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Protein Expression in Orthocortical and Paracortical Cells of Merino Wool Fibers

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Crimp and bulk, important wool fiber properties, are thought to be related to differences in the protein composition of the orthocortex and paracortex. Fiber morphological studies have demonstrated that the paracortex has a higher proportion of matrix and cysteine than the orthocortex. While there is some evidence for the differential expression of genes between these cell types in the follicle, this has not been demonstrated satisfactorily in the mature fiber. Using proteolytic digestion of wool fibers, followed by ultrasonic disruption to obtain relatively pure fractions of both cell types, the KAP3 high sulfur protein family was found to be present in higher concentrations in the paracortex. This significant finding provides an explanation for the higher cysteine content reported in the paracortex. This represents an advance in our understanding of protein expression variation in the orthocortex and paracortex, and how this relates to key physical and mechanical properties of wool fibers.

KEYWORDS: Proteomics; mass spectrometry; electrophoresis

INTRODUCTION

Wool crimp, the number of bends per unit length along the fiber, is an indication of the spinning efficiency of the wool. Thus, the higher the level of crimp in the fiber, the more cohesion there will be between individual fibers after spinning. Fibers with fine crimp usually also have a smaller average fiber diameter. As a result they can be spun into fine yarns of great length for a given weight of wool and hence have a higher market value (*1*). Conversely, the absence of crimp, such as in the Merino felting-luster mutants, results in a fiber that has a high sheen and felts (or matts) seven times faster than normal Merino wool (*2*). For coarser diameter wools the property of bulk is of greater importance, particularly for end-uses such as carpet and hand-knitting yarns, as it imparts a fullness to the yarn and hence improved insulation (*3*). Both properties correlate with fiber curvature (*4*), and hence, there has been considerable interest in the fundamental origin of curvature in the fiber and, in particular, in how this relates to the fiber ultrastructure and protein composition (*5*).

Three types of cortical cells are known to be present in the wool fiber: orthocortical, mesocortical and paracortical (*5*). Orthocortical cells predominate, typically making up over 50% of the fiber cross-section and volume (*5*). Generally, between breeds and within a breed, the proportion of orthocortical cells increases relative to the proportion of mesocortical and paracortical cells as the diameter increases, often with a partial substitution of the paracortex by the mesocortex, particularly in low crimp fibers (*5*). Furthermore, considerable variation in the number and distribution of cell types within a fiber cross-section and along the length of the fiber has been observed (*6, 7*). Fibers of the felting luster mutant Merino have been found to contain paracortical cells only (*8*). High crimp fibers, such as fine Merino wool, usually exhibit a well-defined bilateral arrangement of orthocortical and paracortical cells, with approximately half of the fiber being composed of each type (*9, 10*). The orthocortex is found on the outside (in the cortical region adjacent the convex fiber surface) of the crimp wave, and it has been generally thought that this bilateral segmentation is the cause of crimp (*5*).

Two types of the proteins are found in the cortex of wool: the α-helical intermediate filament proteins (IFPs), which are the main structural element of the wool fiber, and the keratin associated proteins (KAPs) into which they are embedded. Differences in protein expression in the follicle have also been observed between the two halves of the fiber, and this is thought to have an influence on fiber crimp. In Merino follicles, IFP genes are the first to be activated across all cortical cell types, followed by KAP genes, the first of which, the high glycine-tyrosine proteins (HGTPs), appear in the cells in the orthocortical half of the cortex (*11*). The high sulfur proteins (HSPs) appear next in the complementary half of the cortex, while higher up the follicle most cortical cells produce both HGTPs and HSPs. However, the expression of an ultrahigh sulfur protein (UHSP) family appears to be restricted to the half of the cortex that includes the paracortex (*11*). High lateral resolution elemental mapping carried out using TEM/electron energy loss spectroscopy has revealed a higher concentration of sulfur in the

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paracortex than in the orthocortex and the reverse for tyrosine in the orthocortex (*12*).

Various approaches to separating orthocortical and paracortical cells from wool fibers have been made in order to determine the protein composition of the cell types by amino acid analysis. When cortical cells were separated by density gradient centrifugation after selective labeling by gold deposition, the orthocortical cells were observed to contain more tyrosine, glycine, leucine and phenylalanine and less cysteine than the paracortical cells (*13*). However, as the differences in amino acid composition were only of the order of 1 mol %, they were considered unlikely to account for the considerable differences in the properties of the two cortical cell types. Similar results were observed when orthocortical and paracortical cells were liberated either from formic acid-swollen Merino fibers (*14*) or by trypsinolysis followed by ultrasonication (*15*) and subsequently separated by a differential sieving procedure. Disruption of cuticle-free Merino fibers by successive freeze−thawing followed by density gradient centrifugation was used to separate cells from the orthocortex and paracortex, but very little difference was found in the amino acid composition of the two cell types (*16*, *17*). In an alternative approach, involving the swelling of cuticle-free Merino wool fibers in formic acid followed by treatment with trypsin, cells were preferentially released from one side of the cortex, thus enabling the separation of orthocortical and paracortical cells by way of filtration through 35 μm screens (*18*). Once again, the tyrosine content was found to be 1% higher in the orthocortex, while the cysteine content was found to be 2.5% higher in the paracortex. From nonequilibrium-two-dimensional electrophoresis (NE-2DE), the UHSPs were also found to be present in higher concentration in the paracortex, while at least one KAP was found to be unique to the orthocortex (*18*).

Despite these various studies, the exact protein composition of the different cell types in the keratinised fiber is still unclear (*19*). With the advent of modern proteomic techniques there is an opportunity to obtain a more precise picture of the protein composition of the major cell types in the cortex of wool fiber. This study seeks to address these issues, using isoelectric focusing-2DE (IEF-2DE) and mass spectrometry (MS), to identify specific proteins observed to be differentially expressed between the two cell types.

MATERIALS AND METHODS

Materials. Ammonium bicarbonate, ammonium hydroxide, 3-[(3-cholamido-propyl)-dimethylamino]-propane-sulfonate (CHAPS), dichloromethane, ethanol, isopropanol, acetone, guanidine, methanol, potassium hydroxide, sodium phosphate, tris(hydroxymethyl)methylamine (tris), *N*,*N*-dimethylformamide and urea were all obtained from Merck (Darmstadt, Germany). Immobilized pH gradient (IPG) strips were obtained from GE Biosciences (Uppsala, Sweden). Dithiothreitol (DTT), formic acid (FA), Pronase E and 2,2,2-trichloro-1,1-ethanediol (chloral hydrate) were obtained from BDH (Poole, Dorset, England). Teric GN9 was obtained from Orica Ltd. (Auckland, NZ). TPCK trypsin was obtained from Promega Corporation (Madison, WI). Uranyl acetate, tris(2-carboxyethyl) phosphine (TCEP), α-cyano-4-hydroxy-trans-cinnamic acid (HCCA) and lead citrate were obtained from Fluka Chemie GmbH (Buchs, Germany). Acrylamide solution and Colloidal Coomassie Blue G250 were obtained from Bio-Rad Laboratories (Hercules, CA). Osmium tetroxide was obtained from Electron Microscopy Services (Hatfield, PA). TLCK-chymotrypsin and iodoacetamide (IAM) were obtained from Sigma-Aldrich (St Louis, MO).

Methods. *Isolation of the Orthocortical and Paracortical Cells.* Merino wool of approximately 20 μm diameter was scoured with 0.15% Teric GN9 at 60 and 40 °C, then water at 40 and 60 °C, before being further scoured with dichloromethane and ethanol.

Fibers preswollen in FA for 1 h were disrupted by an UltraTurrax shear mixer (IKA-Werke GmbH, Staufen, Germany) in 1 min bursts for periods from 1 to 10 min or disrupted for 30 min with a 750 W Ultrasonic Processor (Cole-Palmer Instruments, Vernon Hills, IL) in a waterbath regulated at 20 °C. The resulting cell preparation was suspended in a 1.45−1.55 g/mL chloral hydrate density gradient and centrifuged at 4000*g* for 30 min (*15*). Alternatively, prior to disruption and separation by density gradient centrifugation, the fibers were stained by immersion in a 2% solution of gold chloride for 10 min then destained in 25% formic acid for periods ranging from 1 to 5 h (*13*).

In another approach, the wool fibers were treated with Pronase E for periods ranging from 3 to 6 days, at 37 °C in a 1% ammonium bicarbonate buffer at pH 8, using a ratio of buffer:enzyme:substrate of 2,000:1:20. The fibers were then disrupted with the 750 W Ultrasonic Processor at 50% power, the process being stopped after 30 min and the isolated cells removed from the fiber residue by filtration through a 125 μm sieve. This process was continued for a maximum of 150−180 min, with more cells being removed by filtration at intervals of 30 min until there was a significant visual reduction in the number of cells released from the fiber residue.

Determination of Cortical Cell Enrichment by TEM. Subsamples of the final preparations were stained en bloc with osmium tetroxide and uranyl acetate, and embedded in acrylic resin (*6*), the fiber residues from the paracortical-enriched preparations being inserted into fine plastic tubing prior to embedding. Fiber transverse sections were cut, grid stained with uranyl acetate and lead citrate (*6*), and viewed by TEM on a Morgagni 286D microscope (FEI, Eindhoven, The Netherlands).

Assessment of the relative proportions of cortical cell types was estimated from the total cross-sectional area of each cell type in a grid section. A boundary line separating the orthocortex and paracortex was added manually using Adobe Photoshop (Adobe Systems Inc., San Jose, CA), and then the images were leveled to the point where the background was white and the fiber turned black. Each image was then imported into analySIS pro 5.0 (Olympus Soft Imaging Systems GmbH, Münster, Germany) and the transverse cross-sectional area of each cell type measured using thresholding and blob area measurement.

IEF-2DE of Orthocortical and Paracortical Proteins. The cortical cell fractions were reductively extracted for 18 h at room temperature with 8 M urea, 0.05 M tris and 0.05 M TCEP at either pH 3.5 or 7.5, and then the extracts were alkylated for 1 h with 1 M IAM or 360 mM acrylamide. Where only the KAPs were required, the concentration of urea was reduced to 2 M. IPG strips were rehydrated with 2 mg/mL of protein in 8 M urea, 4% CHAPS, 0.5% Pharmalyte 3-10 and 0.004% Orange G. Separation in the first dimension was performed on an IPGPhor (GE Biosciences, Uppsala, Sweden) for a total of 115,000 V h (*20*). The proteins were separated in the second dimension on 2 mm thick, 18 × 20 cm, 7.5−17% T polyacrylamide gels on a Protean IIxi tank (Bio-Rad Laboratories, Hercules, CA) at 250 V, 50 mA and 15 W per gel for 6 h. The proteins in the gels were then visualized using Colloidal Coomassie Blue G250 (*21*). Images of the gels were captured with a Nikon D100 digital camera, and the background subtracted with analySIS pro. The images of the gels were aligned and the spots quantitated and matched by PDQuest 7.1 2D gel analysis software (Bio-Rad Laboratories, Hercules, CA). The results were reported as histograms of relative spot volume for each protein spot of interest. In addition, a single factor analysis of variance (ANOVA) was performed on the spots showing the highest variation in intensity to test for significance at the 5% ($P < 0.05$) level.

Sample Preparation for MS Analysis. Spots were excised from gels and destained by washing twice with a solution of 200 mM ammonium bicarbonate and 50% acetonitrile for 1 h at 37 °C (*22*). The spots were then reduced with a solution of 50 mM TCEP and 100 mM ammonium bicarbonate at 56 °C for 45 min. Following this the IAM-alkylated samples were further alkylated with 150 mM IAM while the acrylamide-alkylated samples were treated with 360 mM acrylamide in 100 mM ammonium bicarbonate for 30 min. The gel pieces were crushed, incubated with acetonitrile for 10 min and dried on a Centrivap vacuum centrifugal concentrator (Labconco, Kansas City, MI), before being digested with 2 μg TPCK-trypsin or 1 μg TLCK-chymotrypsin (the latter in 5 mM calcium chloride) in 50 mM ammonium bicarbonate:

N,*N*-dimethylformamide (7:3) for 18 h at 37 °C. Peptides were then extracted from the gel by vortexing for 10 min with a solution of 50% acetonitrile, and 0.5% FA, followed with 80% acetonitrile and then concentrated on the vacuum centrifugal concentrator.

MALDI-Q-TOF/MS and MS/MS Analysis. The concentrated peptide solutions were bound to C18 StageTips (Proxeon, Odense, Denmark) activated using 10 μL of 50% acetonitrile in HPLC-grade water with 0.1% FA, followed by equilibration with 10 μL aliquots of 0.1% FA. The peptides were bound to the StageTips in one pass, desalted using two 10 μL aliquots of 0.1% FA, and finally eluted with 3 μL of 50% acetonitrile containing 0.1% FA. Freshly prepared matrix solution consisting of 40 mg/mL HCCA and 10 mg/mL nitrocellulose dissolved in 1:1 acetone:isopropanol was prepared, and 1 μL was spotted onto a matrix assisted laser desorption/adsorption ionization (MALDI) target plate. Before the matrix solution dried, the peptides eluted from the StageTip were directly applied onto the matrix. The peptides were then allowed to cocrystallize along with the matrix at ambient temperature. MS and MS/MS spectra were collected on a tandem quadrupole time-of-flight (Qq-TOF) mass spectrometer (QSTAR Pulsar *i*, Applied Biosystems, Foster City, CA) (*23*).

LC−MS and MS/MS Analysis. Nanoflow HPLC was performed on an LC-Packings (Amsterdam, Holland) system, consisting of a Famos autosampler, a Switchos column-switching module, and an Ultimate nanoflow pump. Samples were loaded on a precolumn (C18, 0.5 cm, 300 μm ID, 300 Å pore size, 5 μm particles, LC-Packings) at 8 μL/min, after which this column was switched in line with an in-house packed analytical column (Varian MicroSorb C18, 20 cm, 75 μm i.d., 300 Å pore size, 5 μm particles). The elution gradient ran from 5% to 70% acetonitrile (including 0.2% FA) in 45 min, at 150 nL/min. The column outlet was directly interfaced to the QSTAR Pulsar *i* using a Proxeon stainless steel electrospray needle. The QSTAR software was programmed for automatic acquisition of MS-TOF survey spectra, followed by MS/MS-TOF fragmentation of detected $[M + H]^+$, $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ peaks between *m*/*z* 400 and 1200.

Data Analysis. After the MALDI or LC−MS run, peak lists were extracted from the data using the Mascot script for Analyst QS 1.1 (Matrix Science, London, U.K.). These peak lists were then used to search against the NCBInr database using an in-house Mascot server (Matrix Science). The following search parameters were used: Semitrypsin or chymotrypsin was chosen as the proteolytic enzyme; peptide and fragment ion tolerances were set at 0.15 Da; taxonomy was restricted to Laurasiatheria; and two missed cleavages were allowed.

RESULTS AND DISCUSSION

Separation of Orthocortical and Paracortical Cells. A number of approaches have been promoted in the literature for their effectiveness in obtaining purified fractions of orthocortical and paracortical cells, and a number of these were evaluated before settling on the final protocol used in this paper. A process involving treatment of formic acid-swollen Merino wool by an ultrasonic processor (*13*) was found to be unsatisfactory because the fibers demonstrated a resistance to disruption preventing subsequent separation of the cell types by density gradient separation. Shear mixing was found to provide more effective Merino fiber disruption, but left a high proportion of the cells clumped together. Such cell clumping was found to compromise the effectiveness of separation by density gradient centrifugation, as no distinct separation into layers was observed. In a variation of this approach, the fibers were also stained with gold prior to density gradient centrifugation (*13*) but no differential staining between the orthocortex and paracortex was observed, preventing separation via density gradient centrifugation.

As these approaches did not satisfactorily separate othorcortical and paracortical cells, an investigation into a modification of the methods of Kulkarni et al. (*14, 15*) and Dowling et al. (*18*) was undertaken, which eventually proved superior to the other methods tried. This involved digestion of the fiber with a proteolytic enzyme before progressively disrupting it with an ultrasonic probe. For our work Pronase E was substituted for trypsin, as this enzyme was found to be more effective in preferentially digesting the cell membrane complex (CMC) and intermacrofibrillar material on the orthocortical side of the wool fiber (*24*). A number of regimes were tested, but the most satisfactory involved Pronase E digestion of the wool fiber for 6 days, followed by disruption for 30 min with an ultrasonic probe. This was found to preferentially release cells from the orthocortex with approximately 84% of these being of orthocortical origin (**Figure 1a**) and only 1% of paracortical origin, allowing the acquisition of an orthocortical isolate. A further 13% appeared to originate from the cuticle, while the remaining 2% could not be identified. Increasing the sonication time allowed the acquisition of a paracortical cell isolate (**Figure 1b**), as it brought about the progressive removal of orthocortical cells from the fiber residue in conjunction with an increase in the proportion of paracortical cells, to the extent that after treatment for 150 min the residue was found to contain 83% paracortical material (**Figure 2**). A further 14.5% consisted of orthocortical material, while cuticle cells comprised the rest.

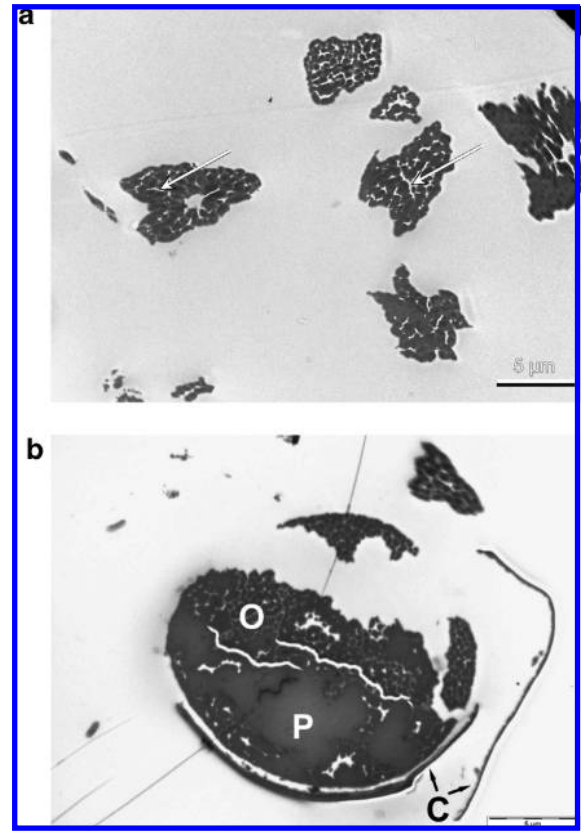

Figure 1. Transmission electron micrographs of the cell preparations obtained by the proteolytic/ultrasonic treatment: (**a**) orthocortical cells (the arrows pointing to where intermacrofibrillar material has been removed from the cell); (**b**) an example of the remnant material from the fiber after removal of the orthocortical cells (O, orthocortex; P, paracortex; C, cuticle arrowed) showing only partial removal of the orthocortex. Other fiber remnants in the paracortical fraction were notable for the complete removal of orthocortical cells.

Protein Expression in the Orthocortex and Paracortex. Duplicate IEF-2DE maps of the wool protein extracts from both the orthocortical and paracortical cells (**Figure 3a**, **3b**) obtained at pH 3.5 showed the characteristic pattern of protein spots as previously observed (*20, 25*). The type I IFPs are clustered around p*I* 5.0 and 45 kDa, and the type II IFPs are located in a long train of spots running from p*I* 5.2 to 6.5, around 60 kDa,

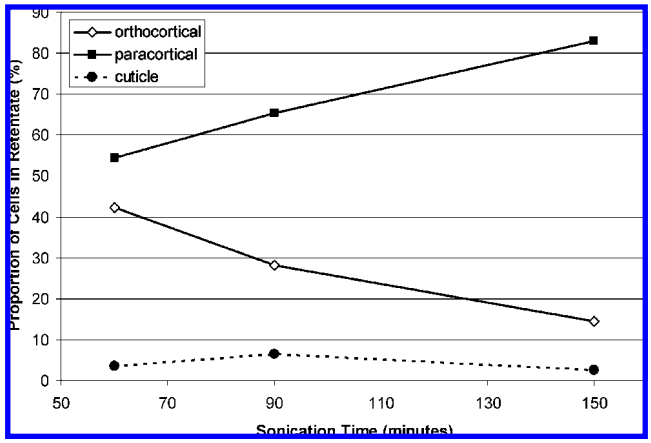

Figure 2. The relationship between the proportion of orthocortical and paracortical cells in the fiber residue with sonication time. The end-point represents the cellular composition of the paracortical isolate.

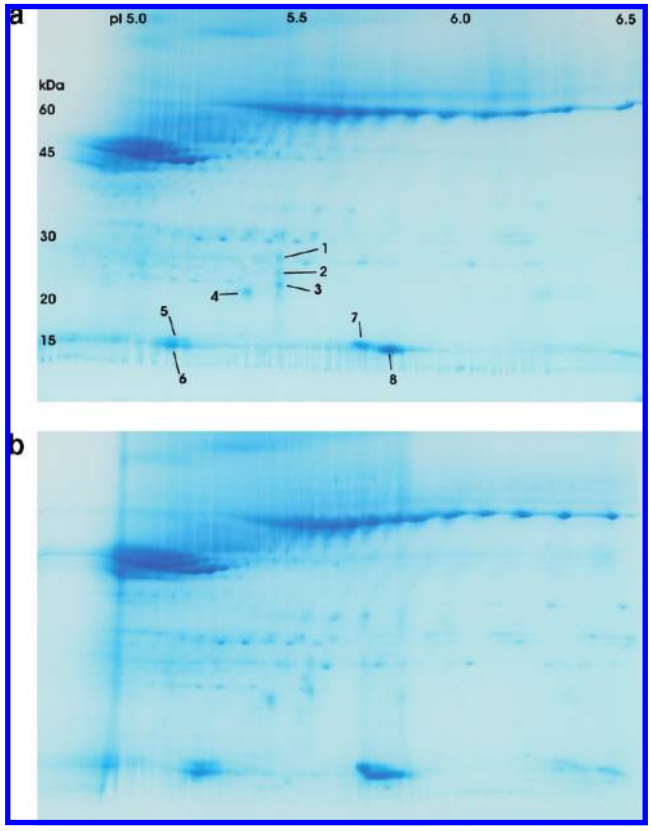

Figure 3. IEF-2DE gels of proteins extracted with TCEP at pH 3.5 from (**a**) orthocortical and (**b**) paracortical cells. The spots labeled in gel **a** were subjected to expression analysis by 2DE gel software and analyzed by mass spectrometry.

while the KAPs are found at lower molecular weight. Two groups of KAPs tended to predominate; a cluster of proteins around p*I* 5.0 between 20 and 30 kDa (spots 1 to 4, **Figure 3**), some of which have previously been identified as being members of the KAP1 HSP family (*20*, *25*). The other group (spots 5 to 8) appears as two trains of spots around 15 kDa. Based on histograms of the spot intensities no significant difference was observed in the protein spots from the KAP1 HSP family (spots 1 to 4) or in the minor trains of spots on the gels. In contrast, the KAPs around 15 kDa were observed to be more prevalent in the paracortex (spots 5 to 8, **Figures 3b** and **4a**). In the case of three of the spots, at least twice as much protein was present in the paracortex than the orthocortex. This

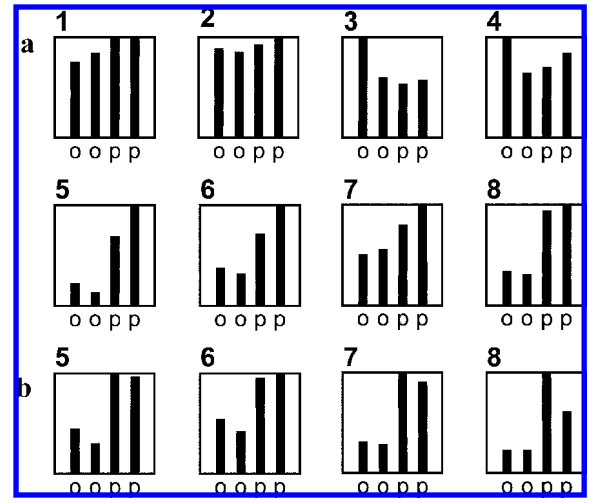

Figure 4. Histogram plots of the relative spot volume in IEF-2DE gels of the HSP proteins extracted with TCEP at pH 3.5 from orthocortical and paracortical cells from (**a**) one set of isolated cells and (**b**) a second set of isolated cells where only the KAP3 protein concentration was determined. In the figure, "o" and "p" represent the duplicate values for the orthocortex and paracortex and the vertical axis is a measure of the spot volume (absorption intensity times the spot area) normalized to the highest spot volume of the set (see **Figure 3a** for spot numbers).

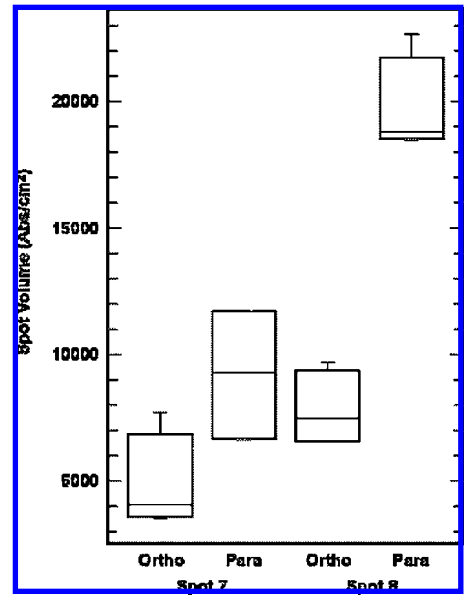

Figure 5. A box and whisker plot of the concentrations of the KAPs in spots 7 and 8 in both orthocortical and paracortical cell fractionations.

difference was observed in a separate sample of Merino wool where the orthocortical cell enrichment was 97% and the paracortical cell enrichment was 75% (**Figure 4b**). An ANOVA, F(3,12), with a *p*-value (probability) of 2.43E−06 was obtained which indicated that the higher level of the protein observed in the paracortex was statistically significant. From the box and whisker plot generated from the above data (**Figure 5**), it was apparent that there was a difference in the levels of the KAPs in spots 7 and 8 between the two cell types. This level of difference was highly significant in the case of spot 8.

When extraction was carried out at pH 7.5, additional trains of spots were observed in the region between the IFPs and the KAPs at 20 kDa (**Figure 6a**). From a comparison of the proteins extracted at this pH from the two cell types there are indications that nine labeled KAPs between 20 and 30 kDa were more prevalent in the orthocortex (**Figure 6b**), while some of the

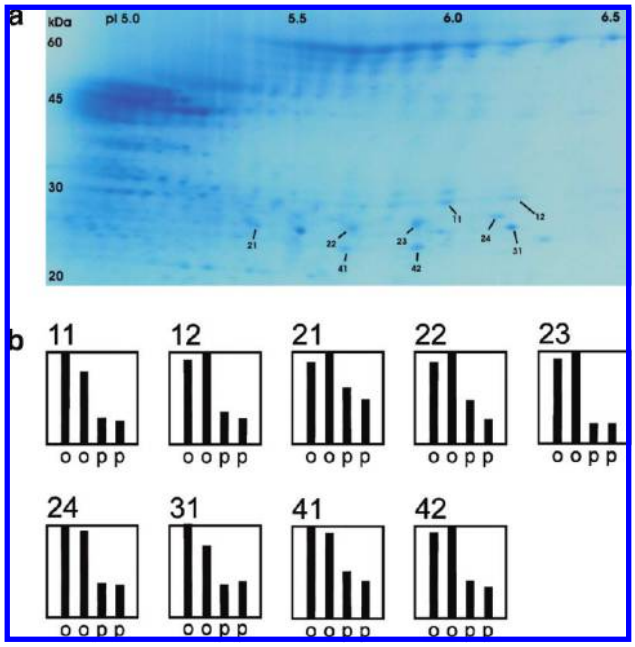

Figure 6. (**a**) IEF-2DE gel region p*I* 5.5 to 6.5 and 20 to 30 kDa of proteins extracted from orthocortical and paracortical cells with TCEP at pH 7.5 and (**b**) histograms of the spots differentially expressed between the orthocortex and paracortex.

minor proteins in the 15 kDa region were more prevalent in the paracortex, with one appearing exclusively in the paracortex (**Figure 7**).

Identification of Proteins by Mass Spectrometry. After in-gel digestion (with either trypsin or chymotrypsin) and extraction, peptides derived from the KAP spots shown by 2DE analysis to be differentially expressed were desalted and concentrated using StageTips, and examined by MALDI-TOF-MS or LC−MS. Selected ions were analyzed by collision-induced dissociation. KAPs typically have only a few basic residues, often followed by proline residues (known to inhibit tryptic digestion), and characteristically generate few ion peaks in MS analysis of a tryptic digest. Therefore, identification is usually based on the occurrence of certain diagnostic peptides, complemented with information from digestion with other enzymes (*20*).

In the case of the spots around 15 kDa (**Figure 3a**), spots 5−8 all contain KAP3.3 and/or KAP3.4: they all showed prominent 609.25 and 2446.13 Da peptides (**Table 1**). The peptide of mass 2446.13 Da was matched to the sequence L^{6}CCSVPTSPATTICSSDKFCR^{26} found in the HSPs KAP3.3 and 3.4. The 609.25 Da peptide was matched to the sequence K^{23}FCR^{26}, a shorter segment of the 2446.13 Da peptide. In spots 5 and 6, no other peptides were found that allowed the differentiation between KAP3.3 and 3.4. However, in spot 6 an additional peptide at 1699.82 Da was found, which was matched to the sequence T^{12}GPATTICSSDKFCR^{26} found only in KAP3.2. Thus, spots 5 and 6 contain KAP3.3 and/or KAP3.4, and spot 6 also contains KAP3.2.

Table 1. Peptide Masses and Amino Acid Residue Sequences Derived from MALDI-TOF-MS/MS Analysis of Peptides Isolated from the Trypic/Chymotryptic Digests of the Spots from the Gel (**Figure 3a**)

spot no.	obsd peptide mass (Da)	sequence	determined by[a]	protein identity
5	609.25	KFCR	F	KAP3.3/3.4
	2446.13	LCCSVPTSPATTICSSDKFCR	M	KAP3.3/3.4
6	609.25	KFCR	F	KAP3.3/3.4
	2446.13	LCCSVPTSPATTICSSDKFCR	M	KAP3.3/3.4
	1699.86	TGPATTICSSDKFCR	M	KAP3.2
7	609.25	KFCR	F	KAP3.3/3.4
	2446.13	LCCSVPTSPATTICSSDKFCR	M	KAP3.3/3.4
	3026.52	LQPTCCCDNRPPPYHVPQPSVPTCF	M	KAP3.3[b]
	1584.65	TQSSCEPCIPSCC	M	KAP3.3[b]
	3025.52	FLEPTCCDNRPPPCHIPQPSVPTCF	M	KAP3.4[b]
	1594.66	TQPSCEPCIPSCC	M	KAP3.4[b]
8	609.25	KFCR	F	KAP3.3/3.4
	2446.13	LCCSVPTSPATTICSSDKFCR	M	KAP3.3/3.4
	1699.86	TGPATTICSSDKFCR	M	KAP3.2
	2144.10	CRCGVCLPSTCPHDISLL	M	KAP3.2[b]
	1197.58	VPDTYVPTCF	M	KAP3.2[b]
	1505.86	LNSSHPTPGLSGINL	M	KAP3.2[b]
	1617.76	IQPGCENVCEPRC	M	KAP3.2[b]

[a] M = MS/MS, F = fingerprint mass. [b] Peptide obtained through chymotryptic digest.

In spot 7, using chymotrypsin as the digestion agent, a number of peptides were found that were derived from KAP3.3 and KAP3.4, respectively (**Table 1**): 3026.52 and 1584.65 Da were matched to residues 42−66 and the C-terminal peptide 86−98 of KAP3.3; while peptides of 3025.51 and 1594.66 Da were matched to residues 41−65 and the C-terminal peptide 86−97 of KAP3.4. In spot 8, the KAP3.2-specific 1699.86 Da peptide was found. In addition, chymotryptic digestion yielded four peptides that were also matched to KAP3.2: residues 25−42 (2144.10 Da), 56−65 (1197.58 Da), 67−81 (1505.86 Da) and the C-terminal peptide 85−97 (1617.76 Da) of KAP3.2. Thus, spot 7 contains both KAP3.3 and KAP3.4, whereas spot 8 contains KAP3.3 and/or KAP3.4 as well as KAP3.2. From this it appears that spots 5 and 7, and 6 and 8 respectively appear to form two extended trains of spots, similar to the trains of spots observed for the IFPs (*20*).

Proteins Associated with Differential Expression in Wool. Previous studies investigating differences in protein expression between the major cortical cell types have relied primarily on amino acid analysis, though without providing details of the purity of each preparation. These methods have provided some information on the amino acid contents of the fiber regions, with the orthocortex shown to contain more tyrosine than the paracortex and less cysteine (*15*, *18*). These findings, combined with gene expression studies lend indirect support for the preferential expression of UHSPs on the paracortical side of the fiber and the HGTPs on the orthocortical side (*26*), but do

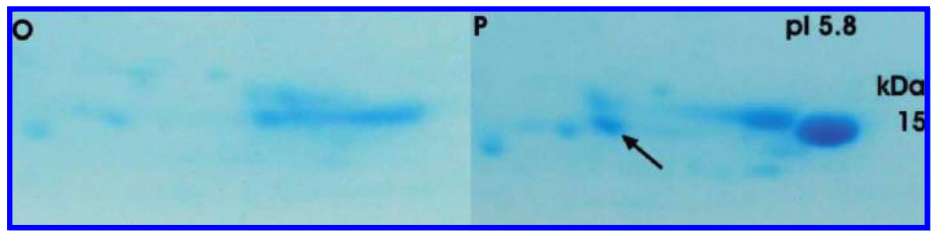

Figure 7. At least one spot in the 15 kDa region (arrowed) was found only in the paracortex, not the orthocortex (O refers to the orthocortex, P the paracortex).

not take into account the fact that the proportion of KAPs is higher in the paracortex (*27*, *28*).

More direct evidence has been obtained from studies employing NE-2DE, in which a broad band of protein material between 30−100 kDa, attributed to the UHSPs, was found to be much more intense in the paracortex (*18*). In the IEF-2DE gels obtained in this study, there is no evidence for any spots in the UHSP region of the protein map being expressed in higher amounts on the paracortical side of the fiber, instead some trains of spots around 30 kDa appear to be expressed in higher concentrations in the orthocortex, though the difference in intensity of the spots in the two cell types was not considered to be significant. However, it is evident that the keratin associated proteins, KAP3.2, 3.3 and 3.4, with their high cysteine contents, are expressed in higher concentrations in the paracortex. We propose that the higher concentrations of cysteine previously observed in the paracortex are directly related to the higher expression of this protein family.

Conclusion. Proteolytic digestion of wool fibers, followed by ultrasonic disruption, has been shown to produce enriched fractions of orthocortical and paracortical cells. A proteomic approach involving the application of two-dimensional gel electrophoresis to locate and identify differentially expressed proteins was used to compare these cell types. Mass spectrometric analysis proved that the differentially expressed proteins all belonged to the KAP3 high sulfur protein family, specifically KAP3.2, 3.3 and 3.4, which were found to be present in higher concentrations in the paracortex. The higher expression of the KAP3 high sulfur protein family in the paracortex is likely to be a significant contributor to the higher cysteine content that has been reported in this part of the wool fiber. This difference in protein composition is considered to be important for the formation of the paracortex, which in fibers showing bilateral segregation of cortical cell types results in high crimp, whereas on its own, as in the Merino fiber-luster mutant, a straight fiber is produced that easily felts or matts. Thus, the KAP3 family might be important determinants of protein crimp. These results represent a step forward in our understanding of subtle protein expression variation in the orthocortex and paracortex of wool fibers, and how this relates to key physical and mechanical properties. Knowledge of which proteins have an influence on crimp will also be important when it comes to searching for markers for breeding programs for sheep with particular wool quality traits.

ABBREVIATIONS USED

IFP, intermediate filament protein; HSP, high sulfur protein; UHSP, ultrahigh sulfur protein; HGTP, high glycine-tyrosine protein; KAP, keratin associated protein; TEM, transmission electron microscopy; 2DE, two-dimensional electrophoresis; IEF, isoelectric focusing; MALDI-TOF, matrix assisted laser desorption/adsorption ionization time-of-flight.

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