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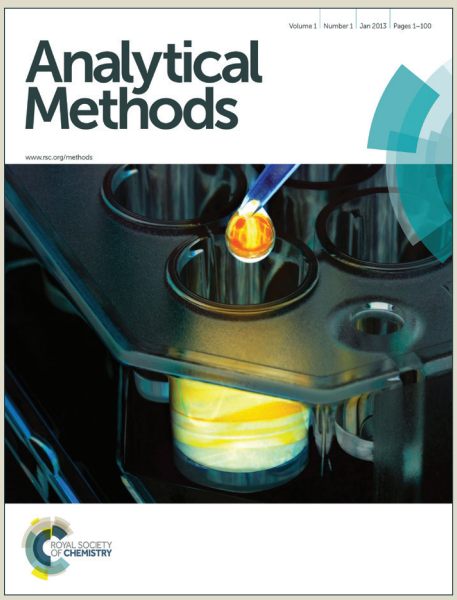

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ARTICLE

Ionic liquid-assisted extraction of wool keratin proteins as an aid to MS identification

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Investigations into the use of the ionic liquid 1-butyl-3-methylimidazolium chloride $[BMIM]^+[Cl]^-$ to extend the mapping of the wool proteome have established that it is complementary to the use of commonly used chaotropic agents such as urea. Extraction of proteins from wool is challenging with only 50-60% extraction possible by traditional methods that utilise chaotropes in the presence of a reducing agent. Incubation of wool at high temperatures in the presence of $[BMIM]^+[Cl]^-$ resulted in large scale disruption of the fibre into cells and sub-cellular fragments. The combination of the proteins identified from the ionic liquid treated wool which was subsequently extracted with urea/thiourea and those identified by urea/thiourea extraction alone resulted in the identification of a larger set of proteins from the wool fibre than would have been found by either method alone. Wool proteins, in particular some trichocyte keratins, were uniquely identified with the ionic liquid/(urea/thiourea) combination, while others such as some of the keratin associated proteins were unique to urea/thiourea alone. We conclude that the ionic liquid/urea/thiourea method provides complementary coverage of the wool proteome when run alongside the traditional urea/thiourea method.

Keywords: cytokeratins, trichocyte keratins, protein extraction, mass spectrometry

1. Introduction

Ionic liquids differ from conventional solvents in that they are composed of ions rather than molecules but still maintain their electrical neutrality. The earliest described ionic liquids were simply salts that melted at very high temperatures; typical examples include the chloride salts of sodium and potassium. The first example of an ionic liquid with a melting point around room temperature, ethyl ammonium nitrate, was discovered in 1914 during an effort to find new explosives[1] but it was not until the 1980s that researchers started to take an interest in more pacific applications.[2]

In recent years ionic liquids have enjoyed renewed interest regarding their potential as solvents, due to their ability to solubilise a wide range of organic, inorganic and organometallic compounds, polymers and gases. They are favoured as solubilising agents due to their low melting points (below 100°C) and their ability to remain liquid over a broad temperature range (up to 300°C) with high thermal stability.[3] Ionic liquids are potentially recyclable, non-flammable and non-volatile and are therefore considered to be 'green solvents'. However, they are also toxic and have low biodegradability.[3]

When it comes to the study of protein systems, ionic liquids have a wide range of potential applications. Many proteins dissolved in ionic liquids behave differently than they do in aqueous solutions. Ethylammonium nitrate, for example, has been found to facilitate the recovery of relatively high concentrations of enzymatically-active hen egg white lysozyme during the refolding process after dilution or dialysis.[4] In the absence of the ionic liquid, both misfolding and aggregation of the protein can occur as a result of disulphide bond reshuffling and exposure of hydrophobic surfaces.

Some enzymatic reactions can be carried out in ionic liquids, particularly reactions involving lipases and proteases that operate better in non-aqueous conditions. An enhancement in the structural stability and resistance to thermal denaturation of these enzymes in ionic liquids has also been reported. The trans-esterification reaction of ethylbutanoate with butanol by lipase B from *Candida antarctica* proceeds slightly faster in an ionic liquid than in *tert*-butyl alcohol

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and butane-1-ol.[5] The specific nature of the anion is important; ionic liquids containing $[BF_4]^-$, $[PF_6]^-$ and $[Tf_2N]^-$ maintain the activity of the enzyme, while $[NO_3]^-$, $[CF_3CO_2]^-$ and $[CF_3SO_3]^-$ inactivate it.[3] Proteins such as human serum albumin and equine heart cytochrome c have been found to retain their higher order structures in mixtures of 1-butyl-3-methylimidazolium chloride $[BMIM]^+[Cl]^-$ of up to 25 vol% in water, though they became denatured when the concentration reached 50 vol%.[6] Reportedly, the 1-butyl-3-methylimidazolium ion $[BMIM]^+$ paired with $[PF_6]^-$ resulted in increased thermostability of a serine protease, cutinase, from *Fusarium solani* than when paired with $[NO_3]^-$.[7]

Researchers have utilised the ability of $[BMIM]^+[Cl]^-$ and dicyanamide ionic liquids to disrupt hydrogen bonds as a means to dissolve biological macromolecules such as starch, cellulose and silk fibroin. Ionic liquids have also been investigated for their utility to dissolve waste wool fibres from the wool weaving industry.[8] These investigations indicated that complete dissolution of wool was possible in $[BMIM]^+[Cl]^-$ at high temperatures.

Traditionally proteins from wool have been extracted with chaotropes such as urea and thiourea in the presence of a reducing agent.[9-12] This method cannot extract all proteins; previous work has shown that only 50-60% extraction is possible. This resistance to extraction is largely because compared to typical animal tissue, the proteins in wool and other hairs are extremely dense and highly cross-linked. Protein extraction may also be influenced by the modifications induced during the keratinisation process.[13,14] Consequently, parts of the fibre remain untouched by standard chaotropic disruption, specifically the cell membrane complex, the intermacrofibrillar material, and the cytoplasmic remnant of the cortex and the cuticle cells.[15] Changing the reductant from dithiothreitol (DTT) to tris(2-carboxyethyl) phosphine (TCEP) has been found to aid in the extraction of the exocuticle and enzyme preparations such as Pronase E are effective in dissolving the CMC, intermacrofibrillar material, cytoplasmic remnant and the endocuticle.[15] While these techniques have improved protein coverage, they have not yet enabled a complete analysis of the wool proteome.

Until recently, wool proteome characterisation has been limited by the availability of sequences in publicly accessible databases,[16] however, this situation has improved following efforts to develop a comprehensive sheep genome.[17] Thus, with the availability of new trichocyte wool keratin sequences and using a new experimental approach it has been possible to refine the map of the keratins in 2D-electrophoretic gels,[12] while differential extraction techniques have been adopted to more completely characterise the low abundance keratin-associated proteins.[18] At the same time, through a mixture of gel and gel-free proteomic approaches, a further 72 complete and 30 partial ovine-specific sequences have been added to the wool protein database and a total of 113 proteins identified.[17] Nevertheless, problems persist in the complete characterisation of the wool proteome. Many of its constituents are highly hydrophobic, so that high chaotrope concentrations are required for solubilisation. The wool proteome is dominated by two keratin families, hindering the identification of minor constituents. Furthermore, although the proteins in the cortex are relatively easy to extract, those in the cuticle are not. Targeted chemical and chemical/enzymatic approaches have partially addressed the cuticle proteome, enabling the identification of some cuticle (especially exocuticle) proteins.[15,19]

A more complete knowledge of the wool proteome will have several beneficial effects. It would assist with the development of keratin-based biomaterials such as drug carriers, scaffolds, films, hydrogel and regenerated fibre for medical and other technological applications.[20-22] It would also provide insight into environmentally and processing-induced protein degradation in wool, thereby assisting in the development of management strategies to minimise damage. Perhaps most importantly, improved proteome information would also fast-track the search for markers for industry-relevant wool quality traits and the development of breeding programmes and molecular biology procedures for the enhancement of favourable characteristics of wool.

$BMIM^+[Cl]^-$, in particular, has been reported to be an excellent solvent for wool by disrupting non-covalent interactions, specifically intermolecular hydrogen bonds.[8] We wanted to further explore this potentially useful characteristic, validate the compatibility of this dissolution method for mass spectrometry analysis, and perform a qualitative evaluation of the efficiency of the procedure with the existing urea/thiourea extraction method.[23]

2. Experimental

2.1 Materials

Ammonium bicarbonate, dichloromethane, DTT, ethanol, acetone, methanol, tris(hydroxymethyl)methylamine (tris) and urea were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from Ajax Fine Chemicals (Taren Point, Australia). Teric GN9 was obtained from Orica Ltd (Auckland, NZ). TPCK-trypsin was obtained from Promega Corporation (Madison, WI, USA). TCEP, LCMS-grade acetonitrile (AcN) and water were obtained from Fluka Chemie GmbH (Buchs, Germany). Empore™ disks (Empore™ Octadecyl C18 47 mm Extraction Disks) were obtained from Supelco (Bellefonte, PA, USA). $BMIM^+Cl$ and iodoacetamide (IAM) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Pharmalyte buffer and 11 cm pH 3-11 non-linear (NL) immobilised pH gradient (IPG) strips were obtained from GE Healthcare Life Sciences (Auckland, New Zealand)

2.2 Sample preparation

De-tipped Merino wool of approximately 20 µm diameter was washed to remove dirt and unbound lipids by scouring with 0.15% Teric GN9 at 60°C and 40°C, with water at 40°C and 60°C, followed by rinsing in dichloromethane and ethanol at a wool:water ratio of 1:40 w/v. The cleaned wool was crushed to a powder in liquid nitrogen.[11] The powdered wool was washed with dichloromethane then air dried.

A series of fractionation steps was initially used to enrich specific families of wool proteins. The first stage involved extracting the wool by shaking with 2 M urea, 50 mM Tris and 50 mM dithiothreitol at pH 9.3 in a wool to extraction buffer ratio of 1:100

w/v in a reciprocal-action shaker for 18 hours at ambient temperature to reduce the dominance of the trichocyte keratins over the keratin-associated proteins (KAPs) in the analysis. The extract was centrifuged at 20,000 *g* for 15 minutes to pellet the fibre residue and the supernatant removed to give Fraction 1.[18] The supernatant was dialysed against eight changes of water using a 3,500 Da MWCO membrane and then freeze-dried. The keratin fraction (Fraction 2) was obtained next by vigorously shaking the fibre residue from the first extraction for 18 hours in 8 M urea, 50 mM tris and 50 mM DTT at pH 9.3 at a residue to extraction buffer ratio of 1:100 w/v. The supernatant from this procedure was then dialysed for 18 hours against eight changes of water and freeze-dried. Fraction 3 was the residue left after the two initial extraction steps.

2.3 Dissolution and digestion of the fractions

Fractions 1, 2 and 3 were individually mixed with $BMIM^+Cl^-$ (w/w ratio: 1:20) and heated with agitation to 99 °C for 18 hours in 1.5 ml sample tubes in a Thermomixer Comfort (Eppendorf, Hamburg, Germany). Each of the fractions was allowed to cool and then re-extracted by vortexing for 18 hours in 7 M urea, 2 M thiourea and 50 mM DTT (v/v ratio: 1:50). The proteinaceous material was precipitated using the chloroform/methanol method[24] and the resulting pellet re-suspended by sonication for 10 minutes in 50 mM ammonium bicarbonate. This was then reduced with 50 mM tris(2-carboxyethyl) phosphine at 56 °C for 45 minutes, followed by vortexing for 30 minutes after the addition of 150 mM IAM. The alkylated protein extract was digested with trypsin for 18 hours at 37 °C at an enzyme:substrate ratio of 1:50 and taken to dryness on a Centrivap centrifugal concentrator (Labconco, Kansas City, MI, USA). Fractions 1, 2 and 3 were also extracted by 7 M urea, 2 M thiourea and 50 mM DTT without the initial $BMIM^+Cl^-$ step, alkylated and subjected to tryptic digestion as described above.

2.4 Isoelectric focussing of tryptic peptides

The tryptic peptides from each fraction were re-dissolved in a solution of 8 M urea, 50 mM DTT and 0.5% Pharmalyte buffer and used to rehydrate 11 cm pH 3-11 NL IPG strips. The IPG strips were focused for 50 kVh with a Protean IEF Cell (Bio-Rad, Hercules, CA, USA). Approximately 1 cm lengths of gel were scraped off the surface of the backing plastic into 1.5 ml microfuge tubes, yielding 10 fractions. The peptides from the gel pieces were progressively extracted with solutions containing 10%, 50% and 80% AcN and 0.5% TFA and then concentrated and cleaned using Empore disks[25]. The peptides were eluted from the Empore disks with 50% AcN, dried down and reconstituted in 35 µL of 0.1% FA.

2.5 MS analysis of wool

LC-MS/MS was performed on a nanoAdvance UPLC coupled to an amaZon speed ETD mass spectrometer equipped with a CaptiveSpray source (Bruker Daltonik, Bremen, Germany). A 2 µl sample was loaded on a C18AQ nano trap (Bruker, 75 µm x 2 cm, C18AQ, 3 µm particles, 200 Å pore size). The trap column was then switched in line with the analytical column (Bruker Magic C18AQ, 100 µm x 15 cm C18AQ, 3 µm particles, 200 Å pore size). The column oven temperature was 50 °C. Elution was with a gradient from 0% to 40% B in 90 min at a flow rate of 800 nl/min. Solvent A was LCMS-grade water with 0.1% FA and 1% AcN; solvent B was LCMS-grade AcN with 0.1% FA and 1% water.

Automated information-dependent acquisition was performed using trapControl v7.1 software, with an MS survey scan over the range m/z 350–1200 followed by three MS/MS spectra from 50–3000 m/z acquired during each cycle of 30 ms duration.

2.6 Data analysis

After each LC-MS/MS run, peak lists were queried against *Ovis aries* in the NCBInr database (date: 14 September 2011) using the Mascot search engine (v2.4, Matrix Science) maintained on an in-house server. The Mascot search parameters included semitrypsin as the proteolytic enzyme with two missed cleavages because semitryptic peptides are seen in wool;[17] error tolerance was set to 0.15 Da for MS and 0.3 Da for MS/MS. Search results were compiled and analysed using ProteinScape 3.1.0 (Bruker) using the ProteinExtractor function. Acceptance thresholds for peptide and protein scores were set at 25 and 80, respectively. The identity score for at least one peptide used for protein identification was calculated by the search engine. Results assessed as being true matches were used for further analysis.

2.7 Microscopy

After ionic liquid treatment, samples for microscopy were taken directly from the sample tubes using a fine glass pipette, either mid tube or at the tube base. Drops of sample were deposited on a cleaned microscope slide, cover-slipped and examined immediately at room temperature using a Leica DM6000B (Leica Microsystems GMBH, Germany) upright microscope using bright-field and differential interference contrast (Normanski) imaging. Images were taken with a Leica D500 digital camera.

3. Results and Discussion

In previous studies wool was crushed under liquid nitrogen in an effort to increase the exposure of the fibre to the extraction solution.[12,18] Examination of the cryo-crushed wool powder revealed discrete, dense wool fragments (Fig. 1a). After the powder was exposed to 99°C for 18 hours in the presence of $BMIM^+[Cl]^-$ a viscous solution formed in which wool powder was no longer visible by eye. Microscopic examination revealed significant changes to the morphology of the wool fragments. These included universal fibre swelling and apparent disintegration of fibres leading, in some cases, to an intriguing "unwinding" effect of cuticle (Figure 1b). When higher temperatures were tried more protein degradation was observed.

A wide range of identifiable structures were observed when wool was exposed to 99°C in the presence of $BMIM^+[Cl]^-$. These included swollen pieces of intact fibre that had a continuous layer of cuticle surrounding cortex cells. Smaller multicellular fragments and single intact cells were common. Sub-cellular fragments that included individual macrofibrils were also observed at high magnification. These observations suggest that $BMIM^+Cl^-$ opens up the fibre structure by disrupting the structural connections (such as the cell membrane complex) that normally hold the fibre together.

Thus, we envisaged that the structural changes in the wool fibre brought about by $BMIM^+Cl^-$ could improve the ingress of chaotropes, such as urea, to parts of the fibre structure that would normally be protected by surrounding un-dissolved material. This increase in surface area would result in a very effective dissolution procedure. As anticipated, additional overnight extraction with a urea/thiourea solution led to obvious further fibre breakdown: after this step the cuticle associated with fibre fragments was only loosely attached or missing altogether (Fig. 1c). A brief centrifugation of this sample resulted in near complete collapse of most fibre fragments into smaller fragments (Fig. 1d), with intact fibre fragments rarely observed.

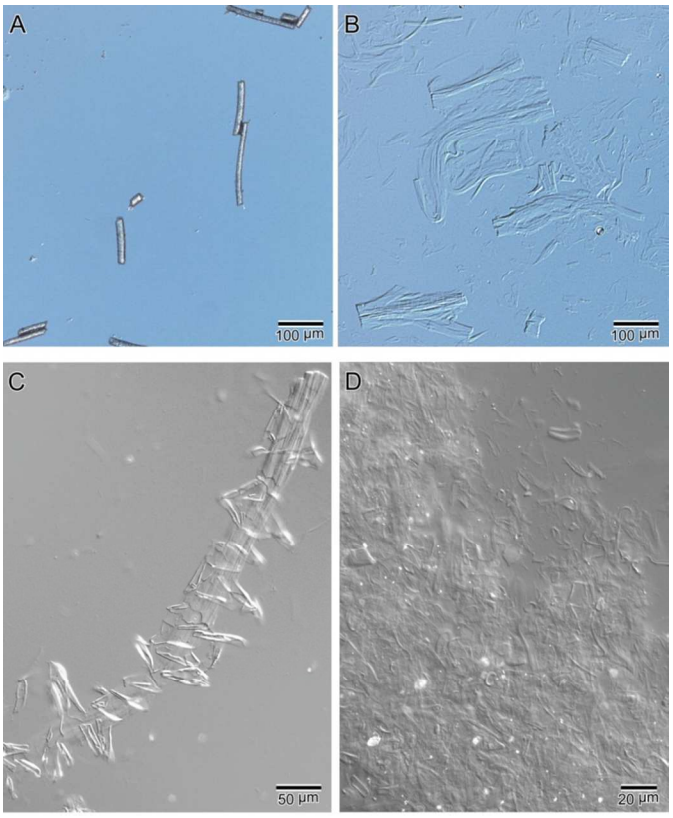

Fig. 1. Effects of BMIM+Cl- treatment on wool. A) Untreated ground wool, B) after treatment, ground wool fragments were swollen compared to untreated ground wool. C) Higher magnification showing cuticle cells typically dissociating from remaining fibre fragments after 18 hours at 99°C. D) Following additional treatment with urea/thiourea and after gentle centrifugation, most fibres dissociated into isolated cells and fragments of cells.

An initial sequential urea fractionation procedure was used in this study for enriching specific families of wool proteins to reduce the complexity of the sample for subsequent mass spectrometric analysis.[18] The 2 M urea fraction (Fraction 1) favours the extraction of keratin-associated proteins (KAPs) over trichocyte keratins. The 8 M urea extract (Fraction 2) was used to enrich the trichocyte keratin proteins. The residue (Fraction 3) left from the above two extraction steps was mainly composed of wool fragments, the cuticle, cell membrane complex, intermacrofibrillar material and cytoplasmic residue.

The three fractions were individually subjected to an extraction step; one set with urea/thiourea alone, and the other with $BMIM^+Cl^-$ followed by urea/thiourea, to evaluate the ionic liquid's effect on the extractability of wool proteins and their downstream characterisation.

As one-dimensional separation using a reversed phase LC does not have enough capacity to resolve the overwhelming number of tryptic peptides that can be generated from the complex wool proteome, multidimensional separation methods are used for higher resolution of peptides to increase the number of identified proteins. Therefore, tryptic peptides generated from the various extraction procedures were subjected to an isoelectric focussing pre-fractionation step because this can outperform SDS-PAGE by up to 27% in the identification of unique peptides[26]. IPG gel strips were chosen over liquid-based isoelectric focussing because fewer carrier ampholytes are required to achieve focussing. Carrier ampholytes are also known to interfere with downstream mass spectrometric analysis.[27] An additional advantage of pre-fractionation by IEF was that approximately 8% of all compounds were selected for fragmentation two or more times, meaning that sufficient MS/MS time (duty cycle) was available to sample all eluting compounds. The false discovery rate was also found to be less than 0.6 in all fractions.

MS analysis of Fraction 1 revealed more identified peptides in the $BMIM^+Cl^-$/(urea/thiourea) extract compared to the urea/thiourea only extraction (control). The number of proteins identified in Fractions 1 and 2 was however similar in both the urea/thiourea control and the $BMIM^+Cl^-$ extractions. A larger number of detected compounds (but fewer compounds corresponding to identified peptides and identified proteins) were found in Fractions 2 and 3, when $BMIM^+Cl^-$ extraction was used in conjunction with urea/thiourea (Table 1, Supplementary Tables 1-6). Both extraction procedures generated peptides mostly in the mass range m/z 1,000-1,500 (Fig. 2). Interestingly, the urea/thiourea only extraction generated more peptides with m/z greater than 1,500, while $BMIM^+Cl^-$ extraction generated more peptides of m/z <1,000 (Fig. 2).

A similar number of trichocyte keratin proteins were identified by both extraction procedures across all fractions (Table 2, Supplementary Tables 1-6). However, the ionic liquid procedure favoured identification of both the KAPs and cytokeratins in the first fraction. These results clearly revealed the complementary nature of the procedures, because the unique peptides generated by these extraction procedures resulted in a larger set of identified proteins than was possible with either of them on their own. A total of 78 proteins, including trichocyte keratins, KAPs and cytokeratins were identified using both procedures (Fig. 3). Of these, 9 were found to be uniquely identified by the ionic liquid procedure and 15 by the urea/thiourea only extraction method, leaving 54 common to both. Furthermore, 106 peptides were found to be unique to the ionic liquid procedure and 140 to the urea/thiourea only extraction procedure, while 180 were common to both (Fig. 4).

Table 1. Total number of unidentified compounds (the number of MS/MS fragmentations carried out for each fraction), compounds corresponding to identified peptides and identified proteins (both keratinous and non-keratinous proteins) in each fraction by ionic liquid/(urea/thiourea) and urea extraction

Fraction	1		2		3	
	Urea/Thiourea	Ionic Liquid/ (Urea/Thiourea)	Urea/Thiourea	Ionic Liquid/ (Urea/Thiourea)	Urea/Thiourea	Ionic Liquid/ (Urea/Thiourea)
Total number of compounds	17924	15771	29803	29745	28756	32800
Number of compounds corresponding to identified peptides	675	692	716	606	869	462
Number of identified proteins	114	113	99	93	180	134

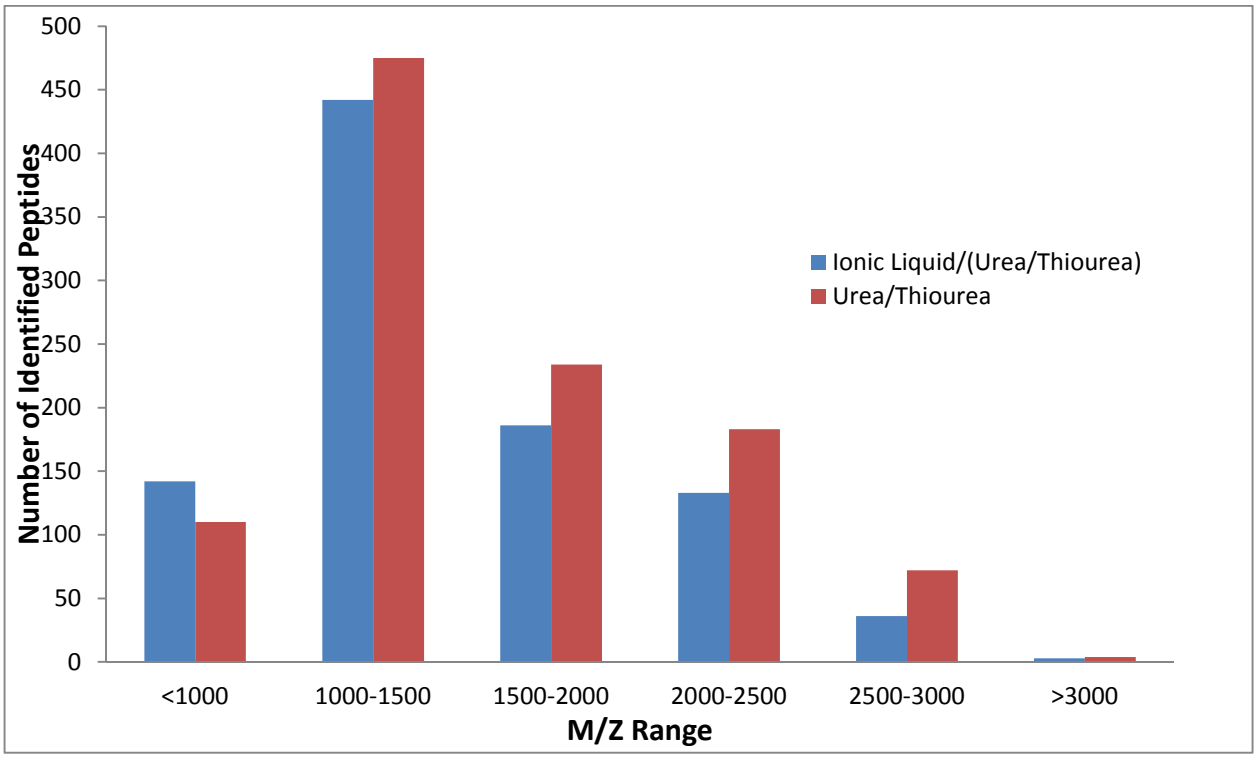

Fig. 2. Distribution of identified peptides (from both keratinous and non-keratinous proteins) by mass in the three combined fractions of the urea/thiourea and ionic liquid/(urea/thiourea) extractions.

Table 2. Comparison of the total number of keratins identified in each fraction using ionic liquid/(urea/thiourea) and urea extraction

	Fraction 1		Fraction 2		Fraction 3	
	Urea/Thiourea	Ionic Liquid/ (Urea/Thiourea)	Urea/Thiourea	Ionic Liquid/ (Urea/Thiourea)	Urea/Thiourea	Ionic Liquid/ (Urea/Thiourea)
Trichocyte keratins	14	14	14	12	13	13
KAPs	18	21	21	13	18	6
Cytokeratins	1	7	2	1	8	5
Total	33	42	37	26	39	24

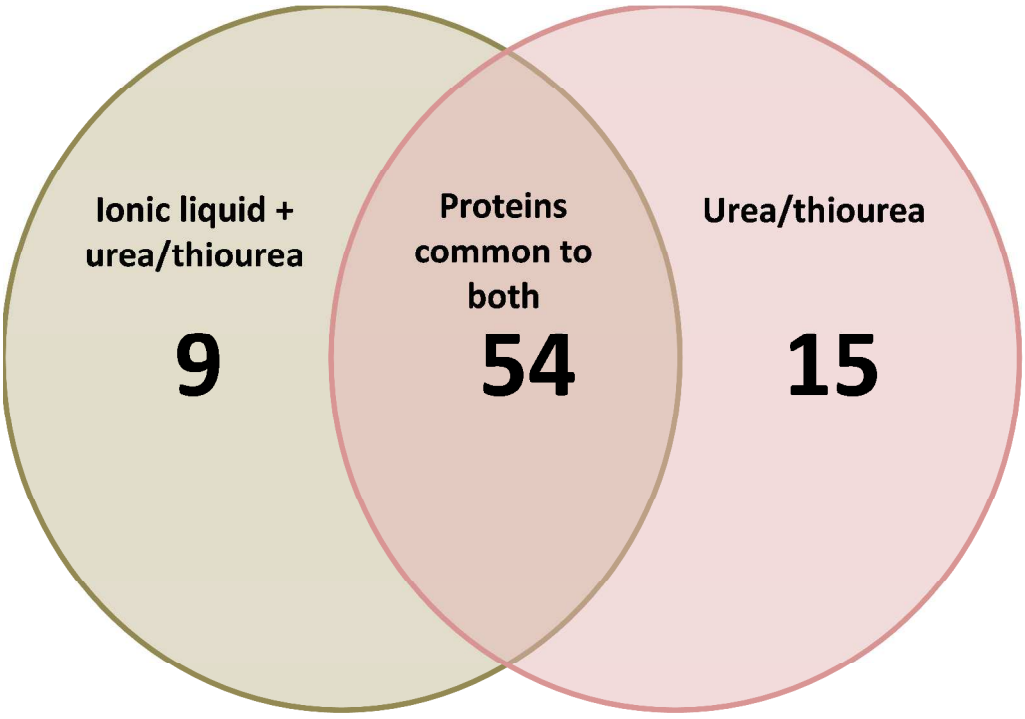

Fig. 3. Unique keratins identified in the combined ionic liquid/(urea/thiourea) and urea/thiourea procedures.

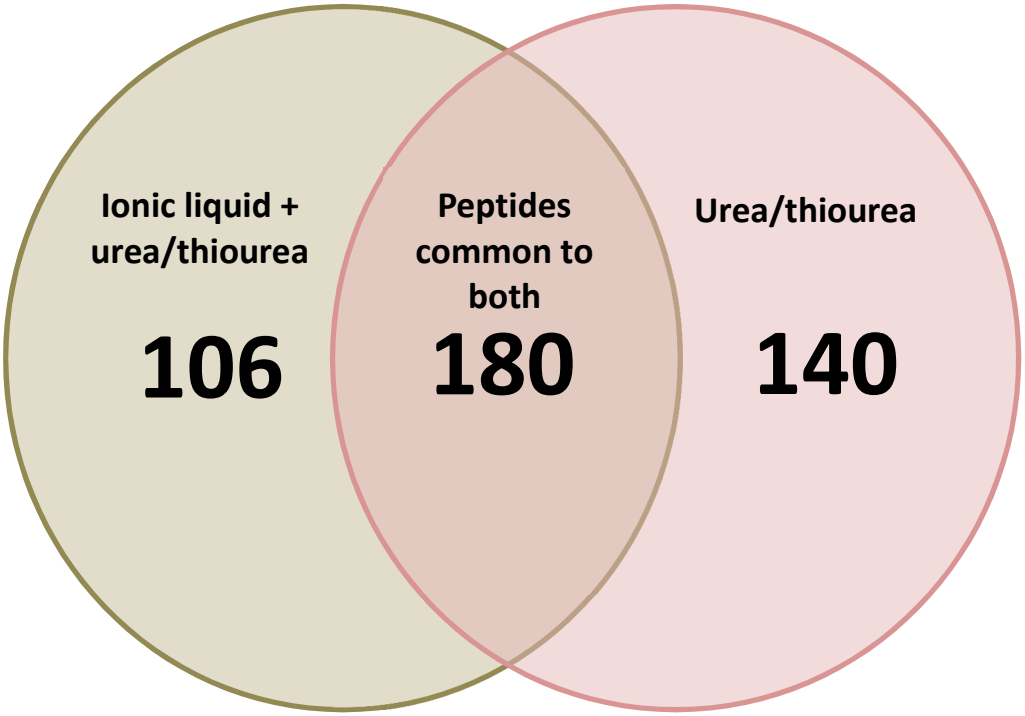

Fig. 4. Unique keratin peptides identified in the combined ionic liquid/(urea/thiourea) and urea/thiourea procedures.

4. Conclusions

While chaotropic agents like urea are able to extract cortical proteins from wool fibres leaving the small, freeze-crushed pieces of fibre unaffected structurally, this study has found that exposure to the ionic liquid, $BMIM^{+}Cl^{-}$ at elevated temperature opens up the fibre for more extensive extraction. Extraction aided by this agent resulted in the partial breakdown of the fibre into smaller fragments that included cells, sub-cellular structures and individual macrofibrils. Qualitative evaluation of wool proteins using two different extraction methods, namely ionic liquid/(urea/thiourea) and urea/thiourea alone, proved to be complementary, with some of the identified peptides and proteins being unique to each of these procedures. Ionic liquid was also found to aid the extraction of cytokeratins. Microscopy results clearly indicated an effective disintegration of the wool fibre including the recalcitrant cuticle, which may have contributed to a better extraction of these cytokeratins. By fractionating the wool extracts, firstly by changing the levels of chaotrope and reductant and secondly by IEF, we were able to reduce the complexity of the samples for analysis by mass spectrometry. The complementary nature of these two approaches thus proved highly effective in obtaining identification of a larger set of proteins, contributing to a better understanding of the wool proteome. This is particularly important in the case of keratins and KAPs from wool because the high degree of homology among proteins from these classes of proteins means that unique identification of any one protein form is highly dependent on finding peptides that are unique to that protein.

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