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Author for correspondence:

Douglas S. Fudge

e-mail: dfudge@uoguelph.ca

Regulation of hard α -keratin mechanics via control of intermediate filament hydration: matrix squeeze revisited

Daniel A. Greenberg and Douglas S. Fudge

Department of Integrative Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada N1G 2W1

Mammalian hard α -keratins are fibre-reinforced biomaterials that consist of 10 nm intermediate filaments (IFs) embedded in an elastomeric protein matrix. Recent work suggests that the mechanical properties of IFs are highly sensitive to hydration, whereas hard α -keratins such as wool, hair and nail are relatively hydration insensitive. This raises the question of how mammalian keratins remain stiff in water. The matrix squeeze hypothesis states that the IFs in hard α -keratins are stiffened during an air-drying step during keratinization, and subsequently locked into a dehydrated state via the oxidation and cross-linking of the keratin matrix around them. The result is that even when hard α -keratins are immersed in water, their constituent IFs remain essentially 'dry' and therefore stiff. This hypothesis makes several predictions about the effects of matrix abundance and function on hard α -keratin mechanics and swelling behaviour. Specifically, it predicts that high matrix keratins in water will swell less, and have a higher tensile modulus, a higher yield stress and a lower dry-to-wet modulus ratio. It also predicts that disruption of the keratin matrix in water should lead to additional swelling, and a drop in modulus and yield stress. Our results are consistent with these predictions and suggest that the keratin matrix plays a critical role in governing the mechanical properties of mammalian keratins via control of IF hydration.

1. Introduction

The appearance of hard, epidermally derived keratin structures was an important step in the evolution of early mammals [1]. Mammalian epidermal appendages, which include structures such as hairs, horns, hooves, claws, quills and baleen, are highly diverse in both structure and function, and are important for thermoregulation, feeding, defence, locomotion and intraspecific competition [2]. All of these structures are made of the same material, hard α -keratin, which consists of two primary protein phases, with fibrous proteins embedded in and covalently linked to a network of amorphous proteins, forming a tough fibre-reinforced composite [3]. While much is known about the development and structure of the hard α -keratins, our understanding of the structural basis of their mechanical properties is lacking, especially in the light of new insights into the mechanical properties of the fibrous component, which belong to the family of cytoskeletal filaments known as 'intermediate filaments' (IFs) [4].

Intermediate filaments (IFs) are a class of 10 nm diameter protein filaments that, in addition to reinforcing the non-living α -keratins, are an important component of the cytoskeleton in most metazoans. In many living cells, IFs form a dense network of filaments that provides passive mechanical support [5–8]. Mutations in IF genes are known to be the cause of several tissue fragility diseases, including the skin blistering disease epidermolysis bullosa simplex [6,9], which underscores the importance of IFs to maintaining the mechanical integrity of cells. Recent work on the mechanical properties of IFs in living cells suggests they are very soft, extensible and tough [10,11], in stark contrast to the relatively rigid cytoskeletal filaments F-actin and microtubules [12,13].

Mechanical testing of isolated IFs using atomic force microscopy confirms that IFs are far softer than F-actin and microtubules [14,15], and able to endure remarkable degrees of tensile strain (approx. 250%) before breaking. These data are consistent with a study of the mechanical properties of IF bundles from the defensive slime of hagfishes, which also showed that IFs can be soft and highly extensible (breaking strain approx. 220%) in water [16].

The discovery that hydrated IFs are soft and highly extensible raises an interesting paradox about the mechanics of hard α -keratins, most of which consist of a high volume fraction (i.e. a majority of the protein in most keratins) of aligned IFs embedded in a protein matrix. How can it be that hard α -keratins remain stiff and relatively inextensible in water, when the IFs that make them up are soft and highly extensible when hydrated? One possible solution to this paradox is that the IFs in hard α -keratins are maintained in a dry state, even when the material is fully immersed in water. This hypothesis was proposed by Fudge & Gosline [17], and was based on the observation that dry hagfish slime threads, which consist of aligned bundles of IFs without an associated matrix, exhibit mechanical behaviour that is similar to that of hydrated hard α -keratins such as wool. While previous models of hard α -keratin mechanics claimed that the IFs are inherently hydration resistant and the matrix is highly sensitive to hydration [3,18], the 'matrix squeeze' hypothesis proposed by Fudge & Gosline suggests the opposite, that IFs are highly sensitive to hydration, and that the less hydration-sensitive matrix protein network elastomerically resists the uptake of water by the IFs under hydrating conditions. The matrix squeeze hypothesis is consistent with the facts that IF proteins tend to be far more hydrophilic than matrix proteins [19], and that hard α -keratins with high matrix contents tend to swell less in water than those with low matrix contents [20].

While the matrix squeeze hypothesis successfully explains several previously puzzling aspects of hard α -keratin mechanics, it also makes several predictions that have not yet been tested. First, it predicts that under hydrating conditions, keratins with high matrix contents should behave more like dry IFs and those with low matrix contents should behave more like hydrated IFs. Specifically, it predicts that modulus and yield stress in water should increase as matrix content increases, because more robust matrix networks should have greater resistance to the swelling and hydration of IFs than less robust ones. Second, it predicts that disruption of the keratin matrix via cleavage of disulphide bonds should limit its ability to resist IF swelling in water and lead to increased swelling, and a reduction in modulus and yield stress. Third, disruption of the matrix should lead to greater changes in these material properties in keratins with high matrix contents than those with low. Fourth, if high matrix keratins have IFs that are 'drier' than IFs in low matrix keratins, then the change in mechanical behaviour between dry and wet conditions should be smaller for high matrix keratins. Here we tested all of these predictions using a comparative approach in which we measured the tensile mechanics and the effects of matrix disruption on several hard α -keratins representing a wide range of matrix contents. The data are consistent with the predictions of the matrix squeeze hypothesis, and suggest that the matrix plays an important role in regulating the mechanical properties of hard α -keratins via control of IF hydration.

2. Material and methods

(a) Hard α -keratins

A variety of hard α -keratin structures were collected from several sources (see acknowledgements for full details). All of the keratins used have published matrix contents (quantified in terms of amino acid residues of matrix protein per 100 residues of total protein), which were measured using various techniques [20,21]. Samples were obtained from live or recently deceased individuals from the following species: short-beaked echidna quill (*Tachyglossus aculeatus*, $n = 2$), white rhinoceros horn (*Ceratotherium simum*, $n = 2$), domestic horse hair (*Equus ferus caballus*; $n = 10$), raccoon hair (*Procyon lotor*, $n = 4$) and blue whale baleen (*Balaenoptera musculus*, $n = 1$). Human hair samples ($n = 4$) were obtained from volunteers between the age of 20 and 22 years whose hair had never been chemically treated. Keratin samples were stored in a dry condition (approx. 30% relative humidity) at ambient temperature (approx. 20°C). For each experiment, samples were randomly drawn from a combined pool of keratins from all individuals. Samples were prepared by creating elongated strips of material using a razor blade for echidna quills, and hairs and fibres were left fully intact. For rhinoceros horn, we used individual keratin tubules isolated from a sample of horn taken from the basal, distal section of horn from a mature rhinoceros. In this area of the horn, the agglutinated tubules are easily separated from the intertubular matrix. We soaked tubules in distilled water and mechanically removed the agglutinated matrix material, after which these isolated tubules were dried in a desiccator at less than 20 per cent relative humidity. Echidna quills were not included in tensile mechanics trials owing to the fact that the IFs are arranged in a highly disordered manner [22], and thus it is difficult to make fair comparisons with other keratins in which the IFs are aligned parallel to the longitudinal axis.

(b) Disruption of the matrix

We disrupted the keratin matrix using an established protocol which cleaves disulphide bonds and prevents their reformation via methylation of free thiol groups [23]. All samples were first treated with acetone to degrease surface lipids [24], which, when left intact, inhibited penetration of the reducing agent in preliminary trials. To reduce disulphide bonds, samples from each keratin type were immersed in a solution of 0.1 M 2-mercaptoethanol in 20 per cent 1-propanol for 48 h. This treatment has been shown to reduce 92 per cent of disulphide bonds in Corriedale wool [25]. Samples were subsequently washed with a 50 per cent 1-propanol solution to remove any remaining 2-mercaptoethanol. After the reduction process, samples were quickly transferred (to minimize oxidation of cysteine) to a solution of 0.1 M methyl iodide in 0.2 M boric acid buffer at pH 8.0 for 24 h [23]. Methyl iodide creates S-carboxy-methylcysteine from free cysteine residues, blocking future cross-linking from occurring in an oxidative environment [26]. At least 50 ml of each solution was used for each 0.5 g of keratin material, in order to maximize reduction [23]. After methylation, samples were again washed with a 50 per cent 1-propanol solution to remove any remaining chemical agents. Control samples underwent an identical treatment but the solutions lacked the active agents 2-mercaptoethanol and methyl iodide.

(c) Swelling trials

Prior to treatment, dry keratin samples were affixed to a section of mesh 3 cm by 6 cm, by either directly tying the samples to it (in the case of hairs) or lashing them down with wire hoops. In the dry state, images of each sample were taken at three demarcated points using a Canon VIXIA HV30 HD digital camcorder

(Canon Inc., Tokyo, Japan) mounted on a Nikon Eclipse T5100 inverted microscope. After control or reduction treatment, samples were kept for a 24 h period in deionized H₂O, and subsequently imaged at the same three locations. This provided a more accurate reflection of swelling, since alcohol solutions can cause additional swelling of keratins [20]. The diameter of each sample was measured from captured images using IMAGEJ v. 1.43 (National Institutes of Health, Bethesda, MD, USA).

(d) Tensile mechanics

Tensile mechanical testing of keratins was conducted under hydrating and dehydrating conditions. Hydrated trials consisted of control and reduced samples immersed in deionized water. Dehydrated trials consisted of unaltered samples tested in air at ambient relative humidity ($29.9 \pm 0.9\%$). Samples were extended longitudinally in tensile mechanical trials using an Instron 3343 universal testing machine (Illinois Tool Works, Glenview, IL, USA). For hydrated trials, specimens were clamped between pneumatic grips and kept submerged in a BioPuls bath containing distilled H₂O at $20.8 \pm 0.3^\circ\text{C}$ (mean \pm s.d.). A 100 N load cell measured the force exerted by specimens as they resisted tensile deformation. Before testing, samples were kept fully hydrated in distilled H₂O at an ambient temperature of approximately 20°C . Prior to testing, samples were held for 5 min in the water bath to counter any dehydration that may have occurred during sample mounting. For dry trials, samples were stored in a desiccator at ambient room temperature and at less than 20 per cent relative humidity.

Specimens were extended at a rate of 1.0 or 0.5 mm min⁻¹ (depending on sample length), corresponding to an approximate strain rate of 0.10 min⁻¹. Initial elastic modulus (Young's modulus, E_i), yield stress (σ_{yield}), yield strain (ϵ_{yield}), break stress (σ_{max}) and break strain (ϵ_{max}) were calculated using INSTRON BLUEHILL software v. 2.9. E_i was calculated as the steepest slope of the stress-strain curve in the linear Hookean region. The yield point was taken as the point on the stress-strain curve at which the initial slope decreased to 80 per cent of maximum. Break strain (ϵ_{max}) was calculated as the strain at which 99 per cent of maximum stress was reached. Sample cross-sectional areas were calculated from images of the samples that were collected as described above for the swelling trials. For tubular samples (i.e. whale baleen), the load-bearing cross-sectional area was calculated from images of sample cross-sections using IMAGEJ. While rhinoceros horn is also tubular, the diameter of the inner tubule was very small, making it difficult to accurately measure and unlikely to greatly affect estimates of stress, and therefore it was not taken into account.

(e) Statistical analysis

To quantify the effects of matrix content and chemical treatment on tensile mechanics and swelling, we developed a linear mixed model with fixed effects of keratin matrix content (% of total protein) and treatment (dry, control or reduced), with species as a random effect. Data were analysed at the species level, with points representing an average of the individual subsamples. The significance of each parameter (matrix content and treatment) was evaluated with Wald tests to determine whether a factor contributed significantly to the overall model. To determine whether there was a relationship between the reduction-induced decrease in modulus (relative to controls) and matrix content, we performed a simple linear regression. For the ratio of wet and dry tensile modulus and matrix content, we used nonlinear regression according to an exponential decay function ($Y = e^{b + a \cdot x}$) fit using nonlinear least-squares estimates of parameters. Data were analysed using the statistical software R v. 2.12 [27], using the 'nlme' package [28] for the linear mixed models.

3. Results

(a) Swelling

There was a significant increase in transverse swelling when keratin samples were treated with the reducing agent, with reduced samples exhibiting an average additional diametrical swelling of $6.34 \pm 1.40\%$ across all keratins compared with hydrated controls (table 1 and figure 1; $t_{105} = 4.52$, $p < 0.001$). The greatest transverse swelling was in reduced rhinoceros horn, which at $43.9 \pm 3.4\%$ (mean \pm 1 s.e.) was very close to the swelling of matrix-free hagfish threads [16], and considerably higher than the un-reduced rhino horn control ($34.3 \pm 1.5\%$). The difference in swelling between control and treated samples of whale baleen was comparatively small at $21.2 \pm 0.9\%$ and $24.5 \pm 0.4\%$, respectively. The lowest average swelling was in control echidna quill at $4.7 \pm 0.3\%$, the keratin material with the highest matrix content, which when treated increased in transverse swelling to $6.4 \pm 0.5\%$. Matrix content was negatively correlated with transverse swelling, with each 1.0 per cent increase in the proportion of matrix proteins: IF proteins decreasing diametrical swelling by $0.56 \pm 0.14\%$ (figure 1; $t_4 = 4.08$, $p = 0.015$).

(b) Tensile mechanics

Tensile mechanical data were consistent with the prediction that, when tested in water, high matrix keratins should behave more like dry IFs, and low matrix keratins should behave more like wet IFs (figure 2). The greatest modulus in water was found in raccoon hair at 1.48 ± 0.23 GPa, which was also the keratin structure with the highest matrix content (45.0%) that was mechanically tested. When treated with a reducing agent, the average raccoon hair sample decreased in modulus by 47.4 per cent (table 1 and figure 2*e*). The keratin with the lowest matrix content, rhinoceros horn, had the lowest tensile modulus in water at 54.4 ± 10.2 MPa, which is almost an order of magnitude lower than any other keratin structure (table 1). Treatment of rhinoceros horn with the disulphide-cleaving agent further reduced its tensile modulus to 23.0 ± 2.4 MPa, which is approaching the modulus of wet hagfish threads, 6.4 MPa [16] and estimates of the modulus of isolated IFs *in vitro* [15]. The relative proportion of matrix:IF protein content was significantly positively related to the tensile modulus of the material in water (figure 3; $t_3 = 5.91$, $p = 0.01$). Treatment with the reducing agent caused a significant decrease in the tensile modulus ($t_{105} = 6.17$, $p < 0.01$), with an average drop of 374 MPa in modulus across all keratins (figure 3). The effect of matrix content on the dry tensile modulus was not significant (figure 3; $t_3 = 1.71$, $p = 0.185$). When keratins were treated with a disulphide-reducing agent, yield stress across all keratins decreased by an average of 10.3 ± 1.1 MPa ($t_{105} = 9.36$, $p < 0.01$). Higher matrix content keratins tended to yield at higher levels of tensile stress (figure 4), but this was not statistically significant ($t_3 = 1.90$, $p = 0.154$). Matrix content had a significant negative effect on yield strain ($t_3 = 3.23$, $p = 0.0483$), but the effect of chemical treatment on yield strain was not statistically significant ($t_{105} = 1.18$, $p = 0.241$). Matrix content had a significant effect ($p = 0.0017$) on the hydration sensitivity of the tensile behaviour, as measured by the ratio of modulus measured in air to that measured in water, with low matrix keratins exhibiting greater hydration sensitivity than high matrix

Table 1. Summary of the mean transverse swelling and tensile mechanical data for each keratin material, including initial tensile modulus (E_i), yield strain (ϵ_{yield}), yield stress (σ_{yield}), break strain (ϵ_{max}) and break stress (σ_{max}), plus the number of samples used for the swelling (n_{sw}) and mechanical (n_{mech}) trials. (Mechanical data for echidna quill are not provided owing to the lack of intermediate filament alignment in this material.)

	matrix (%)	treatment	swelling (%)	n_{sw}	E_i (MPa)	ϵ_{yield} ($\Delta L/L$)	σ_{yield} (MPa)	ϵ_{max} ($\Delta L/L$)	σ_{max} (MPa)	n_{mech}
rhino horn	12.6	control	34.3	14	54	0.084	3.3	0.153	5.3	8
		reduced	43.9	14	23	0.158	2.0	0.856	10.0	4
		dry	—	—	948	0.013	11.1	0.020	14.4	15
horse hair	24.0	control	17.3	11	567	0.056	23.0	0.883	61.1	18
		reduced	24.4	11	290	0.060	13.6	1.323	54.9	16
		dry	—	—	3027	0.036	91.2	0.348	166.6	17
whale baleen	25.0	control	21.2	6	506	0.047	11.2	0.423	24.7	6
		reduced	24.5	6	403	0.030	7.2	0.505	21.0	6
		dry	—	—	2067	0.037	40.5	0.413	79.6	7
human hair	31.5	control	26.5	10	1108	0.030	29.5	0.715	131.8	16
		reduced	31.7	10	641	0.025	15.7	0.820	86.2	11
		dry	—	—	3051	0.046	65.1	0.532	211.1	13
raccoon hair	45.0	control	11.3	5	1477	0.025	27.4	0.532	96.9	8
		reduced	22.1	5	777	0.020	12.3	0.679	44.8	18
		dry	—	—	2810	0.026	58.3	0.281	128.4	14
echidna quill	62.8	control	4.7	10	—	—	—	—	—	—
		reduced	6.4	10	—	—	—	—	—	—

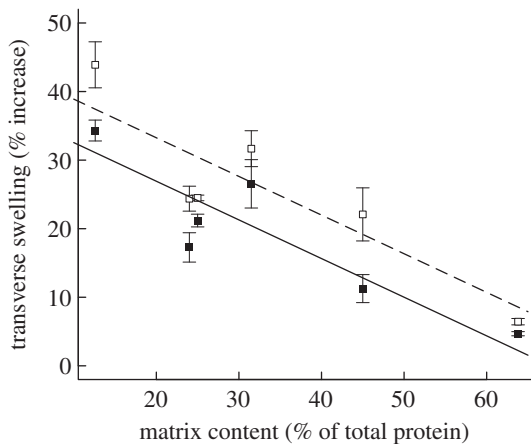


Figure 1. Relationship between keratin matrix content and transverse swelling, measured as the per cent increase in diameter compared with the dry state. The results indicate significantly greater swelling in treated (open symbols) compared with control (black symbols) keratins ($t_{105} = 4.52$, $p < 0.01$), and a negative relationship between matrix content and transverse swelling ($t_4 = 4.09$, $p = 0.015$).

keratins (figure 5). Similarly, the drop in tensile modulus between treated and untreated keratins was positively correlated with matrix content (figure 6; $t_3 = 4.97$, $p = 0.0156$).

4. Discussion

The results presented here from a study of hard α -keratins with a wide range of matrix contents are consistent with several predictions of the matrix squeeze hypothesis. Our results show that high matrix keratins in water behave more like dry IFs, and low matrix keratins in water behave more like wet IFs.

We have also shown that reduction of keratins with disulphide-cleaving compounds leads to significant swelling, a decrease in modulus and a decrease in yield stress across all keratins, with high matrix keratins experiencing the largest absolute changes in mechanical behaviour as a result of reduction.

These results suggest that an important function of the keratin matrix is to maintain IFs in a semi-dehydrated state, so that hard α -keratins can remain stiff even under hydrating conditions. The results also suggest that the modulus of a keratin in water can be tuned via adjustments of the ratio of matrix:IF proteins, with higher matrix contents leading to higher modulus in water. Examination of the relationship between the dry:wet modulus ratio versus matrix content (figure 5) suggests that the benefits of increasing matrix content start to level off at matrix contents around 40 per cent, with higher matrix contents leading to only small changes in the hydration sensitivity. The same curve suggests that hydration sensitivity changes rapidly as matrix contents approach that of rhinoceros horn (12.6%). These data suggest that very low matrix content keratins should be extremely hydration sensitive and may explain why most hard α -keratins have matrix contents that are considerably higher than those in rhinoceros horn [21].

Our results also raise the question of why some keratins have low matrix contents if the matrix is so important to the maintenance of keratin function across a range of humidity. We can think of two possible selective forces that might push keratins towards the low matrix end of the curve. Lower matrix keratins may be able to absorb more energy (i.e. have a higher work of fracture), as the matrix component has been found to have lower fracture toughness than IFs [29], and higher hydration sensitivity may also improve work of fracture of keratins [30,31]. Since work of fracture may be more important than stiffness at high humidity for

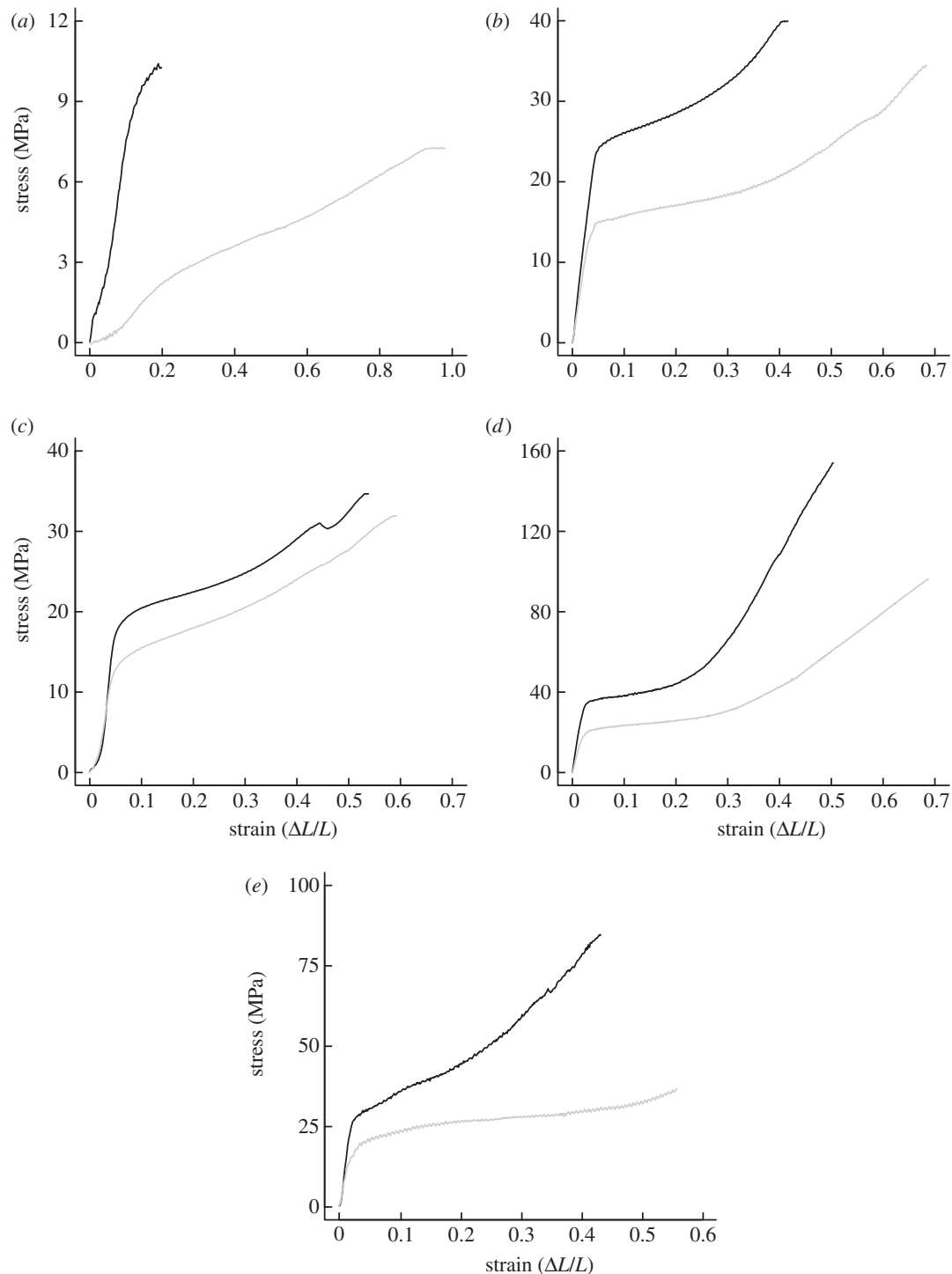


Figure 2. Representative stress–strain curves for control (black lines) and treated (grey lines) keratin samples for (a) rhinoceros horn, (b) horse tail hair, (c) whale baleen bristles, (d) human hair, and (e) raccoon hair.

structures like rhinoceros horn, this could explain the selection for low matrix content. Another possibility is that matrix proteins are more costly for the animals to produce. Sulphur-containing amino acids (such as cysteine, which is highly abundant in matrix proteins) are often one of the most limiting nutrients in a mammal's diet [32,33]. The allocation of these nutrients to keratin structures may thus come at a cost to growth and reproduction. Therefore, the optimal evolutionary strategy would be to have just enough matrix for keratins to maintain their function in the range of humidity experienced in their environment. This may explain why rhinoceros horn and horse hair are both low matrix keratins, as these mammals come from arid environments in southern Africa and central Asia, respectively.

We found that there was a slight, although not significant, trend between dry tensile stiffness and matrix content (figure 3). If the matrix is an elastomeric network, as our model of keratin mechanics suggests, then it should be soft and extensible in water (i.e. rubbery), but stiff and brittle (i.e. glassy) at low relative humidities [34]. The fact that high matrix content keratins are stiffer when dry than low matrix keratins suggests that the dry matrix is stiffer than dry IFs. The mechanics of rhinoceros horn, the lowest matrix sample, show some interesting similarities to the matrix-free hagfish IF threads, including very low hydrated stiffness [16]. Rhinoceros horn was the only keratin to experience greater break stress in reduced compared with control samples. While the hydrated control horn tubules had very

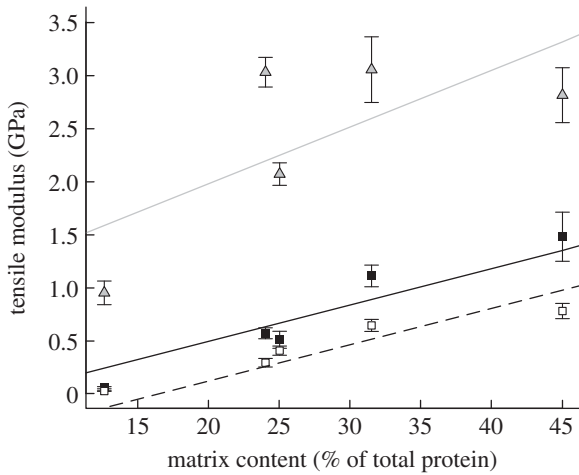


Figure 3. A significant positive relationship was found between matrix content and tensile modulus of keratins in water (black symbols; $t_3 = 5.91$, $p < 0.01$), and there was also a significant decrease in tensile modulus across keratins when the reduction treatment was applied (open symbols; $t_{105} = 6.18$, $p < 0.01$). There was no significant effect of matrix content on the modulus of keratins tested under dry conditions (grey symbols; $t_3 = 1.71$, $p = 0.185$).

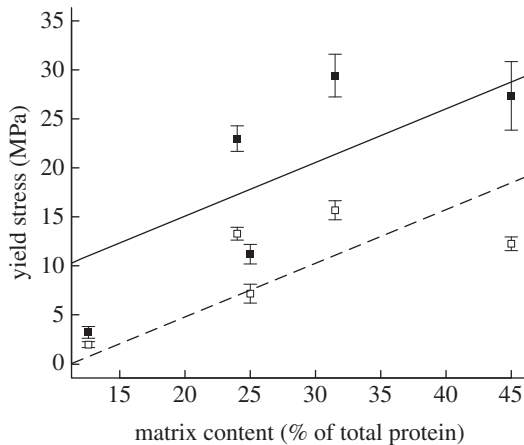


Figure 4. No significant relationship was found between yield stress and matrix content in water (black symbols; $t_3 = 1.89$, $p = 0.154$), but there was a significant decrease in yield stress when keratins were treated with the reducing agent (open symbols; $t_{105} = 9.36$, $p < 0.01$).

low breaking strains (table 1), the reducing treatment may have acted to plasticize the keratin by allowing for increased penetration of water and extensibility. A similar result was found in horse hoof, which shows increased fracture toughness at higher levels of hydration [30].

Our swelling data are consistent with Bendit's finding that swelling correlates negatively with matrix content [20]. This relationship was originally explained by the assertion that greater matrix content would provide less volume for water absorption, based on a model that assumed fixed matrix volume [20]. However, the assumption of fixed matrix volume across all matrix contents is dubious given the large variation in matrix contents across the keratins. A more parsimonious explanation is that the keratin matrix is simply more hydrophobic than the IFs, and therefore swells less in water than the more hydrophilic IFs. This idea was originally proposed by Zahn [19] and revived by Hearle [18]. Another study by Bendit [35] examined keratin matrix content and tensile mechanics and found no significant relationship between the two variables

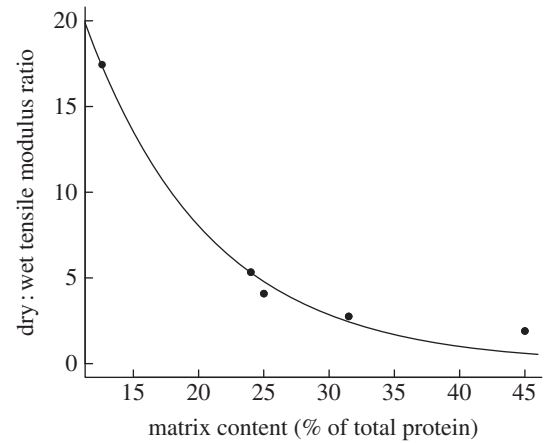


Figure 5. Hydration sensitivity (ratio of tensile modulus in dry and hydrated conditions) of keratins as a function of matrix content ($t_3 = 10.74$, $p = 0.0017$), indicating an exponential decay relationship with large increases in sensitivity at low matrix content and a subsequent plateau of sensitivity at high matrix levels.

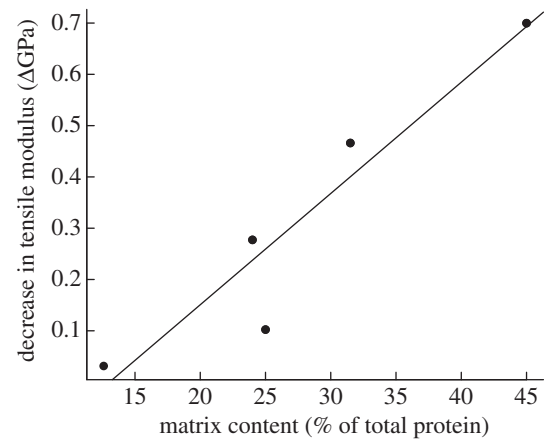


Figure 6. Absolute change in tensile modulus for each keratin resulting from the reduction treatment. Notice that whale baleen bristles (matrix = 25.0%) experienced relatively little change in tensile mechanics when treated with the reducing agent.

for all keratins, and a negative correlation when considering just fibres. There are several methodological differences between our study and Bendit's that may account for the different outcomes. First, in that study various keratins such as echidna quill, human fingernail and cow horn sheath, in which the IFs run perpendicular to the growth axis, were included in the mechanical analysis [22,36,37]. IF orientation in a sample has a strong effect on mechanical properties, and tensile tests that strain keratins perpendicular to the IFs will greatly underestimate modulus. Additionally, we found that degreasing keratins prior to hydration significantly increased swelling and the effectiveness of the reduction treatment. Hydrophobic surface lipids are common on many different mammalian keratins, especially hairs and quills [38,39], and in the light of our results, these lipids may play an important role in reducing the hydration sensitivity of keratins, especially those with low matrix content. In Bendit's study, there was no mention of any attempt to remove surface lipids, which may have dramatically reduced the rate of hydration of the keratins used.

If the matrix squeeze hypothesis is an accurate representation of keratin structure and mechanics, it raises the question of how keratin IFs initially achieve a dry state, since IFs assemble in the aqueous environment of living

keratinocytes. Air-drying is one mechanism by which IFs could be stiffened, and the oxidizing environment of air exposure would also result in the oxidation of sulphhydryl groups, causing the formation of disulphide bonds within the matrix. This model of keratin development is consistent with observations of parturition in the North American porcupine (*Erethizon dorsatum*), which is born with (mercifully for the mother) soft quills that harden within hours of exposure to the air [39]. In keratins produced by juveniles and adults, dehydration and cross-linking probably occur as the epidermal appendage emerges from the skin. IF dehydration via air exposure is not possible in all mammals, however, as some aquatic species, such as cetaceans, experience chronically hydrating conditions.

Baleen whales possess elaborate keratinous baleen plates that grow from their upper palate and are used for straining prey such as plankton and fishes. For baleen, there is no option for air drying, and our data demonstrate that blue whale baleen bristles were less affected by the reduction protocol than other keratins from terrestrial mammals (figure 6). This result is consistent with the idea that IFs in terrestrial hard α -keratins are locked into a dehydrated state after (or during) air-drying via cross-linking of the keratin matrix around them. If the IFs in whale baleen never have the chance to air dry, then one would expect that loosening up the matrix would have less of an effect on their mechanical properties than IFs that have been kept essentially dry by the matrix. The fact that baleen keratin does not behave as if its constituent IFs are fully hydrated (i.e. like wet hagfish slime threads), even after reduction of the keratin matrix, suggests that other mechanisms are probably involved in stiffening this unique material. Recent evidence suggests that high levels of calcification may act to boost the modulus and yield stress of baleen in some species, especially the rorquals [40]. Other keratin structures are known to incorporate calcium phosphate salts [41], possibly with similar effects on their mechanics. Furthermore, air-drying may not be the only way that water can be removed from IFs. Another possibility is that water is actively 'wrung' out of the IFs via syneresis as the keratin matrix is cross-linked and shrinks around the IFs during the final stages of keratinization [17].

While the data presented here are consistent with several of the predictions made by the matrix squeeze hypothesis, there are probably other important mechanisms that govern α -keratin mechanics. For example, disruption of the keratin matrix via reduction and methylation of disulphide bonds did not transform all keratins into soft, extensible, rubberlike materials akin to hagfish slime threads in water. This may have been owing to incomplete reduction or methylation, or possibly owing to the presence of other kinds of linkages that may hold the keratin matrix together, such as dityrosine

bonds. Indeed, one of the main components of the α -keratin matrix is the so-called 'high glycine-tyrosine' protein fraction [42]. Another possibility is that cross-links (disulphide or otherwise) exist within the keratin IFs themselves. However, if our reduction and methylation protocol only acted by reducing cross-links within the keratin IFs, we would not expect a substantial decrease in the yield stress, as we have argued in a previous paper [17]. The yield stress in keratins is indicative of the stress at which hydrogen bonds within constituent α -helices start to fail, and cross-linking should have little effect on this value, especially compared with the globally stabilizing effect of dehydration. Furthermore, the presence of intra-IF cross-links cannot explain the fact that high matrix keratins are stiffer, swell less and experience a greater drop in modulus and yield stress after reduction than low matrix keratins.

Our research indicates that some of the variability in the mechanical behaviour of mammalian hard α -keratins can be explained by the relative abundance of the keratin matrix, which exerts its influence by modulating the degree to which the constituent IFs are allowed to hydrate. The appearance of a keratin matrix capable of resisting IF hydration may therefore have been crucial to the evolution of hard keratins in early mammals. Similarities between the matrix of hard α -keratins and a component in the saurian hard keratins [43] suggest the intriguing possibility that similar mechanisms may be at play in the β -keratins.

5. Conclusions

We have tested several predictions of the matrix squeeze hypothesis, which states that hard α -keratin mechanics in water are influenced by the hydration state of their constituent IFs, which in turn is regulated by the mechanical resistance to swelling and softening provided by an elastomeric keratin matrix. Our results are consistent with this hypothesis and suggest that the hydrated mechanics of hard α -keratins is governed by the presence of the matrix. Our results also suggest that it may be possible to design novel composite materials that take advantage of the molecular tug-of-war between solvent sensitive fibres and a solvent-insensitive polymer matrix.

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