Soluble scute proteins of healthy and ill desert tortoises (Gopherus agassizii)

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Objectives—To characterize protein composition of shell scute of desert tortoises and to determine whether detectable differences could be used to identify healthy tortoises from tortoises with certain illnesses.

Animals—20 desert tortoises.

Procedures—Complete postmortem examinations were performed on all tortoises. Plastron scute proteins were solubilized, scute proteins were separated by use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were analyzed, using densitometry. Two-dimensional immobilized pH gradient-PAGE (2D IPG-PAGE) and immunoblot analysis, using polyclonal antisera to chicken-β keratin and to alligator-scale β keratin, were conducted on representative samples. The 14-kd proteins were analyzed for amino acid composition.

Results—The SDS-PAGE and densitometry revealed 7 distinct bands, each with a mean relative protein concentration of > 1%, ranging from 8 to 47 kd, and a major protein component of approximately 14 kd that constituted up to 75% of the scute protein. The 2D IPG-PAGE revealed additional distinct 62- and 68-kd protein bands. On immunoblot analysis, the 14-, 32-, and 45-kd proteins reacted with both antisera. The 14-kd proteins had an amino acid composition similar to that of chicken-β keratins. There was a substantial difference in the percentage of the major 14-kd proteins from scute of ill tortoises with normal appearing shells, compared with 14-kd proteins of healthy tortoises.

Conclusions and Clinical Relevance—The major protein components of shell scute of desert tortoises have amino acid composition and antigenic features of β keratins. Scute protein composition may be altered in tortoises with certain systemic illnesses.

Materials and Methods

Tortoises and tissue acquisition—Seventeen adult and 3 subadult desert tortoises were obtained from the Mojave and Colorado deserts of California. Adult tortoises were defined as tortoises with microscopic evidence of spermatogenesis or follicular development in the gonads and a midline carapace length (MCL) of >180 mm, indicating they were of reproductive age.19,20 Tortoises were grouped into ill tortoises with a normal-appearing shell (n = 7), tortoises with cutaneous dyskeratosis (6), and healthy tortoises (7).

Six of 7 ill tortoises with a normal-appearing shell were obtained alive for physical examination; tortoises then were euthanatized and necropsies performed. Information on body condition, size, sex, and necropsy results for those 6 tortoises have been reported elsewhere.18 and it included data for 4 tortoises with mycoplasmosis, 1 with urolithiasis, and 1 with multicentric inflammation. The remaining tortoise of this group was a recently deceased female (MCL, 290 mm) whose cadaver was stored on ice prior to necropsy. Necropsy revealed multicentric inflammation; we did not detect evidence of substantial tissue autolysis.

Five of 6 tortoises with cutaneous dyskeratosis were obtained alive and included 3 tortoises reported elsewhere.18 Three other tortoises in this group were males with MCL ranging from 202 to 280 mm. Although the deceased tortoise in this group had been killed recently by a vehicle, necropsy revealed cutaneous dyskeratosis. We did not detect evidence of substantial tissue autolysis.

Seven healthy tortoises, including 1 reported elsewhere,19 had sustained trauma consistent with being struck by moving vehicles. The other tortoises in this group included 3 females with MCL ranging from 170 to 222 mm and 3 males with MCL ranging from 160 to 260 mm. Most of these tortoises were still alive when found and referred to local veterinarians, who euthanatized them and packed the cadavers on ice for shipment. These tortoises underwent a complete necropsy, including gross and microscopic examination of all organ systems, bacterial and Mycoplasma culture of specimens obtained from the choanae and intestine, and evaluation of scute, kidneys, and liver for metals, as reported elsewhere.19,19,19 These tortoises were included in the healthy control group when postmortem examination did not reveal underlying disease and the examinations did not reveal evidence of substantial tissue autolysis. Also, a body condition index (BCI) was calculated as the ratio of body mass to estimated shell volume (MCL X height of shell X width of shell),20 and all tortoises had a BCI of greater than 75% of prime for a healthy tortoise population, which was consistent with healthy tortoises.

During necropsy, the midplastron, centered on the pectoral and abdominal scutes, was cut into multiple 3- to 5-cm pieces and stored at –80°C. Prior to protein extraction, sections were placed into sterile PBS solution (0.15M NaCl, 16 mM Na2HPO4, 2 mM KH2PO4, pH 7.6) for 2 to 3 hours at 4°C. After the scutes softened, the horny layer was separated from the shell and was stored under nitrogen at –20°C.

Protein extraction of scute and dermis—SCute proteins were extracted, using a modification of a protein-extraction procedure for feather keratin.21 Approximately 2 g of scute material was frozen in liquid nitrogen and then minced into fine pieces in an industrial blender. Approximately 55 mg of minced scute was immersed in 5 ml of urea buffer (8M urea, 0.1M tris, 0.001M EDTA, pH 7.4), 200 µl of 5 N KOH, and 145 µl of β-mercaptoethanol, adjusted to pH 10.5. The mixture was stirred overnight for 16 to 24 hours under nitrogen at room temperature (23°C), after which we added 0.75 ml of tris buffer (3M tris, pH 8.5). Final pH was adjusted to 8.3. To each sample, 174 µl of 3M iodoacetic acid was added to alkylate cysteines, and the mixture was incubated at 37°C for 1 hour under nitrogen in the dark. Then, 100 µl of β-mercaptoethanol was added to quench the reaction, and the insoluble fraction was removed after centrifugation. The supernatant was dialyzed22 free of urea against 4 L of 0.05M ammonium hydroxide for 16 hours. Finally, samples were concentrated to a final volume of 1 ml using filter centrifugation.23 Protein samples were stored at –20°C until used for electrophoresis.

After thawing, connective tissue deep to dermal bone of the shell was scraped from the shell and shredded with scissors. Collagen was homogenized and extracted in a neutral salt solvent (1.0M NaCl, 0.05M tris, 20 mM EDTA, pH 7.5) at 4°C for 24 hours.23

Gel electrophoresis and densitometry—Samples of extracted scute protein from all tortoises were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels (10% tris-tricine SDS) were prepared in accordance with established methods.24 Electrophoresis was conducted, using the modified Laemmli SDS-PAGE method.25 Samples of extracted scute protein were diluted to 6 mg/ml with 0.05M ammonia solution, after which the protein concentration was measured, using a protein assay to ensure that protein concentration of all samples was standardized. Samples were diluted further to a concentration of 3 mg/ml, using gel-loading buffer (8M urea, 0.01M tris, 0.001M EDTA, 0.2% SDS, 0.2 mM β-mercaptoethanol, 0.3% bromphenol blue, 2% glycine, pH 8.3). Electrophoresis was conducted in a dual-slab cell.26 Wells of the gel were washed with cathode buffer (0.1M tris, 0.1M tricine, 0.1% SDS, pH 8.25), and 30 µl of overlay buffer (0.06M tris, 20% SDS) was loaded into each well. Anode buffer was 0.2M tris, pH 8.9. Gels were stained (50% methanol, 10% HCl, 0.125% Coomassie brilliant blue R-250) and destained (50% methanol, 10% HCl).27 Molecular weights were determined from the gels. Stained gels were scanned on a flatbed scanner then analyzed by use of an image-analyzer program.28 Relative percentage amount of protein in each band was calculated for all samples.

Two-dimensional gel electrophoresis—Protein samples extracted from scute of a healthy tortoise were subjected to 2-dimensional immobilized pH gradient-PAGE (2D IPG-PAGE). For the first dimension, proteins were separated by isoelectric focusing (IEF) on gel strips according to their charges by use of immobilized pH gradients.29 Extracted proteins (56 µg in 7 µl) were mixed with 150 µl of 9M urea, 4% Triton X-100, 0.3% dithiothreitol (DTT), 2% IPG buffer (pH 3 to 10 nonlinear).30 After focusing by use of an IEF system, gel strips were equilibrated with 50 mM tris-HCl (pH 6.8), 6M urea, 30% (vol:vol) glycerol, 2% (wt:vol) SDS, and 2% DTT for 10 minutes followed by 20 minutes in 50 mM tris-HCl (pH 6.8), 6M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, and a trace amount of bromphenol blue. For the second dimension, proteins were separated by SDS-PAGE as described previously, except that precast tris-gel strips (4 to 20% gradients) were used. Proteins were stained with Coomassie blue or electrobotted.

Immunoblot analysis—Electrophoretic of scute proteins from each of 2 representative healthy desert tortoises, desert tortoises with cutaneous dyskeratosis, and desert tortoises with mycoplasmosis was conducted on tris-tricine-SDS gels and by use of 2D IPG-PAGE, as described previously. Afterwards, proteins were electroblotted31 to a nitrocellulose membrane at 4°C in a solution of 0.024M tris, 0.2M glycine, and 20% methanol, pH 8.3.

After transfer, the nitrocellulose membrane was blocked in a solution (0.01M tris [pH 7.2], 0.15M NaCl, 5% nonfat dry milk, 0.08M Na2SO4) for 1 hour at 23°C, and then it was
incubated with rabbit anti-chicken feather or rabbit anti-alligator scale antisera for 1 hour at 23 C. Anti-chicken antisera reacts with reptilian β keratins. The nitrocellulose membrane was washed in a solution of 0.15M NaCl, 0.01M tris (pH 7.2), and 0.3% polysorbate 20, and then it was incubated with anti-rabbit conjugate for 1 hour. The membrane was washed again, rinsed (0.1M tris [pH 7.2], 1 mM MgCl₂·6H₂O), and developed (0.23M 5-bromo-4 chloro-3-indoyl phosphate, 0.12 mM nitroblue tetrazolium, 0.1M tris [pH 7.2], 1 mM MgCl₂·6H₂O).

Protein extracted from a chicken feather was used as a positive-control sample. Negative-controls included samples of extracted collagen protein from deep dermal tissues of desert tortoises and results of reactions of scute proteins with preimmune rabbit sera.

Amino acid analyses—After SDS-PAGE electrophoresis, protein bands were electroblotted to a blotting membrane in a solution of 10 mM 2-[N-Morpholino] ethane sulfonic acid [pH 6] and 20% methanol. After transfer, the membrane was stained with Coomassie blue to localize proteins. Amino acid composition of the 14-kd protein band was determined by use of a modified vapor-phase hydrolysis method prior to high-performance liquid chromatography. The membrane-bound protein was placed into a 6 × 50-mm hydrolysis tube and vacuum dried in an evaporator. The tube then was purged in a sealed container with 200 µl of 6 N HCl and 1% liquid phenol. For analysis of cysteine composition, 200 µl of 6 N HCL-1% dimethyl sulfoxide were used. Each container was evacuated briefly for 15 to 20 seconds and then flushed with nitrogen for 1 to 2 seconds. This process was repeated 3 times. Tubes were heated in a heating block oven at 120 C for 24 hours following hydrolysis. The membrane was wetted with 10 µl of methanol, and amino acids were extracted twice from the membrane with 50 µl of 20% methanol-0.1 N HCL, dried, and then rehydrated. Samples were loaded into an amino acid analysis system, and amino acids were quantified at a wavelength of 254 nm. Protein matches were determined, using database query programs.

Statistical analysis—The nonparametric Kruskal-Wallis test for completely randomized designs was used for data reported as relative percentage amount of protein for the protein component. Analysis was performed, using statistical software. A value of P < 0.05 was considered significant. The Wilcoxon rank-sum test was used to compare separate pairs of groups to identify groups that differed significantly with regard to the protein component.

Results

The final concentration of protein extracted from each sample of desert tortoise shell and the concentration of extracted collagen protein was approximately 6 to 10 mg/ml. Yields of soluble scute protein were consistent among the 3 groups of tortoises (Fig 1). Seven distinct protein bands with a mean relative protein percentage > 1.0%, ranging from 8 to 47 kd, were identified by use of densitometry (Fig 2). In addition, bands of molecular weight 62 to 64 kd and 67 to 69 kd constituted < 1% of the total protein. In contrast, 2D IPG-PAGE revealed a distinct 62-kd protein, 2 distinct 67-kd proteins, and at least three 14-kd, two 32-kd, and two 45-kd proteins (Fig 3). Mean relative percentage for proteins of 14 to 16 kd ranged from 67 to 75%. Using paired testing, we detected a significant (P = 0.019) difference for the relative percentage amount of protein for the proteins of 14 to 16 kd between the group of healthy tortoises and the group of ill tortoises with normal-appearing shells. The next most abundant proteins in the scute samples were proteins of 32 to 33 kd, which ranged from 13 to 17%, followed by proteins of 45 to 47 kd, which ranged from 2.3 to 4.1%. Proteins of 62 to 64 kd appeared on gels for healthy desert tortoises and desert tortoises with cutaneous dyskeratosis but not on gels for the ill tortoises with normal-appearing shells. Other significant differences were not identified.

On immunoblot analysis, 14-, 32-, and 45-kd proteins of all tortoises tested reacted with both antisera (Fig 4 and 5). Reactivity of these proteins was similar among the representative samples, except that 2 samples from tortoises with mycoplasmia yielded weak reactivity of the 45-kd protein with antisera against avian feather. In the 2D IPG-PAGE gel, all soluble scute proteins of 14, 32, and 45 kd for a control tortoise reacted with antisera against avian feather.

Figure 1—Results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of soluble scute proteins extracted from plastrons of desert tortoises revealing 7 distinct protein bands. Lanes are as follows: MW, molecular weight markers; lane 1, healthy tortoise; lane 2, tortoise with cutaneous dyskeratosis; lane 3, tortoise with mycoplasmia; lane 4, tortoise with urolithiasis; lane 5, tortoise with respiratory tract inflammation; lane 6, healthy tortoise; lane 7, healthy tortoise. Coomassie blue stain.

Figure 2—Histogram of mean percentage of soluble scute proteins extracted from plastrons of 20 desert tortoises. Proteins were separated by SDS-PAGE on 10% tris-tricine gels, and mean percentage protein was determined, using densitometry. □ = Healthy tortoises, x = Tortoises with cutaneous dyskeratosis but not on gels for the ill tortoises with normal-appearing shells.

Figure 3—Results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of soluble scute proteins extracted from plastrons of desert tortoises revealing 7 distinct protein bands. Lanes are as follows: MW, molecular weight markers; lane 1, healthy tortoise; lane 2, tortoise with respiratory tract inflammation; lane 3, tortoise with cutaneous dyskeratosis; lane 4, tortoise with urolithiasis; lane 5, tortoise with respiratory tract inflammation; lane 6, healthy tortoise; lane 7, healthy tortoise. Coomassie blue stain.

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Collagen protein did not react with either antisera, and scute proteins did not react with preimmune rabbit sera. Duplicate amino acid composition analyses for the 14-kd proteins were conducted. Average percentages of each amino acid (in descending order of magnitude) were as follows: glycine, 22.8; cysteine, 10.9; tyrosine, 10.0; leucine, 9.2; proline, 8.4; valine, 8.4; serine, 5.6; alanine, 4.9; glutamine, 4.3; asparagine, 3.6; arginine, 3.6; isoleucine, 2.6; threonine, 2.0; phenylalanine, 2.1; histidine, 1.6; and methionine, 0.1. Matched proteins from databases included β-keratins from chicken claw, scale, and feather. Avian claw and feather keratins ranked second and third, respectively, among 60 possible matches. Avian scale keratin ranked as high as seventh in the data bases. The top match was a hypothetical protein. Other matches that ranked among the top 7 for the databases included silkworm chorion proteins, lutropin β chain, and another β chain.

Discussion

Routine SDS-PAGE electrophoresis of soluble scute proteins obtained from plastrons of desert tortoises revealed 7 distinct protein bands ranging from 8 to 47 kD with a greatest concentration of proteins that were approximately 14 kD. A similar electrophoretic pattern was documented for another tortoise species (Geochelone carbonaria), and the 14-kd protein was the major molecular weight protein reported for Chelydra serpentina, Pseudemys scripta, and Geochelone carbonaria. Five protein bands, ranging from 6.6 to 30 kD, were detected in the carapace scute of Chrysemys sp, with a major component being a 13.2-kd protein. However, it is unknown whether the major protein components or any of the other scute proteins of these chelonian species were composed of β-keratin proteins.

Two-dimensional gels of samples from a healthy tortoise revealed multiple 14-, 32-, and 45-kd proteins and proteins of 62 and 68 kD. Immunoblot analyses of gels by use of SDS-PAGE and 2D IPG-PAGE provided evidence that all 14-, 32-, and 45-kd soluble proteins of desert tortoise scute are β keratins. These 3 protein components constituted approximately 90% of the total soluble scute protein. The 14-kd protein component is in the molecular weight range reported for β keratins in birds and anole lizards. To our knowledge, the other reactive keratin protein components identified during immunoblot analysis (32 and 45 kD) have not been described in other species of reptiles.

Analysis of amino acid composition revealed that the 14-kd proteins had high-ranking matches with β keratin from chicken scale, feather, and claw. Thus, analysis of amino acid composition also supported a β-keratin configuration for the 14-kd protein components. Despite the fact that 2-D IPG-PAGE revealed at least three 14-kd proteins, all proteins reacted with antibody to chicken-feather β keratin. Separation of proteins of avian scute scale by 2D IPG PAGE also revealed multiple proteins of similar molecular weight that reacted...
with antisera specific for β keratin. The high concentration of 14-kd and other β-keratin proteins in desert tortoise scute supports ultrastructural evidence that shells of tortoises are mostly composed of β-type keratins. The shell of a tortoise acts as an essential barrier against the environment and pathogenic organisms, and it has been hypothesized that the molecular complexity of keratins helps provide this barrier. We are not certain of the identity of the soluble scute proteins that did not react with antisera. The molecular weight of avian and certain reptilian β keratins reportedly ranges from 10 to 30 kD, whereas the molecular weight of α keratins of vertebrate epithelia, including reptilian epithelia, ranges from 40 to 70 kD. Matrix proteins also range from approximately 10 to 30 kD.

Statistical analysis revealed significant differences in relative percentage amount of 14- to 16-kd proteins among control tortoises and tortoises with systemic illness. Also, at least 1 protein component (62 to 64 kD) was not detected in ill tortoises with normal-appearing shells, although this may be a reflection of the staining method used to observe proteins in gels. These findings suggest that systemic diseases in desert tortoises may have an effect on the keratinization process by increasing or suppressing expression of soluble scute proteins. It has been suggested that common DNA and protein homologies between keratin and Mycoplasma adherence-associated proteins play a role in postinfectious autoimmune and immune-mediated damage in animals with mycoplasmosis. In humans, certain cutaneous diseases are characterized by disorders of keratinization as a primary defect in keratinization or a secondary keratinization disorder attributable to the disease itself. Expression of keratins K1 and K10 is decreased, whereas that of keratins K6 and K16 is increased, in humans with psoriasis, a disease that affects the keratinization process. Treatment with systematically administered retinoids will reverse these alterations in psoriatic epidermis but will decrease gene expression of keratins K1 and K10 in human keratinocyte cell cultures.

Some of the systemically ill tortoises may have been dehydrated, as evidenced by an increase in plasma concentrations of BUN and uric acid. Because they are reptiles that inhabit the desert, desert tortoises are suitably adapted to long-term imbalances in their water budgets, and they can store up to 30% of their body mass as urine in their urinary bladder for use as a water reserve. It is possible that dehydration affected protein expression and may have been responsible for the differences in relative protein concentration of the 14- to 16-kd proteins. However, dehydration probably would have affected all proteins uniformly, maintaining the relative percentage amount of each protein.

Causes of decreases in the population of desert tortoises are multifactorial and include disease, illegal collection, vehicular trauma, predation, and loss of habitat. Tortoises injured or killed by vehicles may otherwise have been healthy. Measures were taken in the study reported here to ensure the health status of the tortoises in the healthy control group. All tortoises in that group had a BCI indicative of healthy tortoises. Complete postmortem examination was another means of assessing health status. Also, none of the tortoises used in the healthy control group had metal concentrations in scute, kidneys, or liver that were considered anomalous (data not shown). Hematologic examination may have been useful, but broad ranges of hematologic values as a result of seasonal variation, hibernation and estivation, and stage of the sexual cycle preclude the use of hematologic variables to determine definitively whether a tortoise is healthy.

One objective of the study reported here was to determine whether soluble scute proteins of desert tortoises with cutaneous dyskeratosis differed from those of other desert tortoises. The midplastron was chosen for evaluation, because this site is affected most consistently in cutaneous dyskeratosis. Analysis of proteins purified by SDS-PAGE electrophoresis did not reveal proteins that were expressed or suppressed in tortoises with cutaneous dyskeratosis, compared with values for healthy tortoises. Moreover, statistical analysis of relative percentage amount of each protein did not reveal differences between proteins of desert tortoises with cutaneous dyskeratosis and proteins of healthy tortoises. These results may indicate that cutaneous dyskeratosis is not attributable to differences in expression of soluble scute proteins. A larger number of tortoises may be required to detect significant differences, and it is also possible that proteins with small molecular weight or low concentration were lost during extraction or not detected on gels. Coomassie blue staining can be used to detect as little as 0.1 µg of protein/band, whereas silver staining can be used to detect as little as 2 ng of protein/band. In our study, when total concentration of protein loaded onto gels was ≥ 0.25 mg/ml, silver staining produced too much artifact. Samples could have been diluted further; however, the concentration of the major 14- to 16-kd protein was so high that dilution of this protein to accommodate the silver stain resulted in dilution of some of the other proteins beyond their detection limit. Even at lower concentrations of protein, results of Coomassie blue staining, which has been used by others to localize β-keratin proteins, were more consistent than those for silver staining. The absolute optical density value for each protein band may have varied among gels because of varying staining and destaining times. However, only the percentage amount of each protein relative to the total protein per gel (equal to 100%) was determined in an attempt to minimize error attributable to staining variation among gels. Protein concentration of each sample was standardized to 3 mg/ml and verified by protein analysis prior to electrophoresis to minimize variation in the total amount of protein that was loaded. Two-dimensional gel electrophoresis and amino acid sequencing may be useful in identifying subtle differences among scute proteins. Analysis of the results of our study did not rule out possible abnormalities in scute lipids or carbohydrates, and we did not analyze components of the insoluble fraction that were removed during protein extraction.

Waring Blender, Model 33BL79, Dynal Corporation of America, New Hartford, Conn.
β-mercaptoethanol, BioRad Laboratories, Hercules, Calif.
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