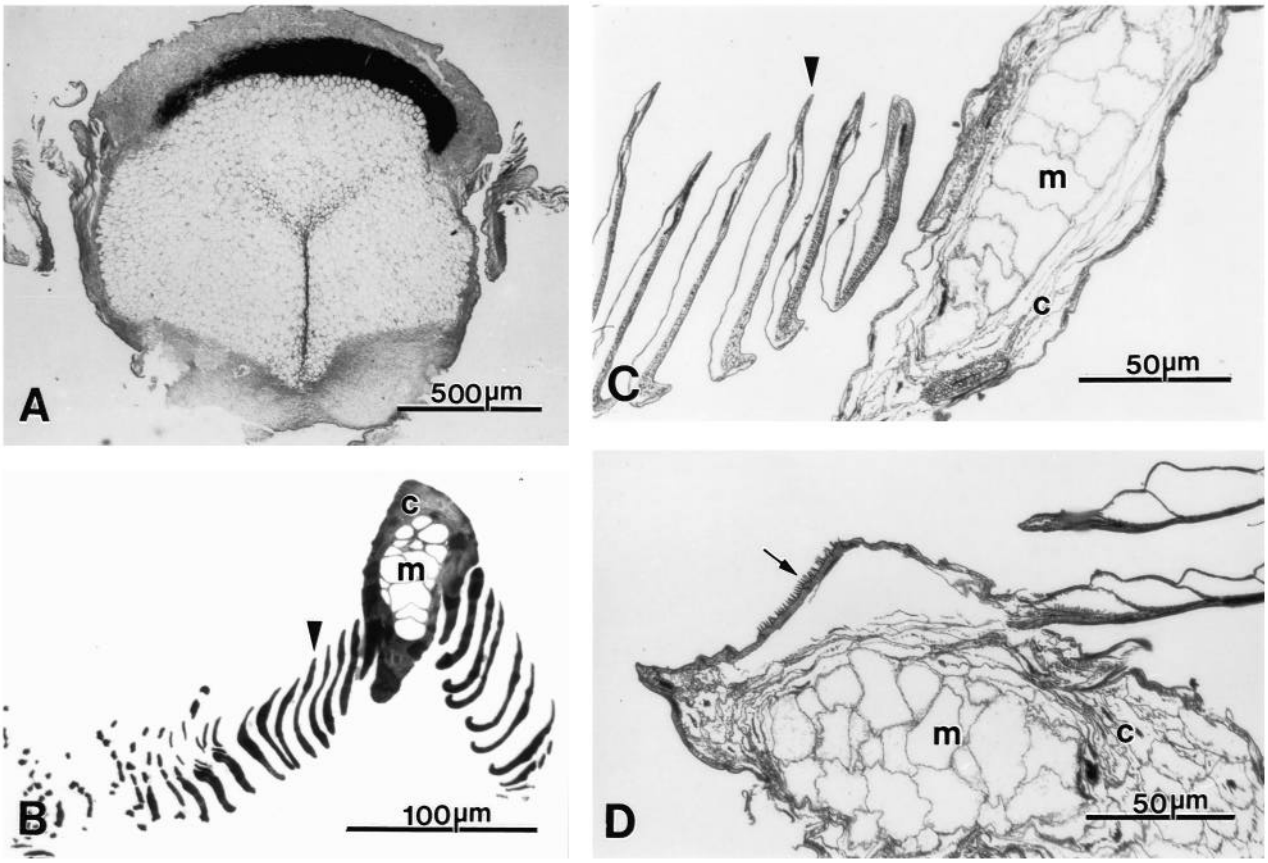

Fig. 5. Pelican feather extracted 6 hours at 100°C with SDS (A,B) or SDS and DTT (C,D). Illustrated are cross-sections of rachis (A) and barbs (B-D), the latter showing the medulla (m), cortex (c) and barbules (arrowheads). The small arrow points to a thickened envelope with attached projections.

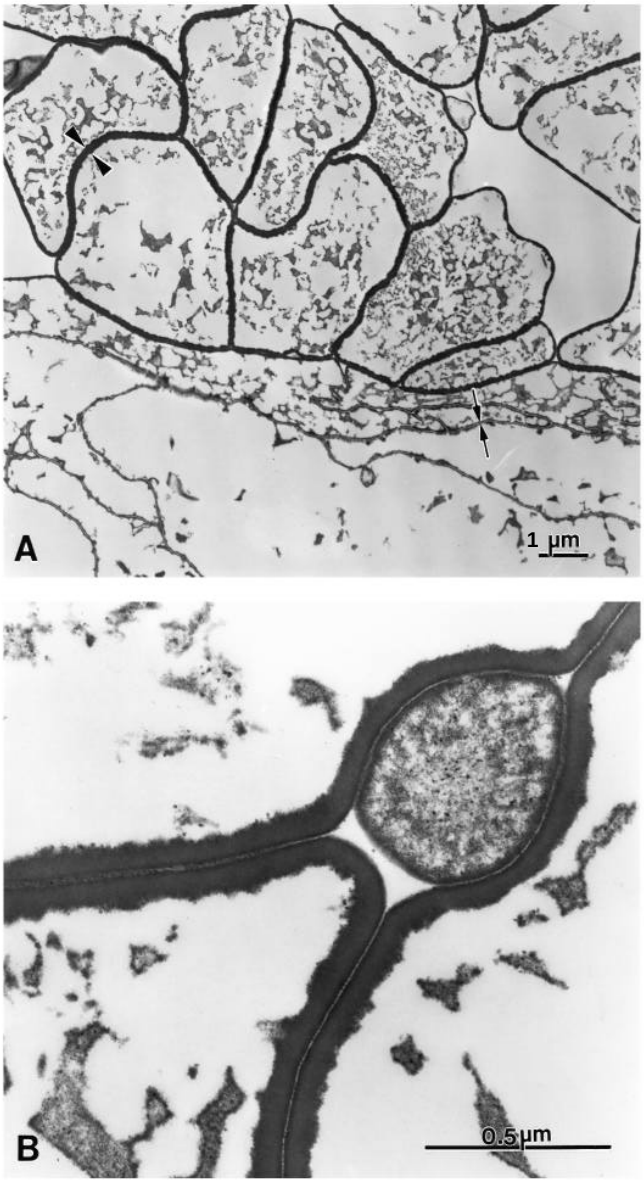

Fig. 6. Pelican feather rachis extracted with SDS and DTT at 100°C for 6 hours. (A), showing the two types of cell envelopes observed, ones resembling the doublet characteristic of mammalian epidermis (arrows) and those considerably thicker (arrowheads). (B), Higher magnification of the latter feature showing a distinct space between cell borders.

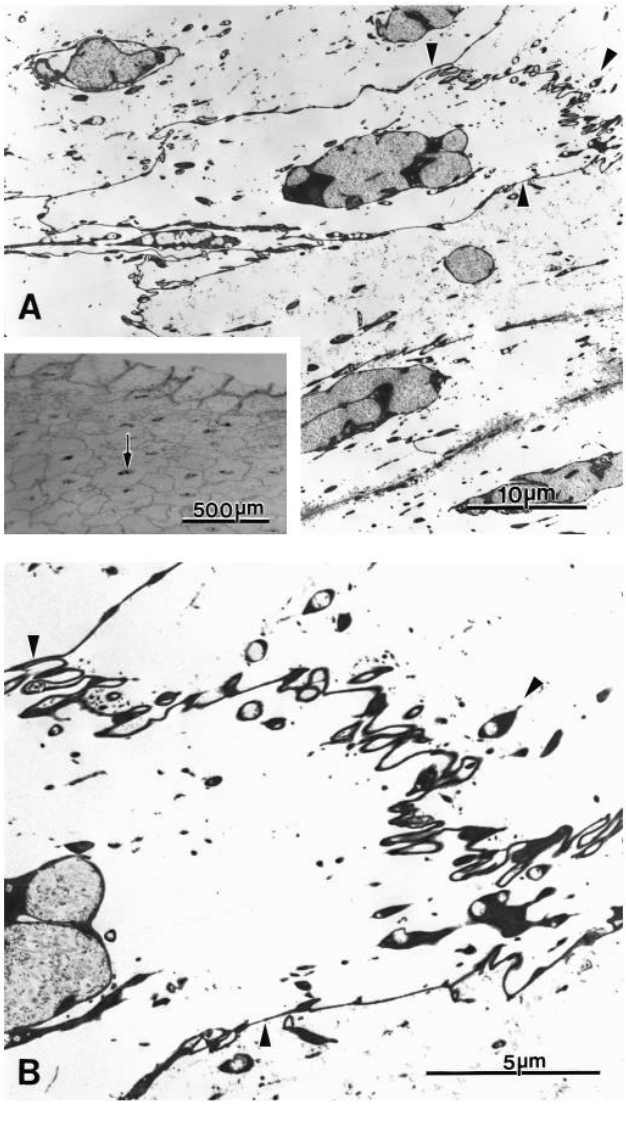

Fig. 7. Hagfish teeth extracted with SDS and DTT at 100°C for 6 hours. (A) Cross-section showing nuclear remnants (n) and cell envelopes (arrowheads). Inset: low magnification showing honeycomb array of cell borders enclosing nuclear remnants (arrow). (B), higher magnification of region bounded by arrowheads in A showing cell envelope convolutions and lack of electron-lucent space between cells.

human callus and nail, one from each apposing cell. Also unlike cells of callus and nail plate, which are enucleate, cells of the teeth had prominent nuclei that resisted extraction.

DISCUSSION

Cornified epidermal appendages, particularly mammalian hair, have long been known to contain a considerable fraction of their constituent protein in a form that is not readily extractable even under harsh denaturing conditions (Ward and Lundgren, 1954), a property that contributes to the remarkable tensile strength these structures display. In hair, the cuticle clearly has a major role in preserving the integrity of the shaft, consistent with its high degree of isopeptide cross-linking (Zahn et al., 1980). The cohesiveness of the cortex is attributable not only to its high content of intermediate filaments and associated matrix proteins but also to interdigitations among the cells (Rogers, 1959a). Cortical cells are separated by a uniform layer of electron-dense material, reportedly highly resistant (like cuticle cell contents) to extraction by sequential treatment with thioglycollic acid and ammonia, which is hypothesized to bind the cells firmly together (Rogers, 1959b). Although variable in continuity depending upon species, the medulla resembles the rest of the hair shaft in stability by virtue of its high content of isopeptide cross-linking (Harding and Rogers, 1971).

From the present work, it is evident that detergent-resistant envelope structures observed in epidermis and hair also occur in feather and horny teeth. Mature avian epidermal cells are known to display cornified envelopes (Matoltsy, 1969). Little has been reported of feather structure in this respect, although intercellular material in seagull feather barbs is extractable under denaturing conditions (Filshie and Rogers, 1962). Histological evidence indicates that certain teleost species can synthesize keratinized epidermis (Wiley and Collette, 1970), suggesting they are capable of cross-linked envelope formation as well. Demonstration of such envelopes in hagfish teeth, presumably derived from oral epithelium, supports the notion that transglutaminase-mediated cross-linking has been associated with epidermis and its appendages at least since the common ancestor of fish and terrestrial animals.

A major difficulty in elucidating the biosynthesis of complex structures such as mature hair and feather is their insolublity. To this end, the substantial isopeptide cross-linking observed, diagnostic of enzyme-mediated stabilization, indicates that identification of responsible transglutaminases and their substrates will be of considerable assistance. The general similarity in amino acid compositions among the extraction-resistant residues studied could reflect similarities in component proteins. However, the composition of the residue from foot callus in this work was considerably different from that reported for cornified envelopes from heat separated foreskin epidermis (Hohl et al., 1991). In a preliminary experiment, the amino acid composition of peeled human arm sunburn extracted and analyzed in parallel (and dorsal thumb blister in a later experiment) was different from callus and virtually identical, including a high glycine content (31%), to that reported for the foreskin envelopes. We speculate that the rather bland compositions observed presently could result from participation of a variety of proteins, perhaps depending upon the anatomic site, physiological state, or stimulus to undergo terminal differentiation, and in any case could reflect modification by endogenous proteolysis.

At present, three intracellular transglutaminases have been reported in hair follicles (Lichti, 1991; Kim et al., 1993). One is primarily membrane-bound and hence could be responsible for envelope formation, while the other two are soluble and thus could account for cytoplasmic and even nuclear cross-linking. The responsibilities may not be so clearly compartmentalized, since the soluble forms could contribute to strengthening of envelopes. Moreover intercellular cross-linking likely occurring in hair cortex and horny teeth cannot readily be ascribed to either isozyme. Such action could be carried out by an extracellular activity yet to be detected in such locations, precedent for which exists in the secreted factor XIII and the glycolipid-anchored isozyme described from rat coagulating gland (Seitz et al., 1991).

Results of the present work demonstrate that the cross-linked features of epidermal appendages can be visualized after a simple extraction with SDS and DTT. Since perturbation of these features may result in decreased cohesiveness or tensile strength, examination of afflicted appendages (such as fragile hair) after extraction may provide a convenient diagnostic test for important structural alterations. Supplementing further study of transglutaminases and their substrate proteins, such a test may prove useful in elucidating development of the appendages and certain disease processes to which they are subject.

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