Keratin made micro-tubes

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KERATIN MADE MICRO-TUBES: THE PARADOXICAL THERMAL BEHAVIOR OF CORTEX AND CUTICLE

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ABSTRACT

Keratin micro-tubes were obtained by heating medullated keratin fibres to temperatures above 230 °C under nitrogen atmosphere, when, as documented by microscopy, the cortex (the core of the fibre) melts from the medulla outwards, followed by pyrolysis of the material through the remaining solid cuticle (shell) layer. The resulted hollow tubes from fibres void of cortical material keep the external cuticle structure, as shown by AFM investigation, and the moisture sorption properties of the initial keratin fibre. Despite similar amino-acid compositions of cuticle and cortex the two morphological components differ significantly in their thermal behaviour, which appears to be a “cortex-cuticle thermal stability paradox”.

KEYWORDS: Keratin fibre; Cuticle; Thermal stability; Micro-tubes

1. Introduction

The hair is a filamentous appendage of the skin of vertebrates, which serves to protect the body against environmental influences. It is made of filamentous proteins, the hard alpha-keratins [1-3]. The keratin fibres have a composite structure, with a core-shell as well as filament-matrix arrangement on various levels of organisation, from the cortex wrapped by cuticle down to the intermediate filament (IF) surrounded by intermediate filament associated proteins (IFAP), also referred to as keratin associated protein (KAP) [1-3].

The keratin fibres exhibit a relatively high thermal stability; the fibre properties remain almost intact until 200 °C [4-6]. The ability of keratins to preserve their properties up to high temperatures, apart from the obvious benefit for body protection, is of interest for the potential to design thermally highly stable proteins [6].

DSC investigations of keratin fibres in the dry state show an endothermic effect, sometime a doublet, which at 10 K/min heating rate ranges from 230 to 260°C [4, 7, 8]. The endothermic effect is generally attributed to the thermal denaturation of the α -helix which makes the crystalline part of the keratin fibre [5, 6, 8, 9].

Cuticle is formed of four layers with different cross-link densities due to their contents of disulphide and iso-dipeptide bonds, namely from the outside, epi-cuticle, a-layer, exo-, and endo-cuticle, respectively [3]. Because the volume fraction of the cuticle is of the order of 10% of the total keratin fibre and because it has a similar chemical structure as the cortex, cuticle contributions are often neglected when hair properties are determined [10]. Our results show, however, that the cuticle has to be understood as a different component of the fibre with very individual properties, which require more investigation.

2. Experimental

As alpha-keratin material we used the commercial, European, brown hair purchased from Kerling Int. Haar Fabrik. L-amino-acids of analytical grade were purchased from Merck. Preparation of cuticle: snippets of hair were mechanically abraded as described in methodology published elsewhere for isolating cuticle from cortex [11]. Microscopic evidence showed that the separation of cuticle was successful.

The heating experiments were carried out on a DSC-7 (Perkin Elmer), using closed aluminium pans, whose lids were pierced with two holes. An empty crucible was used as reference. The DSC device was calibrated using indium and palmitic acid, both of high purity.

Prior to the measurements, the hair samples were cut into 1-2 mm snippets and stored under constant, ambient room conditions (approx 22°C, 55% relative humidity) to ensure invariant water contents. Samples of 7-10 mg were heated with a heating rate of 10 K·min⁻¹ under a nitrogen flow of 20 mL·min⁻¹, for temperatures ranging from 50 to 300 °C. After reaching certain aimed temperatures the heating was stopped and the sample rapidly cooled. This way we were able to collect samples before, at, and after the endothermic peak recorded on DSC.

Thermo-gravimetical analysis (TGA) of amino-acids was performed on an Iris TG209C (Netzsch). Samples of around 10 mg of pure amino-acid were placed in the alumina crucibles and heated at 10 K·min⁻¹ under a nitrogen flow of 20 mL·min⁻¹ from room temperature to 400 °C.

Thermo system FP 90 with FP82 hot stage (Mettler Toledo) and optical microscopy were used to follow the events observed at DSC under normal atmosphere.

Scanning Electron Microscopy photos were taken for gold sputter-coated snippets sampled at temperatures chosen from the DSC curve to lie before, at, and after the peak using a SEM S360 (Zeiss NTS GmbH, Oberkochen) at an acceleration voltage of 15 kV.

Amino-acid analysis was carried out on the snippets before and after the peak. Each sample was hydrolysed in 3 mL 6N HCl at 110 °C for 24 h. The hydrolysed samples were dried in a rotary evaporator under heating. Amino acid analysis was carried out using an analyser type Alpha-Plus II, (Pharmacia).

Scanning force microscopy (SFM) measurements have been performed in Tapping mode (Bruker, ICON) using Nanoscope 8.10 software and OTESPA tips (spring constant 12-10³ N·m⁻¹, resonant frequency 278-357 kHz) under ambient conditions. The samples of

the native hair and of the keratin micro tubes have been fixed on pieces of silica substrates using double-side adhesive tape (Tesafilm®).

Moisture sorption-desorption isotherms were measured on an IGA Sorp Moisture Sorptiometer Analyser (Hidden Analytical), at 25 °C, using a programme of increasing the relative humidity, RH, in steps of 10-15%, from 1% to 95% RH.

3. Results and discussion

The DSC plot in **Figure 1** shows the endothermic effects occurring at around 240 °C. The first effect is attributed to the melting of the crystalline phase, that is the unfolding of the alpha helices, and is termed as the thermal denaturation of keratin fibres [4]. The process is irreversible and kinetically controlled by the surrounding environment, which is the amorphous matrix phase, made of IFAPs, in which the alpha-helices are embedded [4-6].

Figure 1. DSC trace of hair heated with a rate of $10\text{ K}\cdot\text{min}^{-1}$. The temperatures of the peaks and the estimated values of enthalpy are shown on the curve. The dashed lines are the baselines drawn for evaluating the areas of the peaks (enthalpy).

The second effect, less visible in most experiments, is attributed to the pyrolysis of the fibre [4, 5, 9].

We sampled snippets at various temperatures up to $300\text{ }^{\circ}\text{C}$ and examined them in the scanning electron microscope (see **Figure 2**).

Figure 2. SEM images of keratin fibre snippets sampled at the indicated temperatures (heating rate of $10 \text{ K} \cdot \text{min}^{-1}$)

One notices that at temperatures beyond $230 \text{ }^{\circ}\text{C}$ the cortex seems to vanish and the original fibres converted into tubes, consisting only of cuticle. Because the diameter of the tubes matches those of original hair fibres, of around 50 micron in this particular case, we term them “micro-tubes”. At around $300 \text{ }^{\circ}\text{C}$ the micro-tubes appear to become highly brittle and disintegrate into small pieces.

The results shown in **Figure 2** suggest that the thermal stability of cortex and of cuticle differ significantly. Similar results were obtained on fibres sourced from various mammals, indicating that this is a general property of keratin fibres [12].

More detailed information on the surface topography and heterogeneity of the keratin micro tubes was obtained with the SFM measurements. **Figure 3a** shows a three-dimensional topographic image of the ~~cuticle~~ keratin tube surface with clearly preserved scales. The surface ~~structure~~ of the tubes shown in close up images in (**Figure 3b, c**) is relatively homogeneous with sub-100 nm holes (Figure 3b) which presumably assisted the removal of the degraded material of the cortex after pyrolysis. The area close to the scales (**Figure 3 d, e**) exhibits a more heterogeneous character with a noticeable lifting at the scale edge. This Such lifting is clearly seen in the plot in **Figure 3f** which compares

the an averaged topographic profile of a native hair (which has flat plateaus between the scales) with the topographic structure of the keratin micro-tubes. Another observation suggested by the analysis of the cross-sectional profiles of the SFM topography images in **Figure 3f** is the shrinkage with up to 35% of the cuticle. ~~This type of the surface profile indicates that cuticle undergoes partial shrinkage~~ during thermal processing, as derived from the averaged step-height between the residual scales. We note that a detailed statistical analysis would be required for making more precise conclusions on cuticle shrinkage and shape deformation upon thermal treatment. Furthermore, the material of scales appears to shrink to a lesser extent than those of cuticle, leading to a zig-zag form of the topography of tubes compared to native hair. It has also to be underlined that the residual scales are hardly noticeable by SEM (see **Figure 2** at 245 °C), which indicates the usefulness of SFM as a powerful, albeit rarely applied tool for investigating details of hair topography.

Figure 3. SFM images of keratin fibre sampled at 245 °C: **(a)** 3D topography image, **(b)** topography and **(c)** phase images of the area highlighted by the right yellow square in image **(a)**; **(d)** 3D topography and **(e)** phase images of the area highlighted by the left blue square in image **(a)**; **(f)** averaged cross-section of the SFM topography image of a native hair (dashed line) and of keratin tube (solid line). The colour scale bar indicates scales of 70 nm **(b)** and 10° **(c, e)**.

Figure 4. Topographic profiles of the surfaces of a native hair sample (dashed line) and of a keratin micro-tube (solid line).

Figure 5-4. (a) SFM 3D topography image of the inner surface of keratin fibre sampled at 245 °C. (b) Topographic profile of the surface in (a). Cross-section of the topography image showing a characteristic zig-zag profile.

The inner surface of the broken micro-tubes is shown in **Figure 5 4a** and is consistent with the SEM image at 245 °C (see **Figure 2**). According to the phase signal of the SFM images (not shown here for the inner surface), both the upper and the inner surface of the keratin micro-tubes have similar surface characteristic, namely rather homogeneous surface coverage with composed by amorphous materials. Less noticeable on SEM images, the scales with height of 200 ± 50 nm are clearly visible at SFM analysis the inner surface of the tubes with a characteristic zig-zag profile (**Figure 4b**). As such it appears that Presumably, the whole structure of cuticle scales is kept through the thermal treatment and is protruded through the remaining cuticle. It appears that this is the first

indication that the thermal behaviour of the scale-building proteins differ from those of the rest of cuticle.

The amino-acid compositions of fibre, cortex, cuticle and micro-tubes, as given in **Table 1**, are quite similar. By detailed comparison of the composition of tubes with that of the original fibres one notices the loss of Cystine, and Cysteic acid for the tubes, while the amounts of glutamic acid and ornithine increase.

Table 1. Amino-acid composition (in mol / 100 mol) of whole fibre, cuticle, cortex and micro-tubes sampled at 255 °C

Amino acid	Total Fibre	Cuticle	Cortex	Micro-tubes @ 255 °C
Cysteic acid	0.71	1.84	0.68	0.39
Aspartic acid	5.57	3.47	5.73	5.96
Threonine	7.7	4.96	8.08	3.9
Serine	10.78	16.32	11.7	4.24
Glutamic acid	13.44	11.06	13.9	18.09
Proline	8.95	10.92	8.86	12.49
Glycine	6.34	9.66	6.37	10.38
Alanine	4.56	6.06	4.96	8.1
Lanthionine	-	0.46	-	0
Valine	5.67	8.05	6.13	7.97
Cystine	8.83	9.39	8.94	0
Methionine	0.51	0.47	0.29	0.42
Isoleucine	3.38	2.22	2.86	3.39

Leucine	7.46	4.79	6.8	9.24
Tyrosine	2.03	1.29	1.68	2.49
Phenylalanine	2.32	1.67	1.92	2.47
Ornithine		0.38	0.25	1.49
Lysine	2.95	3.56	2.9	2.42
Histidine	1.06	0.57	0.99	1.06
Arginine	7.74	2.87	6.96	5.49

Because of the complete loss of Cystine in the micro-tubes we recalculated (see **Table 2**) the amino-acid composition of cuticle by taking off the amount of Cystine from the components and re-calculating the corresponding percentages for the other amino-acids. The last column in **Table 2** lists the ratio of the amino-acids of the micro-tubes to the recalculated cuticle and highlights (in *Italics*) the values of more than 40% change.

Table 2. Recalculated (neglecting Cystine) amino-acid composition of cuticle compared with those of tubes. The last column indicates the relative change of the amino-acid concentrations for the micro-tubes compared to those in the recalculated cuticle. The ratio values significantly different from 1 are marked *italic*.

Amino acid	Recalculated Cuticle	Micro-tube @ 255 °C	Ratio Micro-Tube : Cuticle
Cysteic acid	2.04	0.39	<i>0.2</i>
Aspartic acid	3.85	5.96	<i>1.55</i>
Threonine	5.50	3.9	0.71
Serine	18.10	4.24	<i>0.23</i>

Glutamic acid	12.27	18.09	<i>1.47</i>
Proline	12.11	12.49	1.03
Glycine	10.71	10.38	0.97
Alanine	6.72	8.1	1.21
Lanthionine	0	0	0
Valine	8.93	<i>7.97</i>	0.89
Cystine	0	0	0
Methionine	0.52	0.42	0.81
Isoleucine	2.46	3.39	1.38
Leucine	5.31	9.24	<i>1.74</i>
Tyrosine	1.43	2.49	<i>1.74</i>
Phenylalanine	1.85	2.47	1.33
Ornithine	0.42	1.49	<i>3.54</i>
Lysine	3.95	2.42	0.61
Histidine	0.63	1.06	<i>1.68</i>
Arginine	3.18	5.49	<i>1.72</i>

The last column of **Table 2** shows the decrease of serine and lysine and the unexpected increase of glutamic acid, leucine and ornithine in tubes compared to the initial composition of the cuticle. It has also to be mentioned that, with the exception of Cystine, there is no other amino-acid which vanishes completely from the composition of the tubes. Joerissen [12] noticed such ratios also for other keratin fibres heated above 200 °C, even if not producing tubes as a result.

Investigating the thermal stability of the individual amino-acids by thermogravimetry (TGA) with $10\text{ K}\cdot\text{min}^{-1}$ we found, in line with results from the literature [13, 14] that all of them decompose mainly within the range of $200\text{--}300\text{ }^{\circ}\text{C}$ (see **Table 3**). Namely, Glutamic, or Aspartic acid as well as Leucine should be decomposed by at least 50% each at the temperature at which the micro-tubes were obtained.

Table 3. Thermal stability in terms of temperature interval of weight loss and percentage of weight loss over that interval as measured by TGA at $10\text{ K}\cdot\text{min}^{-1}$ under nitrogen draft. The last column gives the percentage of amino-acid loss or gain for the micro-tubes compared to the amount in the cuticle (recalculated, as in **Table 2**).

Amino acid	Weight loss (%)	Temperature interval ($^{\circ}\text{C}$)	Amino-acid variation in Micro-Tubes vs Cuticle (%)
Cysteic acid	n.m.	n.m.	-80
Aspartic acid	30%	192-275	+55
	30%	360-451	
Threonine	90%	206-287	-29
Serine	65%	158-262	-77
Glutamic acid	60%	194-376	+47
Proline	100%	192-297	+3
Glycine	50%	212-313	-3
Alanine	100%	267-306	+21
Lanthionine	n.m.	n.m.	n.m.
Valine	100%	220-312	-11
Cystine	85%	206-285	-100

Methionine	100%	260-331	-19
Isoleucine	100%	240-307	+38
Leucine	100%	240-313	+74
Tyrosine	80%	270-433	+74
Phenylalanine	60%	213-294	+33
	40%	294-369	
Ornithine	n.m.	n.m.	354
Lysine	35%	222-285	-39
	30%	285-379	
	30%	379-489	
Histidine	26%	148-197	+68
	35%	247-361	
Arginine	15%	214-283	+72
	55%	283-401	

Note: “n.m.” stands for “not measured”.

The amino-acid composition of the micro-tubes suggests that the thermal stability of the amino-acids in the protein chains of the cuticle is higher than that of the individual molecules. This still does not explain why the same amino-acids arranged in the protein chains of the cortex already undergo pyrolysis at, and beyond, 250 °C, whilst arranged in the chains of the cuticle they remain stable.

In order to understand how the micro-tubes emerge we have examined under an optical microscope the snippets of keratin fibres immersed in silicon oil while heating from room

temperature to 300 °C (with 10 K·min⁻¹) on the hot stage. The silicon oil isolates the snippets from the environment, provides a good thermal medium at temperatures above 200 °C and allows optical examination under the microscope. Two selected snapshots are shown in **Figure 6 5**.

Figure 6 5. Snapshots showing the behaviour of keratin snippets heated in silicon oil at a rate of 10 K·min⁻¹. The temperature of each snapshot is indicated on the photo

It appears that the cortex starts melting within the temperature interval recorded on DSC for the two endothermic peaks (see Fig. 1). The melting process begins from the middle of the shaft, where for coarse keratin fibres the medulla is located, and propagates centrifugally. Since medulla has only traces of Cysteic acid and Cystine, and large amounts of acid and basic amino-acids (Glutamic acid, Lysine, Leucine) compared to rest of cortex, being therefore less cross-linked [15], it is expected that it has a lower thermal stability as a consequence. Following melting, the viscous liquid “boils”, and pyrolysis gases escape through pores of the cuticle, or through the ends of the snippet. At the end of the second DSC endothermic peak the evaporation process is completed and the micro-tubes are obtained.

The snapshots in **Figure 6 5** show also that the first DSC endothermic peak recorded for keratin fibres in the dry state should be interpreted with care; it cannot be ascribed only to the denaturation of intermediate filaments and the pyrolysis process needs to be also considered.

Secondly, the snapshots indicate how the tubes form. When there are no pores available for gas escape the pressure reached inside the fibre may disrupt the cuticle, explaining the broken micro-tubes found among the others.

The snapshots also show that melting of the core of hair shaft at the level of medulla is a prerequisite stage for tube formation. In fact, as **Figure 7 6** shows, we could not detect keratin micro-tubes in thermal treated keratin fibres which do not present medulla, like Yak and fine Merino wool.

Figure 7 6. SEM images of keratin fibre snippets from Yak and Merino fine wool, sampled at 262 °C, well beyond the endothermic peak recorded on DSC at heating rate of 10 K·min⁻¹

The low cross-linked material of medulla is the “weak” place where the process begins. The melting propagates then from centre towards cuticle. Cortex contains 30-40% alpha-helix, ordered (crystalline) material and the rest made of pretty amorphous (or low

ordered) macromolecules [1, 2, 3, 5], which soften and flow with temperature; the cuticle is made of some beta-sheet arranged protein and amorphous cross-linked material [3, 10, 16] which not only does not melt, but becomes even more rigid after thermal treatment [17]. These differences in morphology are very likely the reason for the micro-tube formation and explain also why fine fibres, lacking medulla cells, do not transform into tubes by heating.

Keratin fibres are known to absorb moisture up to 33% of their weight and to exhibit a large hysteresis at desorption (a large difference between the absorbed and desorbed moisture amount at the same relative humidity, RH) supposed to be due to the slow relaxation of the keratin structure [18, 19]. As indicated by the amino-acid analysis, the micro-tubes retain most of their original overall structure. Consequently, we have investigated how much the sorption-desorption properties are changed for the micro-tubes compared to the original hair following the thermal treatment.

Figure 8 7 compares the behaviour of native fibre, separated cortex, separated cuticle and the tubes. The results show that native fibre and isolated cortex behave almost identically, while the isolated cuticle absorbs less moisture than both of them at humidity values higher than 50%. The micro-tubes absorb less moisture than the original cuticle, but, in absolute values, still a significant amount, reaching 20% of the weight at 95% RH.

Figure 8 7. Moisture sorption curves recorded on IGASorp machine for native keratin fibre, isolated cortex, isolated cuticle and tubes obtained at 255 °C, for relative humidity, RH, ranging from 1% to 95%, at 25 °C.

The plot (see **Figure 9 8**) of the differences between the absorbed and desorbed moisture amount at the same relative humidity for the fibre and tubes, respectively, puts into evidence the fact that the micro-tubes show a significantly larger sorption-desorption hysteresis than native fibres, namely, over the mid-range of relative humidity values. This indicates a lower mobility (longer relaxation time) of the protein chains in the micro-tubes which we assume to be due to an increased cross-linking degree of the tubes structure.

Figure 9 8. Differences between absorbed and desorbed moisture content by native hair and micro-tubes, for relative humidity ranging from 1 to 95% RH, at 25 °C

Summing up, by heating alpha-keratin fibres at temperature above 230 °C we achieve producing micro-tubes which retain most of the native protein structure (amino-acids composition), and behave similarly to the native fibres in terms of moisture management.

4. Conclusion

The microscopic investigation of the keratin fibres heated with a controlled programme reveals that the endothermic effects recorded around and beyond 230 °C for the dry fibres are related to a more complex process than just the thermal denaturation of alpha-helices. The melting and volatilisation of the cortical substance, following its pyrolysis, occurs also at that temperature.

Although made of similar proteins the core-shell arrangement of the material in keratin fibres, and the different morphologies of cortex (the core) and cuticle (the shell) lead to different thermal stabilities of the two components. While the cortex, having a third of its protein components organised as crystals, softens, melts and evaporates above 230 °C, the cuticle, made of amorphous cross-linked proteins, withstands temperatures even beyond 250 °C. This paradoxical thermal behaviour of the proteins in cortex and cuticle leads to the formation of protein (keratin) based micro-tubes with preserved but slightly distorted scales as compared to the initial keratin fibre. ~~Our results show that the micro-tubes maintain most of the original amino acid chemical structures of the keratin fibre and its moisture sorption-desorption properties.~~ In summary, micro-tubes produced by heating medullated hair to temperature above 230 °C retain most of their amino-acid composition and also behave similar to native fibres in terms of moisture management.

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