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# Chicken Corneocyte Cross-Linked Proteome

Robert H. Rice,*[,†] Brett R. Winters,[†] Blythe P. Durbin-Johnson,[‡] and David M. Rocke[‡]

[†]Departments of Environmental Toxicology and [‡]Public Health Sciences, University of California Davis, Davis, California 95616, United States

Supporting Information

**ABSTRACT:** Shotgun proteomic analysis was performed of epidermal scale, feather, beak and claw from the domestic chicken. To this end, the samples were separated first into solubilized and particulate fractions, the latter enriched in isopeptide cross-linking, by exhaustive extraction in sodium dodecyl sulfate under reducing conditions. Among the 205 proteins identified were 17 keratins (types $\alpha$ and $\beta$), 51 involved in protein synthesis, 8 junctional, 8 histone, 5 heat shock, and 5 14-3-3 proteins. Considerable overlap among the beak, claw, feather, and scale samples was observed in protein profiles, but those from beak and claw were the most similar. Scale and feather profiles were the most distinctive, each exhibiting specific proteins. Less than 20% of the proteins were found only in the detergent-solubilized fraction, while 34−57% were found only in the particulate fraction, depending on the source, and the rest in both fractions. The results provide the first comprehensive analysis of the content of these cornified structures, reveal the efficient use of available proteins in conferring mechanical and chemical stability to them, and emphasize the importance of isopeptide cross-linking in avian epithelial cornification.


**KEYWORDS:** *beak, claw, detergent extraction, epidermal scale, feather, isopeptide bonding, keratin, transglutaminase*

## INTRODUCTION

Corneocytes of mammalian and avian epidermis and appendages have long been known to constitute chemically resistant protein structures stabilized by disulfide and isopeptide cross-links. Considerable effort has been devoted to identifying the protein components of such structures and how they are assembled. Findings that hair proteins exhibit $\varepsilon$-($\gamma$-glutamyl)lysine isopeptide bonds[1] and that hair follicles express transglutaminase activity[2,3] provided a conceptual framework for understanding the cohesiveness of these structures and their resistance to solubilization. These findings led to an appreciation for the wide distribution of transglutaminase-mediated isopeptide bonding in nature[4] and to continuing interest in related human disease processes.[5]

While many keratins and keratin associated proteins can be solubilized from corneocytes by strong denaturants under reducing conditions, direct identification of non-extractable proteins in them has presented difficulties due to the inability to reverse isopeptide cross-linking so as to isolate the constituents. Isolation and sequencing of individual peptides from proteolytic digests of complex intracellular structures is possible, and sites of cross-linking have been deduced from peptides exhibiting more than a single amino terminus.[6] With great difficulty, a small number of proteins have been identified as corneocyte components from human epidermis[7] and cultured human epidermal cells,[8] and the presence of most has been confirmed immunochemically.[9]

Analogous to those in mammals, chemically resistant corneocyte structures containing $\varepsilon$-($\gamma$-glutamyl)lysine cross-links are visible ultrastructurally in chicken epidermis and bird feather.[10−12] Identifying the protein components of avian corneocytes participating in isopeptide bonding in avian epidermis and appendages would contribute to understanding their development and evolution. Until the recent advent of genomics, permitting compilation of protein and peptide databases, pursuing such analysis has appeared daunting. However, current advances in mass spectrometry and database searching have simplified identification of proteins in complex structures. Successful application of this approach to cross-linked proteins of the human and mouse hair shaft[13,14] has prompted the present analysis of cross-linked constituents of chicken corneocytes. The results provide a comprehensive analysis of the divergence of corneocytes at different anatomic sites.

## EXPERIMENTAL SECTION

### Sample Preparation

Samples for analysis were removed from four retired breeder hens within 2 h of sacrifice. Feather vein was cut free of rachis. Scale tissue was removed from the lower leg, heated 2.5 min in water at 55 °C, and then held 3 min in ice-cold isotonic saline,

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after which the scales were dissected free of dermis.[15] Upon removal, beak and claw were dissected clean of soft tissue after incubating at 100 °C for 5 min in 2% sodium dodecyl sulfate/0.1 M sodium phosphate, pH 7.8. Adventitious material was then removed from each sample (50 mg) by incubation three times at 100 °C for 5 min in this sodium dodecyl sulfate/phosphate buffer. Samples were separated into solubilized and insoluble fractions by extraction for 22 h at 70 °C with sodium dodecyl sulfate/phosphate buffer adjusted to 20 mM in dithioerythritol followed by pulverization with a magnetic stirring bar for 2 h and subsequent centrifugation. This extraction was conducted a total of 5 times, a procedure that stringently removes the solubilizable material from the insoluble cross-linked material in human hair shaft, nail plate, and epidermis.[16] Of the total protein solubilized, the first of these extractions removed 80−90%, the second removed up to 16%, and the third removed the remainder, up to 6%. Little protein was detected in the final two extractions. Detergent-soluble protein and the insoluble protein were reduced separately with dithioerythritol, alkylated with iodoacetamide, precipitated with 2.5 vol of ethanol, and rinsed with 67% ethanol and then 0.1 M ammonium bicarbonate. The protein was resuspended (1−5 mg/mL) in 0.5 mL of fresh ammonium bicarbonate/10% acetonitrile and digested at room temperature for 3 days using a total of 0.14 mg of reductively methylated trypsin[17] added in aliquots at daily intervals. Protein was measured with ninhydrin after digestion with sulfuric acid as described by Schiffman[18] with minor modification. In this way, the residual protein not solubilized by trypsinization was measured as 3−10% of the starting protein in solubilized or particulate fractions from feather and scale.

A total of 32 samples were submitted for mass spectrometric analysis arising from beak, claw, feather, and scale, each divided into solubilized and particulate fractions, from four individual chickens. Salts and polypeptides that resisted elution from C18 reverse phase columns were depleted from samples by solid phase extraction with Aspire RP30 C18 desalting tips (Thermo) rinsed exhaustively in order with 60% acetonitrile, 0.1% trifluoroacetic acid, the sample digest, and 0.1% trifluoroacetic before the cleaned sample was eluted with 60% acetonitrile. The samples (adjusted to equal peptide amounts by $A_{280}$) were then directly loaded onto an Agilent ZORBAX 300SB C18 reverse-phase trap cartridge that, after loading, was switched in-line with a Michrom Magic C18 AQ 200 $\mu$m × 150 mm nano-LC column connected to a Thermo-Finnigan LTQ iontrap mass spectrometer through a Michrom Advance Plug and Play nanospray source and CTC Pal autosampler. The nano-LC column was used with a binary solvent gradient; buffer A was composed of 0.1% formic acid, and buffer B was composed of 100% acetonitrile. The 120 min gradient consisted of the steps 2−35% buffer B in 85 min, 35−80% buffer B in 23 min, hold for 1 min, 80−2% buffer B in 1 min, then hold for 10 min, at a flow rate of 2 $\mu$L/min for maximal separation of tryptic peptides. An MS survey scan was obtained for the $m/z$ range 375−1400, and MS/MS spectra were acquired from the 10 most intense ions in the MS scan by subjecting them to automated low energy CID. An isolation mass window of 2 Da was used for the precursor ion selection, and normalized collision energy of 35% was used for the fragmentation. A 2 min duration was used for the dynamic exclusion.

## Protein Identification

Tandem mass spectra were extracted with Xcalibur version 2.0.7. All MS/MS samples were analyzed using X!Tandem (The GPM, thegpm.org; version TORNADO (2010.01.01.4)). X! Tandem was set up to search the GallusUn_20110630_no-DOfb database (60902 entries) assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 1.8 Da. Iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine and tryptophan, sulfone of methionine, tryptophan oxidation to formylkynurenin of tryptophan, and acetylation of the N-terminus were specified in X!Tandem as variable modifications. Scaffold (version Scaffold_3_00_08, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90% probability as specified by the Peptide Prophet algorithm.[19] Protein identifications were accepted if they could be established at greater than 90% probability and contained at least 2 identified peptides. The false discovery rate was 2% for proteins and 0.1% for peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[20] Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principles of parsimony. Numbers of unique peptides (minimum two for identification) were tabulated as a basis for further analysis. A protein was considered present when detected in at least two of the four samples from either the solubilized or particulate fraction of at least one sample category (beak, claw, feather, scale). As previously noted,[16] isopeptide cross-linked peptides would not be detected in database searches since the sites of protein cross-linkage are not known. Since only a small fraction of lysines (<20% in epidermal callus) participate in these linkages, identifications are hampered to only a small degree.

## Statistical Analysis

Poisson regression allowing for overdispersion (specifically, quasipoisson regression) was used to model the number of unique peptides for each protein as a function of body part (beak, claw, feather, or scale) of the chicken.[21] For proteins where the global test of a body part effect was significant (indicating that differences in number of unique peptides existed between at least one pair of body parts, but not which ones), posthoc testing of all pairwise comparisons was conducted using the Tukey HSD method. Data from the solubilized and particulate fractions were analyzed separately. Proteins that were absent in more than 75% of the 32 samples were excluded from the analysis, leaving 100 proteins in the analysis from the original 224. All analyses were conducted in R, version 2.13.0,[22] using the function glm. Hierarchical clustering was performed using the hclust function in the R statistical software environment using the complete linkage method as described in http://nlp.stanford.edu/IR-book/html/htmledition/single-link-and-complete-link-clustering-1.html.[23]

## Proteomics Data Set

The data associated with this manuscript may be downloaded from ProteomeCommons.org Tranche using the following hash:

sF/sHwzqsFZOZwEdIjSAJnsqYEWQfJkRUkdMqrG-Xswk3iLiHbaq/Y5eiUFdL8neeKb40UZUpq4Rs2LbiQCs-kO3ol2+0AAAAAAAAD3Q==

The hash may be used to prove exactly what files were published as part of this manuscript's data set, and the hash may also be used to check that the data have not changed since publication.

## RESULTS

Of the total protein in the samples, 7.1 ± 0.6% was in the particulate fraction in feather, 5.8 ± 0.6% in scale, 2.5 ± 0.5% in beak, and 1.5 ± 0.3% in claw. A compilation of results from all particulate and solubilized samples revealed a total of 205 identified proteins originating from various intracellular compartments (Supplemental Table S1). Of these, 81% corresponded to known proteins in mammals. In addition to keratins (17), numerous ribosomal proteins (38), initiation/elongation factors (13), junctional proteins (8), histones (8), heat shock proteins (5), and 14-3-3 proteins (5) were identified. When they were stratified for their presence in the particulate fraction only, solubilized fraction only, or in both fractions, 13−19% were seen only in the solubilized fraction, while 34−57% were seen only in the particulate fraction, and 24−53% were seen in both fractions depending on the sample category.

The most proteins were observed in samples from feather (143) and beak (140), while fewer proteins were identified in the claw samples (97) and the fewest in scale (52) as illustrated in Table 1. These constituents were subjected to hierarchical clustering analysis. For this purpose, the proteins were compared on the basis of presence in any fraction. In the extreme case, a protein found only in the solubilized fraction from one sample category and only in the particulate fraction of another sample category (two such cases observed) were not considered a mismatch. A compilation of mismatches in pairwise comparisons is given in Table 2. In this way, the claw and feather samples displayed the fewest mismatches (53), while scale and feather displayed the most (129). A hierarchical representation of the mismatch distances is given in Figure 1.

**Table 1. Proteins Detected in Chicken Corneocytes[a]**

| sample | particulate (%) | solubilized (%) | both (%) | total (%) |
|---|---|---|---|---|
| beak | 52 (37) | 21 (15) | 67 (48) | 140 |
| claw | 97 (34) | 33 (13) | 51 (53) | 97 |
| feather | 82 (57) | 27 (19) | 34 (24) | 143 |
| scale | 22 (42) | 9 (17) | 21 (40) | 52 |

[a]A protein was counted as detected when at least two unique peptides in at least two samples of a given category and fraction were identified.

**Table 2. Mismatches in Pairwise Comparisons of Sample Categories[a]**

| category | claw | feather | scale |
|---|---|---|---|
| beak | 53 | 107 | 97 |
| claw | | 101 | 65 |
| feather | | | 129 |

[a]A protein was considered present whether it was detected in the particulate or solubilized fraction.

Visual inspection of the profiles revealed considerable differences among the proteins in expression level according to sample category. Some of the more striking contrasts are illustrated in Figure 2. Among the prominent proteins (those with many unique peptides), a dozen showed distinctly different values in the feather samples compared to the others (Figure 2A,B), while a half dozen displayed striking differences in the scale samples compared to the rest (Figure 2C), and several showed higher levels in the beak and claw compared to feather and scale (Figure 2D).

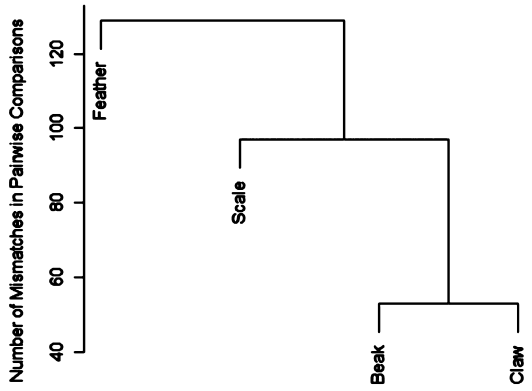


**Figure 1.** Hierarchical clustering of sample categories based on mismatches among the 205 detected proteins.

To document such differences more accurately, the solubilized and particulate protein fractions were analyzed separately for significant differences among the four sample categories (Supplemental Table 2). As summarized in Tables 3 and 4, each category (beak, claw, feather, scale) displayed significant pairwise differences. Of the 100 proteins amenable to such analysis, 21% exhibited significant differences on average in each fraction, and the differences ranged from 18 to 24% for the sample categories averaged between the solubilized and particulate fractions.

The higher yield of TGM3 (transglutaminase 3) in beak and claw compared to feather and scale (Figure 2D) raised the possibility that proteins could differ in distribution between solubilized and particulate fractions in sample categories due to different levels of enzymatic isopeptide cross-linking activity. Among the 35 proteins with the highest number of unique peptides detected, half were expressed in all four categories in sufficient amount to offer meaningful comparison. Four keratins exhibited variable degrees of inclusion in the particulate fraction but were not indicative of lower transglutaminase activity in feather and scale (Figure 3A). Overall, the degree of incorporation into particulate matter was comparable in the categories among several junctional and histone (Figure 3B), cytosolic (Figure 3C), and uncharacterized (Figure 3D) proteins, probably reflecting the presence of redundant transglutaminases. For example, low peptide levels of TGM6 were detected in the insoluble fraction of beak samples. Although TGM1 was not identified in this work, a membrane-associated activity in chicken epidermis is readily observable.[24]

## DISCUSSION

Initial[25] and numerous subsequent determinations of amino acid composition of chemically resistant protein from the integument have indicated that constituents differ among species and even, in humans, among anatomic sites. Indeed, the distinct protein profiles of human hair shaft and nail plate bear out earlier findings of differences in their keratin expression.[26] The present data demonstrate considerable differences in protein profiles among the terminally differentiated cells of cornified avian epidermal structures. Similar study of the rachis will be of interest to provide a more complete picture of


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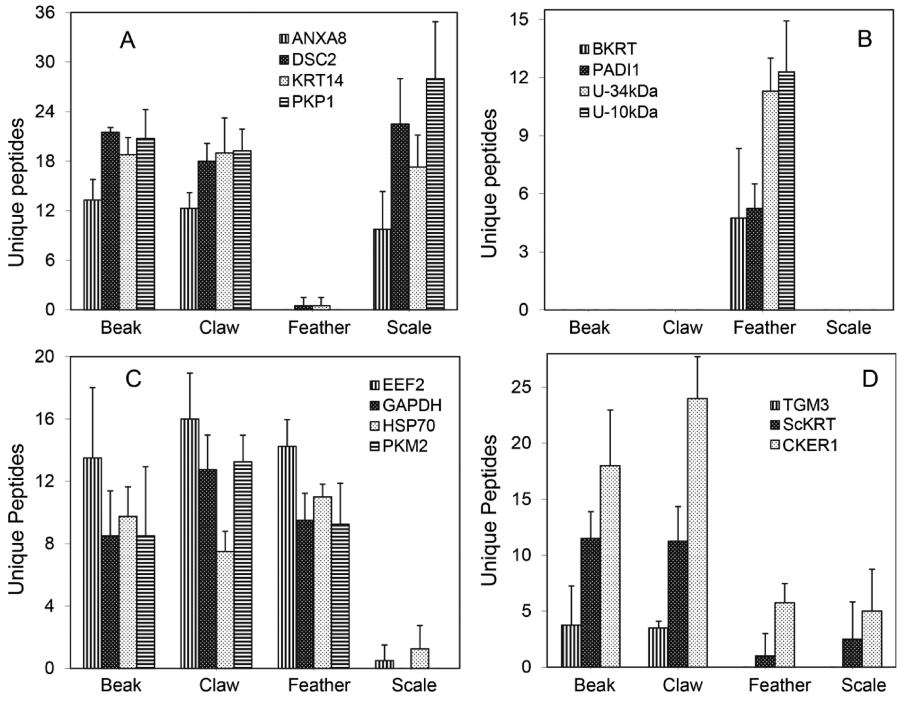


**Figure 2.** Comparison of protein expression in beak, claw, feather, and scale samples. The numbers of unique peptides are given as means and standard deviations of four samples, one from each of the four chickens. For simplicity, since many of the proteins were found in both solubilized and particulate fractions, only one larger fraction is shown. Panel A illustrates four proteins (particulate fractions) expressed at very low levels in feather samples compared to the others, while panel B illustrates four (solubilized fractions) detected at much higher levels in feather then the other samples. Panel C shows four proteins (particulate fractions) that were expressed only at low levels in scale compared to the other sample types. Panel D shows 3 proteins (solubilized fractions) present at higher levels in beak and claw than in feather and scale. Standard abbreviations used are ANXA8 (annexin A8), CKER1 (claw keratin 1), DSC2 (desmocollin 2), EEF2 (eukaryotic elongation factor 2), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), HSP70 (heat shock protein 70 kDa), KRT14 (keratin 14), PADI1 (protein arginine deiminase 1), PKP1 (plakophilin 1), PKM2 (pyruvate kinase M2) and TGM3 (transglutaminase 3). Other abbreviations are BKRT ($\beta$-keratin), U-34 kDa (uncharacterized, 34 kDa), U-10 kDa (uncharacterized, 10 kDa), and ScKRT (scale keratin).

**Table 3. Significant Differences in Expression Levels among Prominent Particulate Proteins**[a]

| category | claw | feather | scale |
|---|---|---|---|
| beak | 14 | 19 | 22 |
| claw | | 23 | 23 |
| feather | | | 22 |

[a]Among the 100 proteins that were absent in less than 75% of the samples, the 74 particulate proteins exhibiting significant global differences ($P < 0.05$) by quasipoisson regression in numbers of unique peptides were subjected to pairwise comparisons. The values tabulated show the numbers of pairwise differences.

**Table 4. Significant Differences in Expression Levels among Prominent Solubilized Proteins**[a]

| category | claw | feather | scale |
|---|---|---|---|
| beak | 26 | 29 | 25 |
| claw | | 17 | 13 |
| feather | | | 16 |

[a]Among the 100 proteins that were absent in less than 75% of the samples, the 78 solubilized proteins exhibiting significant global differences ($P < 0.05$) by quasipoisson regression in numbers of unique peptides were subjected to pairwise comparisons. The values tabulated show the numbers of pairwise differences.

divergent pathways within the feather. These profiles provide a valuable source of biochemical markers for investigating developmental processes such as differential gene induction as a result of epithelial-mesenchymal interactions. Use of specific markers may permit more precise analysis of manipulations of stem cells and keratinocyte cultures,[27] perhaps leading to deeper understanding of the evolution and regeneration of these appendages.[28]

The evolution of tetrapod epidermis and appendages as teleost fish adapted to survival on land has provided an intriguing puzzle. Positioned between the marine and the mammalian lineages, birds provide a good contrast to the rest of the reptile lineage. Developmental and morphological studies over many years have led to the conclusion that claw and then feather in avians evolved from scale.[29,30] Biochemical evidence for such inferences has relied heavily on studies of the $\beta$-keratins, which have been proposed to function similarly to the keratin-associated proteins in hair[31] and whose continued evolution likely assisted establishment of powered flight.[32] Present results are compatible with the proposed evolutionary pathway but identify other constituents contributing to evolution of present day structures. Moreover, a common feature in scale and appendages is the considerable degree to which many proteins participate in isopeptide bonding. This observation contrasts with a current model of isopeptide cross-linking in human epidermal corneocytes that assumes a small number of components.[33]

A recurring theme in evolution is the adaptation of pre-existing components for novel functions. An excellent example is the recent finding in reptile genomes of hair-like keratins, previously thought to be an innovation of the mammalian


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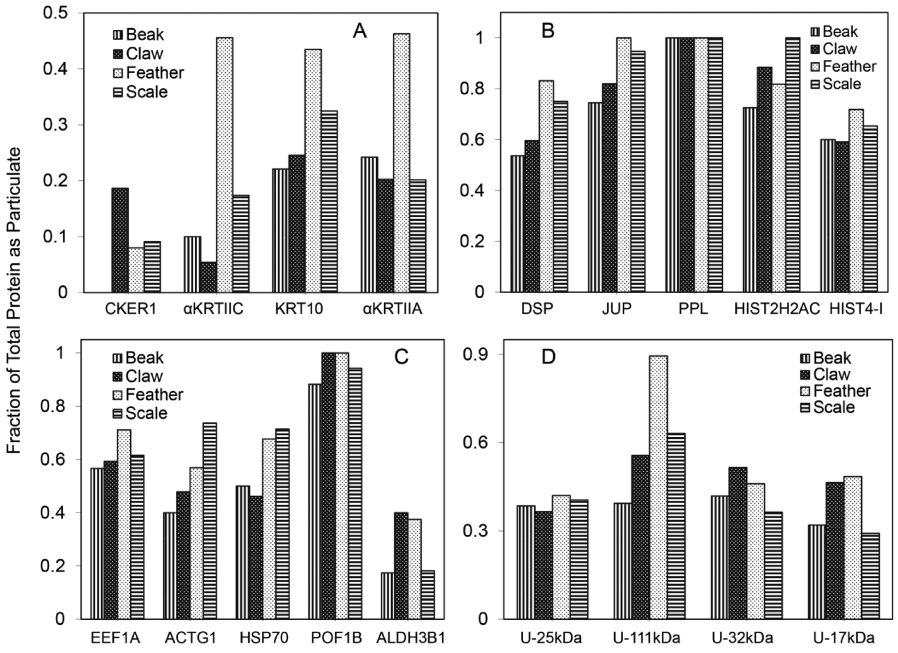


**Figure 3.** Distribution of prominent proteins between particulate and solubilized fractions from beak, claw, feather, and scale. Illustrated are the fractional amount as particulate protein from several (A) keratins, (B) histones and junctional proteins, (C) cytosolic proteins, and (D) uncharacterized proteins. The latter have chicken Uniprot accession numbers E1BZZ9 (U-25 kDa), F1NHU4 (U-111 kDa), F1NNW3 (U-32 kDa), and E1C8E5 (U-17 kDa). Standard abbreviations used are ACTG1 (cytoplasmic actin 2), ALDH3B1 (aldehyde dehydrogenase 3B1), CKER1 (claw keratin 1), DSP (desmoplakin), EEF1A (eukaryotic elongation factor 1A), HIST2H2AC (histone 2H2AC), HIST4-I (histone 4-I), HSP70 (heat shock protein 70 kDa), JUP (junctional plakoglobin), KRT10 (keratin 10), αKRTIIA (alpha keratin IIA), αKRTIIC (alpha keratin IIC), POF1B (premature ovarian failure protein 1B), and PPL (periplakin).

lineage.[34] Beyond the morphological changes that occurred in their diversification, the use of isopeptide cross-links that stabilize epithelial structures appears to have been an important factor in developing the cornified features of terrestrial epidermis. This feature appears to have long been present in teleosts, since it is inferred by morphology in contact organs and breeding tubercles of numerous species[35] and the lip of carp[36] as a protection from abrasive damage, and it is known to occur during egg envelope hardening.[37,38] However, agnathans (hagfish, lamprey) exhibit cornified teeth[39] with abundant isopeptide cross-links,[11] suggesting the process of cornification went through even earlier adaptation. Tracing the molecular evolution of transglutaminases and their substrates in such aquatic species may provide a valuable perspective on adaptations permitting life on land. In the chicken, as in mammalian nail plate and hair shaft,[26] isopeptide bonding permits efficient use of available proteins to help confer great mechanical and chemical stability to cornified structures. Thus transglutaminase diversification appears to have provided an important contribution to evolution of epithelial cornification and the emergence of terrestrial tetrapods.

## ■ ASSOCIATED CONTENT

### Supporting Information

Profiles of protein constituents in insoluble and solubilized fractions and statistical testing of insoluble and solubilized fractions This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Tel: 530-752-5176. Fax: 530-752-3394. E-mail: rhrice@ucdavis.edu.

### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

(1) Harding, H. W. J.; Rogers, G. E. ε-(γ-Glutamyl)lysine cross-linkage in citrulline-containing protein fractions from hair. *Biochemistry* **1971**, *10*, 624−630.

(2) Chung, S. I.; Folk, J. E. Transglutaminase from hair follicle of guinea pig. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 303−307.

(3) Harding, H. W. J.; Rogers, G. E. Formation of the ε-(γ-glutamyl) lysine cross-link in hair proteins. Investigation of transamidases in hair follicles. *Biochemistry* **1972**, *11*, 2858−2863.

(4) Folk, J. E.; Finlayson, J. S. The ε-(γ-glutamyl)lysine crosslink and the catalytic role of transglutaminases. *Adv. Protein Chem.* **1977**, *31*, 1−133.

(5) Iismaa, S. E.; Mearns, B. M.; Lorand, L.; Graham, R. M. Transglutaminases and disease: lessons from genetically engineered mouse models and inherited disorders. *Physiol. Rev.* **2009**, *89*, 991−1023.
(6) Steinert, P. M.; Marekov, L. N. The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope. *J. Biol. Chem.* **1995**, *270*, 17702−17711.
(7) Nemes, Z.; Steinert, P. M. Bricks and mortar of the epidermal barrier. *Exp. Mol. Med.* **1999**, *31*, 5−19.
(8) Robinson, N. A.; Lapic, S.; Welter, J. F.; Eckert, R. L. S100A11, S100A10, annexin I, desmosomal proteins, small proline-rich proteins, plasminogen activator inhibitor-2, and involucrin are components of the cornified envelope of cultured human epidermal keratinocytes. *J. Biol. Chem.* **1997**, *272*, 12035−12046.
(9) Steinert, P. M.; Marekov, L. N. Initiation of assembly of the cell envelope barrier structure of stratified squamous epithelia. *Mol. Biol. Cell* **1999**, *10*, 4247−4261.
(10) Matoltsy, A. G. Keratinization of the avian epidermis. An ultrastructural study of the newborn chick. *J. Ultrastuct. Res.* **1969**, *29*, 438−458.
(11) Rice, R. H.; Wong, V. J.; Pinkerton, K. E. Ultrastructural visualization of cross-linked protein features in epidermal appendages. *J. Cell Sci.* **1994**, *107*, 1985−1992.
(12) Alibardi, L. Structural and immunocytochemical characterization of keratinization in vertebrate epidermis and epidermal derivatives. *Int. Rev. Cytol.* **2006**, *253*, 177−259.
(13) Lee, Y. J.; Rice, R. H.; Lee, Y. M. Proteome analysis of human hair shaft: From protein identification to posttranslational modification. *Mol. Cell. Proteomics* **2006**, *5*, 789−800.
(14) Rice, R. H.; Rocke, D. M.; Tsai, H.-S.; Lee, Y. J.; Silva, K. A.; Sundberg, J. P. Distinguishing mouse strains by proteomic analysis of pelage hair. *J. Invest. Dermatol.* **2009**, *129*, 2120−2125.
(15) Macdiarmid, J.; Wilson, J. B. Separation of epidermal tissue from underlying dermis and primary keratinocyte culture. *Methods Mol. Biol.* **2001**, *174*, 401−410.
(16) Rice, R. H. Proteomic analysis of hair shaft and nail plate. *Int. J. Cosmet. Sci.* **2011**, *62*, 229−236.
(17) Rice, R. H.; Means, G. E.; Brown, W. D. Stabilization of bovine trypsin by reductive methylation. *Biochim. Biophys. Acta* **1977**, *492*, 316−321.
(18) Schiffman, G. Immunological methods for characterizing polysaccharides. *Methods Enzymol.* **1966**, *8*, 79−85.
(19) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **2002**, *74*, 5383−5392.
(20) Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **2003**, *75*, 4646−4658.
(21) McCullagh, P.; Nelder, J. A. *Generalized Linear Models*, 2 ed.; Chapman & Hall/CRC: London, 1989.
(22) R Development Core Team *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2011.
(23) Manning, C. D.; Raghavan, P.; Schütze, H. *Introduction to Information Retrieval*; Cambridge University Press: New York, 2008.
(24) Bures, D. M.; Goldsmith, L. A. Localization of transglutaminase in adult chicken epidermis. *Arch. Dermatol. Res.* **1978**, *262*, 329−332.
(25) Matoltsy, A. G., The membrane of horny cells. In *Biochemistry of Cutaneous Epidermal Differentiation*; Seiji, M., Bernstein, I. A., Eds.; University of Tokyo Press: Tokyo, 1977; pp 93−109.
(26) Rice, R. H.; Xia, Y.; Alvarado, R. J.; Phinney, B. S. Proteomic analysis of human nail plate. *J. Proteome Res.* **2010**, *9*, 6752−6758.
(27) Vanhoutteghem, A.; Londero, T.; Ghinea, N.; Djian, P. Serial cultivation of chicken keratinocytes, a composite cell type that accumulates lipids and synthesizes a novel beta-keratin. *Differentiation* **2004**, *72*, 123−137.
(28) Chuong, C. M.; Randall, V. A.; Widelitz, R. B.; Wu, P.; Jiang, T. X. Physiological regeneration of skin appendages and implications for regenerative medicine. *Physiology* **2012**, *27*, 61−72.
(29) Alibardi, L. Claw development and cornification in the passeraceous bird zebrafinch (*Taeniatopygia guttata castanotis*). *Anat. Sci. Int.* **2009**, *84*, 189−199.
(30) Greenwold, M. J.; Sawyer, R. H. Genomic organization and molecular phylogenies of the beta (beta) keratin multigene family in the chicken (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*): implications for feather evolution. *BMC Evol. Biol.* **2010**, *10*, 148.
(31) Alibardi, L.; Dalla Valle, L.; Nardi, A.; Toni, M. Evolution of hard proteins in the sauropsid integument in relation to the cornification of skin derivatives in amniotes. *J. Anat.* **2009**, *214*, 560−586.
(32) Greenwold, M. J.; Sawyer, R. H. Linking the molecular evolution of avian beta ($\beta$) keratins to the evolution of feathers. *J. Exp. Zool.,Part B* **2011**, *316*, 609−616.
(33) Candi, E.; Schmidt, R.; Melino, G. The cornified envelope: a model of cell death in the skin. *Nat. Rev. Mol. Cell. Biol.* **2005**, *6*, 328−340.
(34) Eckhart, L.; Valle, L. D.; Jaeger, K.; Ballaun, C.; Szabo, S.; Nardi, A.; Buchberger, M.; Hermann, M.; Alibardi, L.; Tschachler, E. Identification of reptilian genes encoding hair keratin-like proteins suggests a new scenario for the evolutionary origin of hair. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 18419−18423.
(35) Wiley, M. L.; Collette, B. B. Breeding tubercles and contact organs in fishes: their occurrence, structure and significance. *Bull Am. Mus. Nat. Hist.* **1970**, *143*, 143−216.
(36) Tripathi, P.; Mittal, A. K. Essence of keratin in lips and associated structures of a freshwater fish Puntius sophore in relation to its feeding ecology: Histochemistry and scanning electron microscope investigation. *Tissue Cell* **2010**, *42*, 223−233.
(37) Oppen-Berntsen, D. O.; Helvik, J. V.; Walther, B. T. The major structural proteins of cod (Gadus morhua) eggshells and protein crosslinking during teleost egg hardening. *Dev. Biol.* **1990**, *137*, 258−265.
(38) Ha, C. R.; Iuchi, I. Enzyme responsible for egg envelope (chorion) hardening in fish: purification and partial characterization of two transglutaminases associated with their substrate, unfertilized egg chorion, of the rainbow trout, Oncorhynchus mykiss. *J. Biochem.* **1998**, *124*, 917−926.
(39) Dawson, J. A. The oral cavity, the 'jaws' and the horny teeth of *Myxine glutinosa*. In *The Biology of Myxine*; Brodal, A., Fänge, R., Eds.; Scandinavian University Books: Oslo, 1963; pp 231−255.