Soft epidermis of a scaleless snake lacks beta-keratin

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Beta-keratins are responsible for the mechanical resistance of scales in reptiles. In a scaleless crotalus snake (*Crotalus atrox*), large areas of the skin are completely devoid of scales, and the skin appears delicate and wrinkled. The epidermis of this snake has been assessed for the presence of beta-keratin by immunocytochemistry and immunoblotting using an antibody against chicken scale beta-keratin. This antibody recognizes beta-keratins in normal snake scales with molecular weights of 15-18 kDa and isoelectric points at 6.8, 7.5, 8.3 and 9.4. This indicates that beta-keratins of the stratum corneum are mainly basic proteins, so may interact with cytokeratins of the epidermis, most of which appear acidic (isoelectric points 4.5-5.5). A beta-layer and beta-keratin immunoreactivity are completely absent in moults of the scaleless mutant, and the corneous layer comprises a multilayered alpha-layer covered by a flat oberhautchen. In conclusion, the present study shows that a lack of beta-keratins is correlated with the loss of scales and mechanical protection in the skin of this mutant snake.

Key words: snake; scaleless; beta-keratin; ultrastructure; immunocytochemistry, immunoblotting.

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The epidermis of reptiles and birds (sauropsids) produces two major groups of keratins, termed alpha- and beta-keratins. These proteins present different chemical and physical properties and tissue localizations (Baden and Maderson, 1970; Fraser *et al.*, 1972; Baden *et al.*, 1974; Wyld and Brush, 1979, 1983; Landman, 1986; Fraser and Parry, 1996; Sawyer *et al.*, 2000; Alibardi, 2003). Alpha-keratins represent the intermediate filament family (40-67 kDa in molecular weight), present in most cells, including those specialized for the formation of the stratum corneum. Beta-keratins are small proteins (10-22 kDa) that constitute the bulk of protein in terminal differentiating corneocytes of hard corneous layers of scales, scutes, claws, beak and feathers.

The importance of the beta-layer, the hard corneous layer of snake epidermis, for the mechanical resistance of the skin in reptiles has been indicated in previous studies on a colubrid scaleless snake (*Pituophis melanoleucus catenifer*), a mutant in which scales are reduced or completely absent (Licht and Bennett, 1972). These studies indicated that the skin in this mutant resembled that of a newborn mouse. The water permeability of the skin was unchanged in comparison to that of normal snake scales, while the mechanical resistance of the skin was much reduced. It is unknown whether the mutation affects the epidermis or the dermis of this scaleless snake.

The histological study showed that the skin of the mutant colubrid snake resembled the inner scale surface or hinge region of normal scales (Licht and Bennet, 1972). A thick beta-layer with a basal columnar epidermis and a loose mesenchymal dermis was missing in this mutant. The corneous layer was replaced by a thin beta-layer and a thick alpha-layer.

Detailed ultrastructural and immunological analysis of the epidermis of scaleless snakes can better identify the missing elements in mutated

scales, in particular the complex epidermal layers of snake epidermis (Maderson, 1985; Tu *et al.*, 2002; Alibardi and Toni, 2005). Another goal of the present study is to search for the presence of beta-keratins in a scaleless snake (a crotalus) using the known cross-reactivity with an antibody produced against chicken scale beta-keratin (Sawyer *et al.*, 2000; Alibardi and Sawyer, 2002, Toni and Alibardi, 2007). This study will help in future molecular studies on mutations in the gene for beta-keratin in snakes.

Materials and Methods

Immunocytochemistry and electron microscopy analysis

Moults from one normal and three scaleless mutant crotalus snakes (*Crotalus atrox*) were fixed for 3-4 hours in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4, dehydrated and embedded in Bioacryl Resin (using the chemical components indicated in Scala *et al.*, 1992). Two samples of lateral, dorsal, and ventral moults from the normal individual were used in the present study. In the scaleless specimens one sample in each studied area (lateral, dorsal and ventral moults) was analysed.

Using an ultramicrotome, 2-4 μm thick sections were collected on gelatine chromoalum-coated slides, and were incubated for 20 minutes in 0.05 M TRIS buffered saline at pH 7.6 containing 2% bovine serum albumin and 5% normal goat serum (to block non-specific antigenic sites). Sections were incubated overnight at 4°C with Beta-1, a rabbit polyclonal antibody against chicken beta-keratin (kindly supplied by Dr. R.H. Sawyer, University of South Carolina, Columbia, USA, see Sawyer *et al.* (2000)) diluted 1:200 in buffer (the controls were incubated with buffer only). Sections were rinsed and incubated for 1 hour at room temperature with a goat anti-rabbit FITC-conjugated secondary antibody (Sigma, St Louis, MI, USA) diluted 1:50 in the above buffer, rinsed and observed under a fluorescence microscope.

Thin sections (40-90 nm) of the skin or moults were collected on nickel grids for ultrastructural immunocytochemistry. Sections were incubated at room temperature for 10 minutes in Tris buffered saline at pH 7.2, containing 0.1% Triton X-100 and 1% cold-water fish gelatine (to block non-specific antigenic sites). Sections were then incubated overnight in the same buffer at 4°C with the Beta-1 antibody at a dilution of 1:200 (controls were incubated with the buffer only). After rinsing in buffer, grids were incubated for 1 hour at room temperature with the secondary anti-rabbit antibody conjugated to 10 nm gold particles (Sigma, St Louis, MI, USA) at 1:40 dilution in the same buffer. Grids were rinsed in the buffer, then in distilled water, dried, lightly stained with 2% uranyl acetate, and observed under the electron microscope (CM-100 Philips, Eindhoven, The Netherlands).

Protein extraction

Moults from normal and scaleless snake *(C. atrox)* were utilized for protein extraction and electrophoretic analysis. Moults were cleaned in 1% SDS and extensively rinsed with double distilled water. The extraction of beta-keratin from moults was done using the method of Sybert *et al.* (1995): the epidermis was homogenized in 8 M urea/50 mM Tris-HCl (pH 7.6)/0.1M 2-mercaptoethanol/ 1 mM dithiothreithol (DTT)/1 mM phenylmethylsulphonyl fluoride and the homogenate was left overnight stirring at room temperature. Particulates were removed by centrifugation at 10,000 g for 10 minutes and the protein concentration was determined by the Lowry method. Proteins were analysed by one- and two-dimensional electrophoresis and by immunoblotting using Beta-1 antibody.

SDS-PAGE and western blot analysis

For one-dimensional electrophoresis, proteins (50 μg each lane) were separated in a tricine-SDS-PAGE system as described by Schagger and Von Jagow (1987), using 10% acrylamide in the concentration gel and 16% acrylamide in the separation gel. Before initiating the two-dimensional electrophoretic separation of proteins, 2% CHAPS (Sigma, USA) and 1% carrier ampholyte mixture, pH 3.5-10 (GE Healthcare, U.K.) were added to the protein samples. The isoelectrofocusing (IEF) was carried out using 7 cm long dry polyacrylamide gel strips with an immobilized pH gradient (pH 3-10) and the Ettan IPGphor II IEF System (GE Healthcare, U.K.). A 150 μg sample was loaded as described by the manufacturer. The following protocol was used: electrically assisted re-hydration for 12 hours (120 ½A/strip at 20°C), followed by IEF with steps of ½ h 500 V, ½ h 1000 V, ½ h 8000 V (gradually), 1 h 8000 V. The hydrated strips con-

taining proteins separated according to their pI were kept at 50 V until loaded on the second dimension. Before the start of the second dimension, the strips were equilibrated in 6 M urea, 30% glycerol, 50 mM Tris pH 6.8, and 2% DTT for 10 minutes Strips were briefly rinsed in double distilled water and equilibrated in 6 M urea, 30% glycerol, 50 mM Tris pH 6.8, and 2.5% iodoacetamide for an additional 10 minutes. The second dimension was carried out in a MiniProtean III electrophoresis apparatus (Biorad, USA) using 15% polyacrylamide gels and a current of 30 mA per gel. Wide Range (M.W. 6,500-205,00) molecular weight markers (Sigma, USA) were used. After electrophoresis, gels were stained with Coomassie Blue or immunoblotted onto nitrocellulose (Hybond C+ Extra, GE Healthcare, U.K.). Membranes were stained with Ponceau Red to verify the protein transfer and incubated with Beta-1 primary antibody (dilution 1:3000) and HRP-conjugated secondary antibodies (Sigma, USA) (dilution 1:1000) diluted in TBS-TWEEN + 5% non-fat milk powder. Detection was performed by using the enhanced chemiluminescence procedure (ECL, GE Healthcare, U.K.).

Results

Moults of normal crotalus specimens showed a thick, pale pigmented beta-layer, with a dark alpha-layer beneath (Figure 1 A). The beta-layer was immunofluorescently labelled by the Beta-1 antibody (Figure 1 B).

Available samples of scaleless crotalus snake were completely devoid of scales in the lateral and dorsal regions of the body, reduced in other dorsal areas, and scaled in the ventral part where large gastrosteges were present. In moults from the lateral region the corneous layer was folded, and a paler outer layer was associated with a thicker, dark layer (Figure 1 C). No immunofluorescence labelling was seen in this epidermis using the Beta-1 antibody (Figure 1 D).

The mutant moult surface showed sparse rounded scales in lateral areas (Figure 1 E) while dorsal areas were almost devoid of scales. The ultrastructural examination of moults showed that the surface was irregular to undulated, and formed an oberhautchen-like layer (corresponding to the superficial part of the pale layer seen with the light microscope). Beneath the latter was a multi-layered mesos layer (very narrow cells with lipid inclusions, contributing to most of the pale layer seen on the light microscope), and a variable layer of alpha-cells (Figure 1 F-H), contributing to the lower, dark part of the moult seen with the light microscope. The oberhautchen-like, mesos and alpha-cells were not labelled with the Beta-1 antibody (Figure 1 F-H). In contrast, in normal scales, Beta-1 labelling was seen over the oberhautchen, merged with the beta layer, and disappeared in the underlying mesos- and alpha-layers (Figure 2).

After protein extraction, the one-dimensional protein separation of moults of both normal and scaleless Crotalus atrox stained with Ponceau red or Coomassie Blue, showed most protein bands in the 45-60 kDa range (typical of alpha-keratins; Figure 3, A1-3, B1 and C1). The comparison of both one- and two-dimensional electrophoresis patterns between normal and scaleless snakes revealed the absence in the latter of proteins at 15-18 kDa (the range of beta-keratins; compare A1 with A2-3; B1 with C1; B2 with C2). This result was confirmed using the Beta-1 keratin antibody after one-dimensional electrophoresis (Figure 3, A4-6). Moults from the lateral region of the scaleless body (which were mostly scaleless) did not produce bands in the beta-keratin range (Figure 3, A5). From the ventral side of the scaleless body (where the large gastrostege were present) the pattern (Figure 3, A6) resembled that of normal snake (Figure 3; A4), although the beta-keratin band at 15-17 kDa was less intense than that of normal scales.

A clear difference was also seen after two-dimensional protein separation and immunoblotting (Figure 3, B2-3 and C2-3). In normal *C. atrox*, the two-dimensional protein pattern stained with Coomassie Blue showed mainly acidic proteins at 50-60 kDa with pI at 4.5-5.5, while minor spots were seen at 62-64 kDa with pI at 6.4-7.0 (Figure 3, B2). The Beta-1 immunoblotting performed on the two-dimensional separation of normal *C. atrox* proteins revealed spots at 14-17 kDa with pI mainly at 6.8, 7.5, 8.3, and 9.4 (Figure 3, B3). Minor spots were seen at 24-25 kDa (pI 8.2), at 54 kDa (pI at 5.2), and 64-66 kDa (pI 4.8, 6.0 and around 7.0).

In comparison, from moults of the lateral region of the scaleless snake, some protein bands were seen in the 50-66 kDa range using Comassie blue, but not in the 14-20 kDa range (Figure 3, C1 and C2). Using the Beta-1 antibody, only weakly labelled spots at 50-60 kDa and faint spots at 15 and 20 kDa were seen (Figure 3, C3).

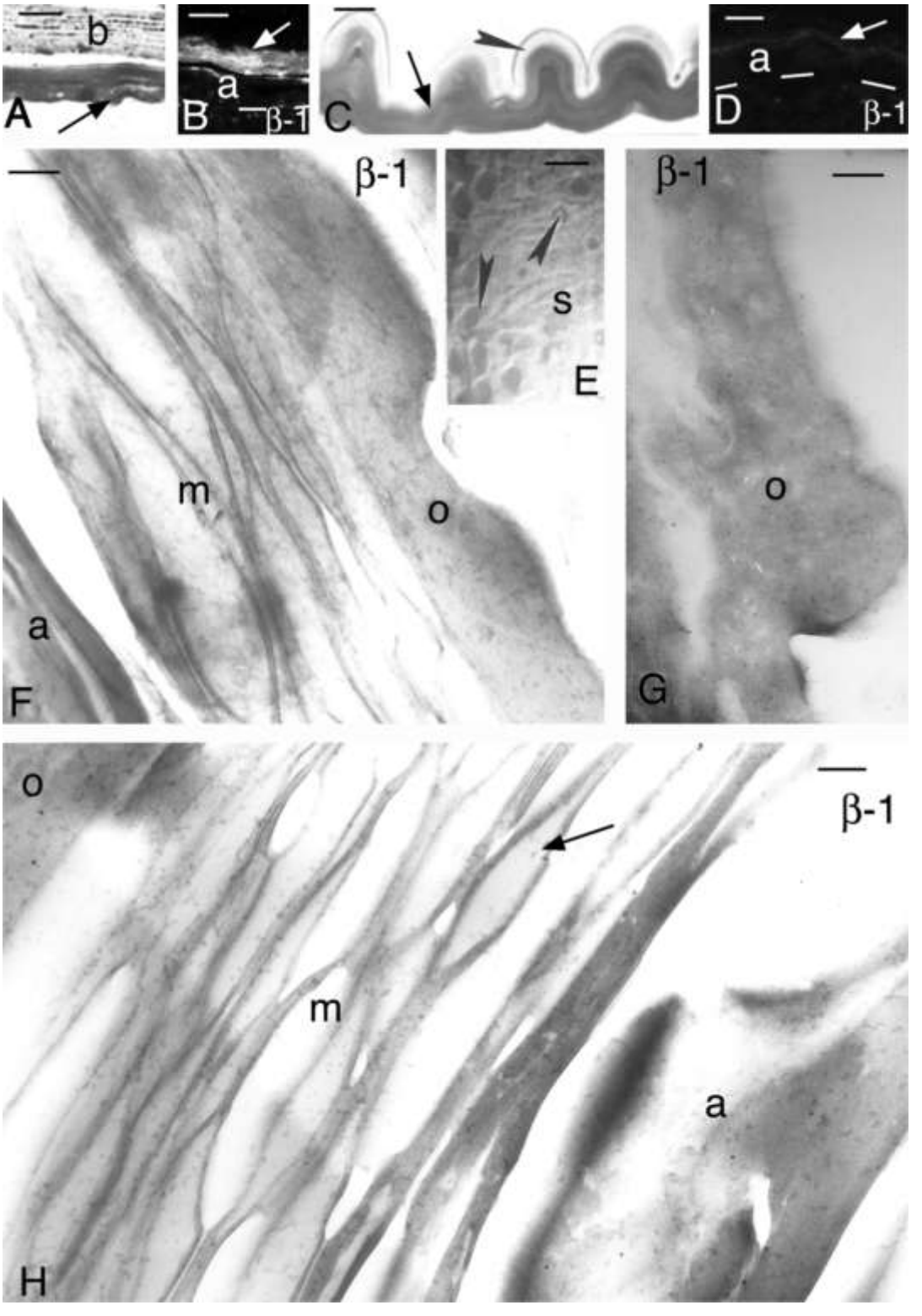

Figure 1. A, moult of normal scale showing the beta-layer (b) and the dark alpha-layer with the incisions/undulations of the mature clear layer (arrow). Toluidine blue. Bar 5 μm. B, Beta-1 immunofluorescence labelling; beta-layer (arrow) is positive while the alpha-layer (a, underlined by dashes) is negative. Bar, 10 μm. C, moult of scaleless epidermis with a pale superficial layer (arrowhead) and dark alpha-layer (arrow). Toluidine blue. Bar, 5 μm. D, Beta-1 immunonegative upper layer (arrow) and alpha-layer (a) of scaleless moult (dashes underline the alpha-layer). Bar, 10 μm. E, gross aspect of the smooth surface (s) alternated with small scale imprints (arrowheads) present on the lateral surface of the scaleless snake body. Bar, 0.5 mm. F, G, H, electron micrographs of moults immunogold-labelled with Beta-1 antibody. F, superficial part of scaleless snake moult showing undulating oberhautchen layer (o), mesos-layer (m) and alpha-layer (a). All layers are devoid of gold particles. Bar, 100 nm. G, detail of oberhautchen tip (o) of scaleless snake which is immuno-negative for the Beta-1 antibody. Bar, 100 nm. H, detail of immunonegative mesos-cells (m) of scaleless snake with lipid inclusion (arrow) and alpha-cell (a). o, superficial oberhautchen layer. Bar, 100 nm.

Discussion

During skin morphogenesis in reptiles, as in birds, dermal-epidermal interactions are required to produce normal scales (Dhouailly, 1975; Maderson, 1985; Sawyer *et al.*, 2000; Sawyer and Knapp, 2003; Alibardi, 2004). In the scaleless snake, the lack of beta-cells and beta-keratin shows a dramatic effect on the skin. The present ultrastructural study has shown a lack of beta-keratin accumulation and packing in the epidermis of the mutant snake. The result is the loss of a distended and stiff texture of the outer scale surface, which virtually disappears.

Most beta keratins, identified with the Beta-1 antibody in the 15-17 kDa range, are basic proteins (pI-7.2-9.4) that probably combine with acidic cytokeratins in cells of the differentiating beta-layer, as indicated for other species of snake (Toni and Alibardi, 2007). Some beta-keratins in reptilian epidermis, including that of snakes, are composed of 3-8 different types of glycine-proline-rich proteins, which appear to bind to cytokeratins (Dalla Valle *et al.*, 2005, 2007; Alibardi *et al.*, 2006; Alibardi and Toni, 2006). Beta-keratins replace most of the cytokeratin bundles in differentiating beta-cells, forming a dense and compact mass of corneous material of high mechanical resistance (Alibardi and Toni, 2006). It is likely that the genetic defect present in the skin of the mutant snake used in the present study inhibits the formation of scales, and indirectly also of the compact beta-layer.

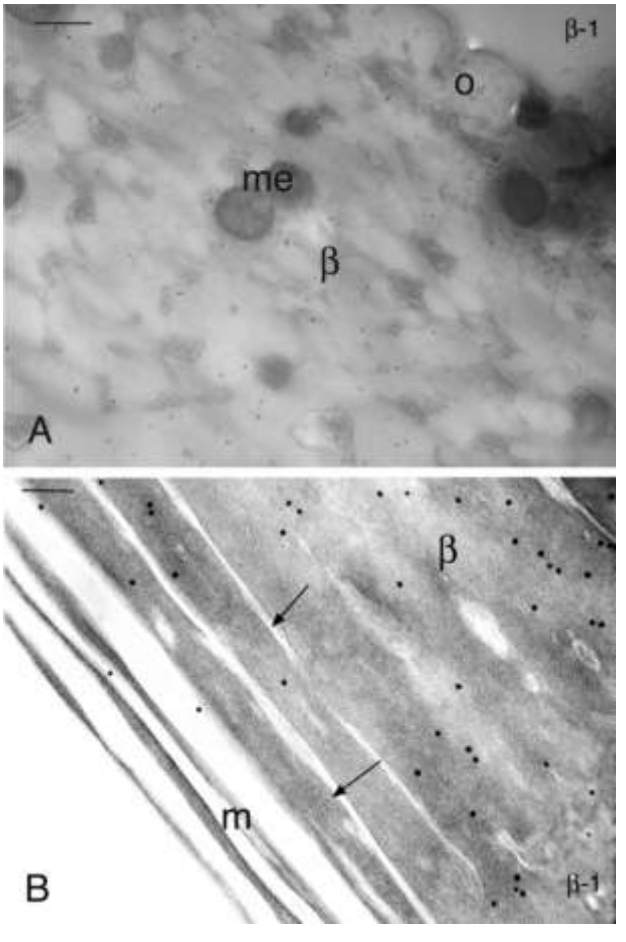

Figure 2. Ultrastructural view of normal moults after immunolabelling using the Beta-1 antibody. A, diffuse labelling in the mature beta layer which is merged with the short oberhautchen spinulae. Bar, 250 nm. B, lower part of the immunolabelled beta-layer which shows the transition with the mesos layer (immunonegative, arrows). Bar, 100 nm. β, beta-layer; m, narrow cells of the mesos-layer; me, melanosomes; o, oberhautchen.

The present study has shown in both one-dimensional and two-dimensional immunoblots some reactivity of protein bands/spots in the molecular weight and pI range for alpha-keratins (40-70 kDa, Figures 3). This result is probably due to the large amount of alpha-keratins (40-68 kDa) present in moults (Figure 3). The latter are made by both alpha- and beta-layers in normal snakes, and mainly by the alpha-layers in the scaleless snake. The large amount of protein localized in this range of molecular weights probably produces a non-specific capture of the antibody. Also, the presence of a large amount of glycine-rich sequence in some alpha-keratins of moults (AE2/AE3 positive, probably K1/K10-like cytokeratins), may be partially recognized by the Beta-1 antibody (Alibardi and Toni, 2006). This non-specific staining can be detected with variable intensity when extracted proteins are present in relatively high amount. In comparison, the smaller amount of protein in the molecular weight range typical of beta-keratins shows a high immunoreactivity, comparable to or higher than that of proteins localized in the alpha-keratin size range. This indicates that these small proteins have more affinity with the Beta-1 antiserum. Finally, another possibility is that some polymeric forms of beta-keratins remain associated with alpha-keratin at higher molecular weight. Despite the above short-comings our study has shown that proteins in the range for beta-keratins (13-18 kDa) are absent in the scaleless snake.

The presence of a fibrous dermis in scales of the colubrid scaleless snake *Pituophis melanoleucus catenifer* indicated that here the dermis is also defective (Licht and Bennet, 1972). Similarly, it is therefore likely that in scaleless *C. atrox* the lack of

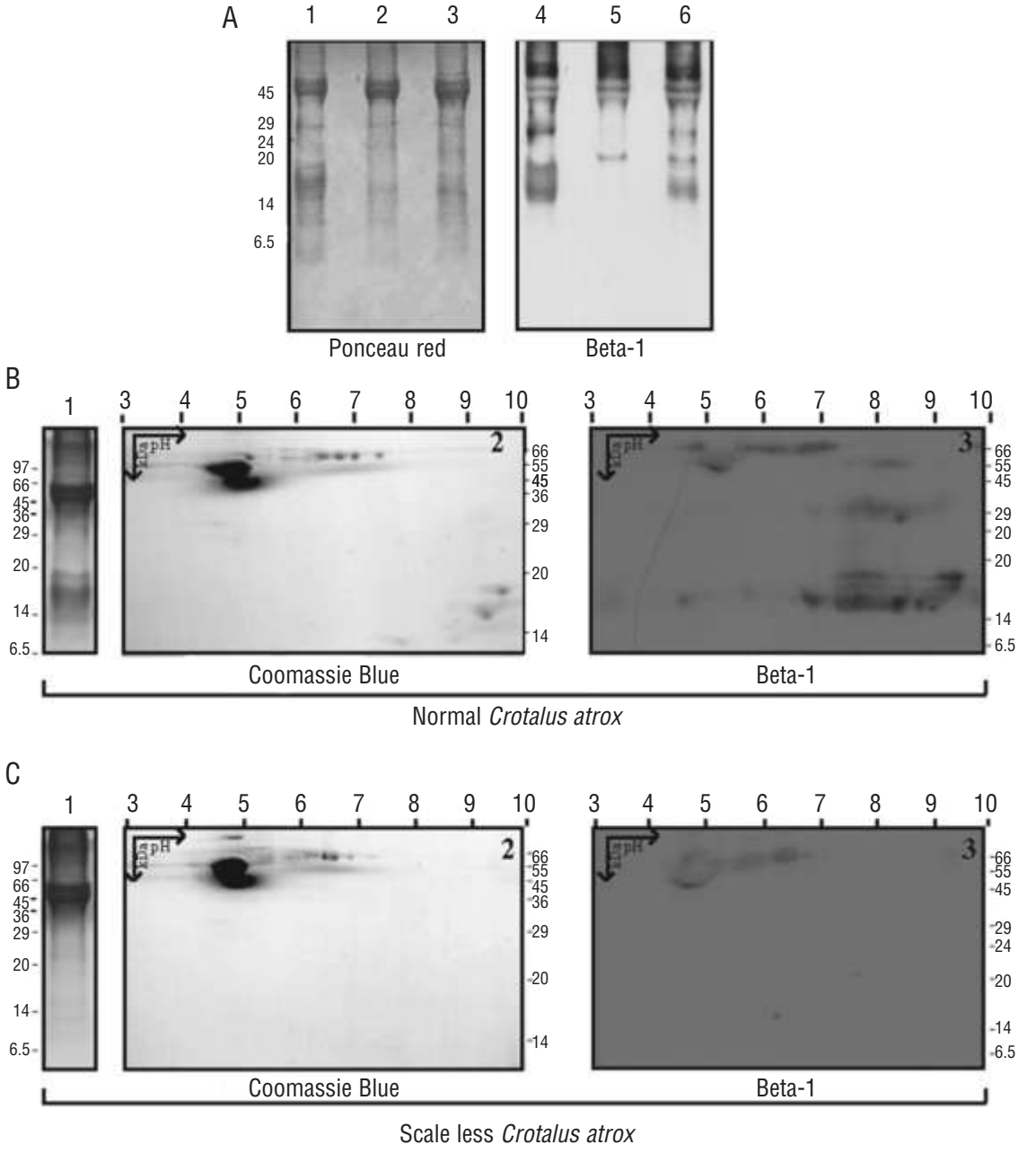

Figure 3. A, Protein patterns (50 μg each lane) separated in tricine SDS-PAGE stained by Ponceau red (lanes 1-3), and corresponding immunoblots (lanes 4-6) of normal scales (lanes 1, 4), and scaleless *C. atrox* (lanes 2, 3, 5, 6) moults. Lanes 2, 5, lateral scaleless *C. atrox* moults (where no scales are present); lanes 3,6, ventral scaleless *C. atrox* moults (where some scales are present). Molecular weights are indicated. B, C, One- and two-dimensional protein patterns (1 and 2 respectively) stained by Coomassie Blue, and Beta-1 two-dimensional immunoblot (3) of normal (B) and scaleless (C) snake moults. 15% polyacrylamide gels were used, with 50 and 150 μg of proteins loaded in each one- and two-dimensional gel, respectively. Molecular weights and pH values are indicated in ordinate and in abscissa, respectively.

beta-keratin may derive from the absence of a competent dermis that normally induces scale morphogenesis. This effect has been shown for a chick scaleless mutant, where alteration of scale morphogenesis leads to a modification of epidermal histogenesis, with loss of beta-layer formation (Sawyer *et al.*, 2000; Sawyer and Knapp, 2003). However, a direct mesenchymal-epidermal interaction responsible for inducing the beta-layer has never been directly demonstrated in any reptilian skin. Some experiments on lizard skin cultivated *in vitro* have indicated that the epidermis is independent from the dermis as far as cytodifferentiation is concerned (Flaxmann *et al.*, 1968). The epidermis can form

successive epidermal generations, including the beta-layer. However, the influence of soluble dermal factors present in the culture medium used in the above study has not been assessed, and therefore a dermal influence cannot be ruled out.

In moults of the scaleless snake *C. atrox* utilized in the present study, only an oberhautchen-like layer appears to be present, probably representing the remnant of the thin beta-layer. This thin layer was probably described also in the colubrid snake *Pituophis melanoleucus catenifer* (Licht and Bennet, 1972). The low amount of beta-keratin bands at relatively high molecular weight (33 kDa) may be derived from this narrow layer, although the immunocytochemical study showed no immunolabelling.

In the scaleless snake, mechanical resistance of the skin is limited and the epidermis becomes folded and pliable, as in the epidermis of mammals (Licht and Bennet, 1968). Without an impliant beta-layer, the remaining alpha-layer has insufficient rigidity to resist forces of deformation acting on the skin. The above data indicate that the small beta-keratins (13-15 kDa), typical of normal scales and of the epidermis of other reptiles (Sawyer *et al.*, 2000; Alibardi *et al.*, 2004; Alibardi and Toni, 2006, Toni and Alibardi, 2007), contribute to the formation of a stiff lamina covering the dorsal scale surface.

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