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INVESTIGATING AMINO ACID RESIDUE-LEVEL DAMAGE USING NOVEL PROTEOMIC APPROACHES, WITH APPLICATION TO WOOL PROTEINS

A thesis

submitted in partial fulfilment

of the requirement for the degree of

Doctor of Philosophy

at

Lincoln University

by

Anita J. Grosvenor

Lincoln University

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Declaration

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Contribution of authors

Dr. James Morton and Dr. Jolon Dyer were the supervisors for the PhD research reported in the above publications. Anita Grosvenor planned and performed the majority of the experimental and wrote the reports.
Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Ph.D.

INVESTIGATING AMINO ACID RESIDUE-LEVEL DAMAGE USING NOVEL PROTEOMIC APPROACHES, WITH APPLICATION TO WOOL PROTEINS

by

Anita Josephine Grosvenor

Abstract

Damage to wool is derived from the modification of its constituent proteins, as the dry matter of wool is principally made up of protein. A significant component of protein damage consists of modifications to the amino acid side chains. In wool, these modifications can lead to lowered quality in the form of reduced strength and elasticity (phototendering), and to undesirable colour changes (photobleaching and photoyellowing). These limitations hinder the competitiveness of natural wool fibres against synthetic counterparts in carpet and apparel manufacture and consumer appeal. Better approaches to resolving these limitations will arise from an increased understanding of the process of protein damage in wool at the molecular level.

Wool damage occurs during on-farm production, processing, and over the product lifespan with the consumer. During processing, elevated temperatures and chemical treatments are applied to the fibre, while the primary degradative influences experienced during subsequent product life are abrasion and light exposure.

This study used novel proteomic techniques to investigate the heat- and light-induced degradation of synthetic model peptides and of intermediate filaments derived from wool. Electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI) mass spectrometric techniques were used to comprehensively characterise the degradation of model peptides containing tryptophan and tyrosine. A total of sixteen residue side-chain degradation products were detected and confirmed using tandem mass spectrometry and detailed de novo
sequencing. The relative abundance of parent peptides and their degradation products was determined using two mass spectrometric approaches: a label-free approach, based on observed parent ion abundance, and an isobaric-labelling approach, based on the abundance of the reporter fragment ions of a commercially available isobaric tag (iTRAQ) in tandem mass spectra.

The influence of reactive oxygen species was apparent by the UVA-, UVB-, and hydrothermally-induced modifications observed in the model peptides. The deduced oxidative degradation pathways shed light into the mechanisms behind protein damage under these conditions. In the UVA and UVB-irradiated proteins, the modifications observed were consistent with the hydroxyl radical playing a key role, as well as the involvement of peroxynitrite. The observation of reactive oxygen species-generated modifications in the hydrothermally-damaged peptides was particularly noteworthy, as the heat-induced generation of reactive oxygen species is not well recognised or reported.

When wool-derived intermediate filament proteins were exposed to hydrothermal insult, the formation of residue-level modifications was also observed after reverse phase high performance liquid chromatography (HPLC) and MALDI mass spectrometric analysis. The presence of oxidative modifications confirmed the involvement of reactive oxygen species. Using quantitative isobaric iTRAQ labels, the degradation of a number of marker peptides and the formation of selected modified products was monitored. Such molecular-level markers of damage provided sensitive and specific evaluation of the type and extent of protein damage experienced. It is anticipated that utilisation of these markers will provide a sensitive and effective tool for determining and tracking protein damage at the molecular level, as well as facilitating validation and optimisation of protection or repair strategies for wool and other protein fibres.

Although this research focussed on understanding protein degradation in wool, the understandings gleaned from the work on model peptides and the molecular damage marker approach may be extended to a variety of other substrates in which protein degradation is a concern. These include a number of living tissues, in which protein damage plays a role in the development of disease states, and other protein-based substrates such as skin, hair, leather, and protein-based foods.

**Keywords**

Protein oxidation; Wool damage; Mass spectrometry; Keratins; Photo-oxidation; Hydrothermal damage; Residue modification; iTRAQ
Acknowledgements

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The whole Protein Quality & Function team at AgResearch has helped me enormously by providing a wonderful and friendly environment in which to perform research. I owe Stefan Clerens my special thanks for lots of help in experimental planning and scientific writing, but particularly in navigating the complexities of mass spectrometric equipment and software – Stefan knows more about proteomics than any one person rightly should! Thanks also in this regard to Santanu Deb-Choudhury and Henning Koehn, who never stinted when I needed advice regarding mass spectrometry. I would like to acknowledge my positivity boosters, James Vernon, Richard Walls and Duane Harland, who were always there with a kind and listening ear when I encountered problems.

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**Abbreviations used**

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<td>AAA</td>
<td>amino acid analysis</td>
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<tr>
<td>CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
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<td>deam</td>
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<td>dopa</td>
<td>dihydroxyphenylalanine</td>
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<td>EC</td>
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<td>HGTP</td>
<td>high glycine tyrosine protein</td>
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<td>HOHICA</td>
<td>3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indol-2-carboxylic acid</td>
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1 Introduction

1.1 Research context and goals

The subject of protein damage is one that concerns many. It touches all of us at a personal and a commercial level. Our health, the biological products we rely on, our agricultural exports and our food are all affected by the processes of protein degradation. Medical problems such as cataracts, premature ageing of the skin, stress and atherosclerosis are linked to the degradation of tissue proteins. Animal-derived textiles such as wool, leather and fur, and even some cosmetics, are protein-based, and the deterioration of their constituent proteins leads to reduced quality and consumer satisfaction. Our comfortable New Zealand (NZ) lifestyle relies heavily on the success of our protein-based agricultural exports, and the characteristics of high protein components of our diet such as meat, milk and fish are impacted adversely by the deleterious effects of protein degradation.

Unsurprisingly, a great deal has been discovered about the roles of proteins in these areas of life, yet much remains to be learned, especially with regard to how these complex molecules interact with the environment to which they are exposed during the lifetime of the organism, or of the protein-based product. New Zealand researchers have discovered much about the effects of protein modification on the characteristics of wool (McMahon & Speakman 1939; Simpson 1994; Plowman et al. 2003), due to the significance of this natural product to our heritage and our economy.

This research programme sought to advance knowledge regarding protein degradation, with particular reference to wool proteins, by the development of a robust mass spectrometry (MS) based approach to characterise and quantify protein degradation. Reliable protocols were developed to characterise, locate, track and quantify specific degradation products. Quantification methodologies, such as differential isobaric labelling in combination with high performance liquid chromatography (HPLC) and MS analysis techniques were applied to evaluate damage in synthetic peptides and wool proteins. Specific residue modifications were identified, located within the primary structure and relatively quantified. The protocols thus developed will assist future fundamental wool research in assessing and tracking damage and in developing procedures to ameliorate damage associated with wool protein degradation.
The primary research goals were:

- The comprehensive mass spectrometric characterisation of amino acid residue side-chain degradation in model peptides
- The development of MS-based protocols to allow the quantitative observation of modifications to specific residues
- The location and characterisation of protein degradation products in wool at a primary structural level
- The development of robust proteomic-based methodologies for the quantification of wool protein damage

This thesis reports the application of label-free and isobaric labelling quantitation methods developed for MS to tracking protein degradation, and the formation of primary and secondary products. This provided new insight into the pathways of formation of residue modifications and into the chemical species or physical stresses that give rise to specific changes in wool proteins. Techniques such as these are required for future research targeting the problems of value retention that affect the NZ wool industry. Reliable quantitation tools will enable accurate evaluation of amelioration strategies, fast-tracking the development of novel preventative and protective wool treatments.

1.2 Thesis structure

This thesis comprises eight chapters. Building on this introductory chapter, Chapter 2 provides a comprehensive up-to-date literature review, setting the context for research into protein damage, and a history of notable research in this area. The analytical techniques used within this research field are also detailed. Section 2.3, ‘Protein primary level degradation in wool’, focuses specifically on research performed to investigate residue degradation in wool, and is presented in the form of a review article published in the Proceedings of the 12th International Wool Research Conference, 2010.

Chapter 3 describes some of the experimental methodology used within this research in more detail than allowable in the following chapters, which are written in the style of journal articles. The experimental chapters are Chapter 4, ‘Profiling of residue-level photo-oxidative damage in peptides’, which is published in Amino Acids; Chapter 5, ‘Isobaric labelling approach to the tracking and relative quantitation of peptide damage at the primary structural level’, published in...
the Journal of Agricultural and Food Chemistry; Chapter 6, ‘Hydrothermal damage in peptide and protein side-chains’, which has been submitted to the Journal of the Science of Food and Agriculture; and Chapter 7, ‘Determination and validation of markers for wool oxidative damage’. A discussion of the results and overall conclusions drawn from these experimental papers are brought together in the final Chapter 8.
2 The impact and characterisation of protein damage

2.1 Why be interested in protein damage?

Proteins are present in all living things, and are consequently also constituents of many biologically derived commercial products, such as meat, milk and wool. Small changes in the chemical composition of these remarkable molecules can lead to surprisingly large changes in structure and function, both in vivo and in any products from these organisms. The term “protein damage” encompasses a huge range of chemical changes. These changes occur in both living organisms and protein products and can be observed on a macro-scale via associated changes in cellular behaviour, solubility, aggregation, colour and physical properties. At the molecular level, even modifications to single amino acid residues may impair cellular function and impart properties characteristic of protein damage. Oxidative modifications represent a class of protein damage that is of particular relevance in a wide array of substrates, due to its links with age-related degeneration and exposure to radiation.

Understanding the processes by which proteins are damaged and how the proteins are affected is of interest to researchers in such diverse fields as nutrition (Baptista & Carvalho 2004), nutraceuticals (Lee et al. 2004), age-related pathology (Linton et al. 2001; Keller 2006), and cosmetics (Sander et al. 2002).

2.1.1 Protein damage within living tissues

Proteins within living tissues are particularly susceptible to damage via oxidative processes. Reactive oxygen species (ROS) are released during processes such as respiration, apoptosis, cell signalling, and host defence, and cause tissue damage and pathogenesis when present at quantities above that which the cell can regulate (Novo & Parola 2008). They are also produced when photosensitisers are triggered by light (termed photo-oxidation) (Davies & Truscott 2001). ROS and reactive nitrogen species (RNS) are highly reactive nucleophiles, and include the hydroxyl radical, peroxynitrite, singlet oxygen, superoxide, peroxides and hypochlorous acid. These species are known to both directly and indirectly promote oxidation in proteins, lipids, carbohydrates and nucleic acids. In proteins, the peptide backbone can be attacked to form backbone peroxides, individual residue side-chains can be oxidised, or the backbone may be cleaved after ROS attack.
of glutamyl, aspartyl and prolyl side-chains (Berlett & Stadtman 1997). Residues that are particularly susceptible to oxidation include the sulfur-containing cysteine and methionine (which can form the reversible oxidation products, disulfides and methionine sulfoxide) and aromatic residues such as tyrosine, tryptophan, phenylalanine, and histidine. Widely reported oxidation products of these amino acids include kynurenine, N-formylkynurenine (NFK), hydroxytryptophan, dihydroxyphenylalanine, nitrotyrosine, hydroxyhistidine and dimer formation (Berlett & Stadtman 1997; Domingues et al. 2003; Żegota et al. 2005). Accumulations of these oxidative products over time can induce dramatic changes in protein structure and behaviour; this is especially evident in tissues such as the eyes and skin, which are chronically exposed to light, resulting in cataracts and accelerated skin ageing (Fisher et al. 2002; Sander et al. 2002; Park et al. 2006; Widmer et al. 2006; Williams 2006).

Unsurprisingly, a diverse range of age-related processes are thought to correlate to the degenerative effects of protein residue modification (Dean et al. 1997; Linton et al. 2001; Sohal 2002). Some researchers even suggest there may be a link between protein oxidation levels and the longer lifespan of females (Kayali et al. 2007). Increased levels of protein oxidation and associated cross-linking are observed in the neural tissue of individuals with Alzheimer’s disease (Keller 2006), which disrupts cellular homeostasis (Giasson et al. 2002). Diabetes mellitus is also linked with oxidative stress, and associated protein oxidation, which has been observed in the form of cysteine oxidation, nitrotyrosine and other oxidised amino acids (Boisvert et al. 2010; Karachalias et al. 2010).

Lens tissues are transparent to damaging wavelengths of electromagnetic radiation (UVA and UVB), and the constituent proteins are replaced only very slowly, if at all, causing the effects of protein damage to be cumulative. Cataract formation is strongly associated with protein degradation, particularly oxidative stress-induced modifications (Spector 1995; Williams 2006). Protein aggregation, cross-linking, peroxide formation, and the formation of coloured amino acid residue products have been observed in nuclear cataracts (Parker et al. 2004; Mizdrak et al. 2008). The free water soluble ultraviolet filters present in the young lens (such as the tryptophan derivatives, kynurenine and 3-hydroxykynurenine) may, in the older lens, oxidise and bind to proteins, becoming photosensitisers, which produce ROS during exposure to light (Mizdrak et al. 2008). Due to downstream effects, photo-oxidation results in protein degradation in excess of that caused directly by UV wavelengths. Cataracts have been found to contain oxidation products of cysteine, tyrosine (such as dihydroxyphenylalanine (dopa), and dityrosine), tryptophan (such as
NFK), methionine, and histidine, as well as deamidation, cross-linking, truncation and methylation (Balasubramanian et al. 1990; Hoehenwarter et al. 2006; Hains & Truscott 2007).

2.1.2 Protein-based products in New Zealand

The detrimental effects of protein damage are not limited to living tissues. Commercially important animal-derived products such as wool, milk and meat rely on the integrity of their constituent proteins to assure high quality and monetary value. As a nation specialising in agricultural exports, New Zealand’s economy relies heavily on protein-based materials. Combined, the export value of protein-based products to New Zealand in 2008 was NZD 13,312 million; 34% of the total agricultural and forestry exports (MAF 2009). The wool industry contributed significantly to this total, with NZD 844 million received from wool and wool products alone. It follows that the degradation of the major constituents in protein-based products, which is a critical factor in their quality and marketability, is of economic importance to New Zealand.

Protein damage in protein-based materials reduces product life-span and performance, impacting negatively on consumer perception of quality. Damage to proteins in commercial products is primarily due to thermal and chemical exposure (which can be categorised as processing-induced damage), and to exposure to photo-oxidative and wear-and-tear type insults during the product life-span (product-life damage) (Millington et al. 2009). For example, protein deterioration in milk products while on the shelf causes a loss in nutritional value (Evangelisti et al. 1999; Baptista & Carvalho 2004) and is associated with negative flavour, colour formation and reduced product stability (Coulter et al. 1948; Thorsen 1958). During thermal exposure (processing-induced), lysine within milk is degraded and reacts with accessible carbohydrates to eventually form advanced glycosylation endproducts (AGEs) (Millington et al. 2009).

The protein-based product of primary interest in this project, cleaned (scoured) wool, has a very high protein content (Plowman 2003), supplemented by small quantities of lipids, nucleic acids and cellular remnants. The protein component consists primarily of what are loosely termed keratins. These are forms of intermediate filament proteins (IFPs), a class of protein high in sulfur (by virtue of a high cystine residue content) that contributes to the structure of many other materials such as skin, hair and nails and to the cytoskeleton of many other cell types. The keratin-associated proteins (KAPs) make up the majority of the other proteins found in wool. Consequently, most damage observed to wool fibres and products is brought about by changes to the wool proteins. Protein degradation gives rise to changes in colour (both yellowing and fading),
and reductions in strength and resilience (Wang & Pailthorpe 1987; Millington & Church 1997). This is discussed in more detail in Section 2.3.

Protein damage chemistry, especially in combination with lipid chemistry, is also critical to meat quality, since proteins and lipids interact to influence nearly all meat quality attributes. In fact, protein degradation is associated with all of the key meat quality traits: flavour, colour, texture and nutrition (Spanier et al. 2004; Mancini & Hunt 2005; Kerry & Ledward 2009).

While this thesis reports work performed with a focus on wool protein degradation, the principles and methodologies underlying the investigations are pertinent to protein damage in a number of materials.

2.2 Research to date on damage-induced protein modification

Improvements in protein product performance and in our understanding of ageing and oxidative degradation mechanisms require knowledge of the specifics of protein damage (Davies 2003; Kayali et al. 2007; Kinoshita & Sato 2007).

With the advent of molecular biology and proteomics techniques, the DNA and protein sequences of many organisms have been determined in whole or in part, representing substantial progress in understanding the functioning of cells. A progression of this has been the characterisation of post-translational protein modifications – which cannot be simply determined by genetics, and which may be endogenously or exogenously induced. Two important components of post-translational modifications comprise the majority of protein damage, namely, side-chain modifications and proteolytic cleavages.

Underlying protein damage is evident at the macro-scale in a variety of observable ways. Although important in living (dynamic) systems, studies of protein damage have been weighted heavily towards (relatively static) protein-based materials, such as wool and hair, and towards tissues such as lenses and skin, where damaged proteins are only slowly, if ever, replaced. Protein damage in these structures is not only easier to detect, but also has a large impact on their function.

Researchers have long recognised the adverse effects of protein residue degradation on wool quality, particularly with regard to photoyellowing and photobleaching, which have a large impact on consumer perception of wool products due to their effect on colour. These colour changes primarily occur during early product life, when consumers are the most sensitive about quality.
The source and formation of chromophores associated with photoyellowing has therefore been of considerable interest to researchers and has been debated widely over the last few decades (Asquith & Brooke 1968; Goddinger et al. 1994; Millington 2006a) – see Section 2.3. Effective and targeted strategies to prevent photodegradation will rely on the identification of the modified species and on an understanding of the mechanisms of their formation. Comparably, research investigating the protein modifications present in cataractous lenses has revealed the formation of a number of coloured products, in addition to protein cross-linking and aggregation (Hood et al. 1999; Linetsky et al. 2004).

Unwanted colour changes are not the only deleterious effects of protein damage in wool. For example, photo-tendering refers to the decreased tensile strength and embrittlement imparted to wool after exposure to light (during the product life-span), thought to be related in part to the degradation of cystine cross-links within wool during treatment (Steenken & Zahn 1984). Even prior to exposure to light, however, wool proteins are routinely damaged during processing steps such as scouring, carding, drying, fulling, dyeing, setting and assorted other treatments. Fibre weakening during high temperature stock dyeing (commonly performed for wool destined for use in worsted or semi-worsted carpets) is especially important (Holmes-Brown & Carnaby 1982). In contrast to irradiation damage, which occurs primarily over a product’s lifespan (in carpets, garments, and car upholstery), chemical and heat damage to fibres is commonly caused during processing, prior to sale. In practice, it is very likely that these processes contribute in synergistic fashion throughout the product’s lifespan to form complex degradation patterns.

Detailed investigation into the various forms of degradation in wool and in other materials has been limited and hindered by the lack of sensitive techniques available to researchers. To progress this research, there is a need for reliable information and techniques to assess the chemistries involved in wool protein degradation.

2.2.1 The chemistries of side-chain modification

Residue side chains and the peptide backbone can be disrupted by the action of ROS or, in the case of tryptophan and tyrosine residues or other bound chromophore groups, direct UV absorption (Davies 2003; Stadtman & Levine 2006; Igarashi et al. 2007). The key chromophores in wool are tryptophan, tyrosine and phenylalanine; these display maximum absorption from 270-286 nm (Yang et al. 1985). Modification caused by direct absorption by the protein components is termed Type I photo-oxidation. Other degradative oxidative pathways are initiated by the formation of ROS, which can be generated within cells or in external systems by the reaction of
excited state oxygen with sensitisers, or by the irradiation of a photosensitiser (Type II photo-oxidative processes) (Lee et al. 2004; Wei et al. 2007). In Type II photo-oxidation, and in oxidative pathways mediated by processes other than light, ROS such as singlet oxygen react with electron-rich molecular sites, forming peroxides and other reactive intermediates, which go on to form more stable oxidised products (Huvaere & Skibsted 2009). The chemistry of reactions of ROS with the susceptible residues, tyrosine and tryptophan, is described in detail in section 4.4.2.

ROS production is known to be prevalent in cellular systems, but even in non-cellular materials such as wool, ROS play an important role regarding their performance over their lifespan. ROS may be oxygen-centred radicals (containing one or more unpaired electrons) or oxygen-centred non-radicals. The radicals include superoxide anion (O$_2^-$), hydroxyl radical (OH), alkoxyl radical (RO$^.$), and peroxyl radical (ROO$^.$). The non radicals include hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (1O$_2$). Other reactive species are nitrogen-centred, like peroxynitrite (ONOO$^-$), nitric oxide (NO$^.$) and nitric dioxide (NO$_2$) (Lee et al. 2004).

The superoxide anion may be photo-generated (Ishibashi et al. 2000) or formed in cellular systems. It contributes to the formation of other ROS such as hydrogen peroxide (2 O$_2$ + 2H$^+$ $\rightarrow$ H$_2$O$_2$ + O$_2$), hydroxyl radical (O$_2^-$ + H$_2$O$_2$ $\rightarrow$ ‘OH + OH$^-$ + O$_2$) or singlet oxygen or can react with them to form more reactive products, such as peroxynitrite, which can then go on to form hydroxyl radical and nitric dioxide (ONOO$^-$ + H$^+$ $\rightarrow$ ‘OH + NO$_2$).

Hydroxyl radical is very reactive to proteins and other biological molecules. It has been detected in wet irradiated wool (Millington & Kirschenbaum 2002; Wang et al. 2009). Hydrogen peroxide is not as reactive as other ROS, but is both generated within and directly applied to wool during wet processing (Millington & Maurdev 2004). Singlet oxygen reacts with susceptible residues in proteins (Davies 2003) and is produced by dry irradiation of wool (Smith 1992). It can also be produced in aqueous systems in the presence of photosensitisers (Vileno et al. 2004). Peroxy radicals can be formed from the UV irradiation of protein peroxides (ROOH $\rightarrow$ ROO$^.$ + H$^+$ + Fe$^{3+}$ $\rightarrow$ ROO$^.$ + Fe$^{2+}$ + H'). Peroxynitrite may be formed by the reaction of nitric oxide and superoxide (O$_2^-$ + NO$^.$ $\rightarrow$ ONOO$^-$).

2.2.2 Progress in damage assessment

Historical approaches to the quantification of protein degradation were limited to holistic techniques. A review of methods used to characterise damage to wool proteins is presented in Section 2.3. Holistic tests for protein deterioration in other materials included the detection of
furosine in milk (Baptista & Carvalho 2004), the observation of protein aggregation and denaturation through native and SDS-PAGE (Davies & Delsignore 1987), protein extractability tests for detecting protein changes in stored fish meat (Shenouda 1980), and the observation via amino acid analysis of damage indicators such as lysinoalanine in alkali-treated proteins (Schwass & Finley 1984). Assays have been designed to measure artefacts of protein oxidation, such as protein hydroperoxides using the ferric-xylene orange method (Gay & Gebicki 2003), tryptophan degradation using changes in fluorescence (Balasubramanian et al. 1990), carbonyls (which are widely acknowledged as markers of oxidation) using derivatisation with dinitrophenylhydrazine (Levine et al. 1994; Nakamura & Goto 1996; Berlett & Stadtman 1997; Howard et al. 2007), and advanced oxidation protein products (Witko-Sarsat et al. 1996).

The detection of oxidation progressed so that protein damage could be localised to individual proteins using carbonyl derivatisation combined with electrophoresis and immunoblotting (Levine et al. 1994; Nakamura & Goto 1996). An assay for nitrotyrosine, a specific marker of RNS protein modification, was developed using derivatisation and high performance liquid chromatography-electrochemical detection (HPLC-EC) (Shigenaga et al. 1997) and may also be determined through ELISA (Khan et al. 1998).

Investigating protein damage with mass spectrometry has enabled rapid progress in the characterisation of protein damage at the amino acid residue level. This has allowed the identification of modifications to individual amino acids in wool proteins – enabling both the chemical modification, and its location within a protein to be established (Dyer et al. 2006b; Dyer et al. 2010).

MS is characterised by high detection sensitivity and molecular specificity (Dass 2001), and, in conjunction with soft-ionisation techniques such as electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI), is used widely in proteomic research. In brief, MS identifies compounds by means of their mass, or, more accurately, by their mass-to-charge ratio (known as m/z). Proteins separated using HPLC (either intact, or enzymatically digested into short lengths, peptides) are formed into charged ions in an ion source. In a time-of-flight (TOF) mass analyser, these ions are then accelerated in an electric field of known strength. The speed of the ion’s flight is determined by its mass-to-charge ratio. A similar principle applies to quadrupole mass analysers. Ions of a given mass range may be isolated and fragmented within the mass spectrometer. The masses of the fragments may be used, jigsaw-style, to calculate the structure and formula of the parent molecule. This is termed tandem MS (or MS/MS). In this way, the
amino acid sequences of peptides fragmented in the mass spectrometer may be determined (see Figure 2.1).

Figure 2.1  Schematic of the processes involved in mass spectrometric analysis of proteins.

Due to the complex heterogeneous composition of extracts from biological materials, conventional large scale proteomic research has involved sample fractionation prior to MS. Until recently, this relied principally upon the high resolution separation technique of two-dimensional electrophoresis (2DE), separating proteins on the basis of their isoelectric point (pI, the pH at which they contain no net charge), molecular mass, and abundance (Görg et al. 2004). Several limitations of 2DE separation restrict its use in protein quantification, such as limited reproducibility, a bias against proteins exhibiting extremes in size, pI or hydrophobicity and against low-abundance proteins (Rabilloud 2002), co-migration (Peng & Gygi 2001), a lack of automation (Rabilloud 2002), and difficulties in analysis (Görg et al. 2004). Consequently, HPLC systems, which can be linked directly or closely with MS analysis, have become a preferred means of achieving sample fractionation (Yan & Chen 2005). Forms of HPLC separate compounds based on polarity, hydrophilic interaction, size and charge (Suder 2008).

MS, as applied in conventional proteomics, has primarily been aimed at the qualitative identification, characterisation and sequencing of proteins, peptides and post-translational modifications, whereas quantification of these species has only become possible relatively recently. An MS spectrum provides detailed information on the masses of sample components, but does not, on its own, provide sufficient information for quantifiable comparison of different peptides, as the signal intensities of particular ions do not necessarily correspond to their proportions in the sample (Wenschuh et al. 1998). This variability of peptide signals occurs because the ionisation of particles in MS is influenced by their chemical composition and by interfering ions in the sample (Wenschuh et al. 1998; Muller et al. 2002). As a consequence, much effort has been applied in developing strategies to circumvent these problems, and recent
advances in proteomic mass spectrometry have provided a range of approaches targeted at quantifying protein differences between sample populations (see Section 2.2.3.1).

The application of ESI-MS combined with HPLC separation was recently applied to furthering the understanding of wool protein degradation. This demonstrated the suitability of these techniques for the task at hand: numerous light-induced modifications were identified in wool proteins, including eleven derived from tryptophan (hydroxytryptophan, NFK, hydroxyformylkynurenine, kynurenine, hydroxykynurenine, carbolines, tryptophandiones and nitrotryptophan) and three derived from tyrosine or phenylalanine (dopa, dityrosine and hydroxydityrosine). These modifications were located within the primary structure of proteins utilising bioinformatic analysis of known wool protein sequence data (Bringans et al. 2006; Dyer et al. 2006b).

2.2.3 MS-based approaches to quantitation

As briefly described in Section 2.2.3, MS spectra do not provide inherently quantitative data. This has been addressed in a variety of ways, primarily with the use of isotopic and isobaric variants.

In MS, peptides are distinguished on the basis of their mass/charge ratio \((m/z)\), meaning that if two samples (both containing Peptide X) are combined without any modification, it is not possible to decipher how much of the Peptide X signal comes from Sample A and how much from Sample B. Consequently, a variety of methods have been developed to alter the mass of peptides from Sample A, without otherwise changing their behaviour substantially from that of the peptides in Sample B. This is known as isotopic labelling, and it makes use of non-radioactive isotopic variants such as \(^{18}\text{O}/^{16}\text{O}, ^{13}\text{C}/^{12}\text{C}, \) and \(^{1}\text{H}/^{2}\text{H}.\) These isotopically distinct atoms can be incorporated into labels that are added to peptides, so that heavy and light forms of the labelled peptides are present in one sample or the other. In MS, the peaks of the labelled peptide ions are now separated by a known number of Daltons (atomic mass units), allowing the samples to be quantitatively compared using peak area or signal intensity.

There are numerous techniques available to achieve quantitation in mass spectrometry using isotopic sample differentiation. Most rely on the derivatisation of side-chain functionalities on residues with isotopically-distinct labels. Some exploit ubiquitous groups for labelling, such as the amine and carboxyl groups present at \(N\)- and \(C\)-termini, respectively (Chakraborty & Regnier 2002; Zhang et al. 2002; Brancia et al. 2004; Guillaume et al. 2006; Regnier & Julka 2006; Mirza et al. 2008). These are known as global tagging approaches, as each protein or peptide will be labelled. Some techniques even use isotopic incorporation in growth media, prior to extraction
(Jensen et al. 1999). Non global techniques rely on the derivatisation of specific residues. Cysteine may be targeted using isotope-coded affinity tags (ICAT), which employs isotopic tags combined with biotin affinity chromatography (Gygi et al. 1999; Griffin et al. 2003; Hansen et al. 2003), and by various isotopically distinct alkylation agents (Gehanne et al. 2002; Sechi 2002; Niwayama et al. 2003; Pasquarello et al. 2004; Vosseller et al. 2005). Tryptophan-specific labelling approaches also exist (Kuyama et al. 2003; Matsuo et al. 2006).

Isotopic labelling remains popular, but suffers from a few drawbacks (Julka & Regnier 2004). By creating double peaks in MS for each peptide, the spectral complexity is greatly increased. In addition, isomers can behave differently in HPLC, causing some heavy and light isomers to differ in their retention times – this is highly problematic for automated on-line LC-MS, where quantitation relies on identical column retention times.

2.2.4 Isobaric tags

The chemistries involved in generating isotopic labelling strategies led to the development of isobaric labelling strategies. This type of approach also uses isotopes, but in such a way that the total mass of the label is equal in each sample. Isobaric approaches are distinct in that they rely upon mass differentiation (and thus quantitation) in MS/MS (after fragmentation).

An isobaric tag is constructed with a reactive group (to attach to the peptide), a cleavable reporter section (the ion used for quantitation that is released during MS/MS, and has a different mass per sample) and a balance group (which is heavier or lighter in order to compensate for the reporter section). To reiterate, the mass-identical tags allow peptide and proteins to elute simultaneously and to form a single ion peak in MS. MS/MS fragmentation causes the release of distinctive and quantifiable reporter ions.

A small variety of isobaric labels have been reported: Tandem mass tagging, which comes in two forms, allowing the labelling of two samples (Thompson et al. 2003; Dayon et al. 2010); iTRAQ labelling, which is now available in four-plex and eight-plex forms (Ross et al. 2004; Ow et al. 2008); DiART reagents, which come in six forms (Zeng & Li 2009), and DiLeu reagents, which are four-plex (Xiang et al. 2010). A similar strategy, which does not make use of cleavable reporter ions, has been reported as isobaric peptide termini labelling (IPTL) (Koehler et al. 2009). The most popular of these, commercialised by ABSciex (Foster City, CA), are the iTRAQ reagents, with their potential for multiplexing. The reporter ions released by iTRAQ reagents have masses of 113.1, 114.1, 115.1, 116.1, 117.1, 118.1, 119.1 and 121.1 Da.
It is anticipated that the application of sensitive qualitative and quantitative mass spectrometric techniques to the analysis of protein degradation in commercial products such as wool will provide significant advances in our understanding of the chemistries involved in protein damage. In turn, this will allow targeted ameliorative or protective treatments to be developed.

2.3 Protein primary level degradation in wool

Protein degradation lowers the value of wool and wool textiles by reducing product life-span and performance, which impacts negatively on consumer perception of quality. When wool proteins are damaged after exposure to sunlight, processing, finishing treatments, or heat, the fibres may discolour, become more brittle, lose tensile strength or otherwise develop undesirable characteristics (Gullbrandson 1958; Sweetman 1967; Milligan 1980; Holmes-Brown & Carnaby 1982).

Proteins, which are the chief constituents of wool, are composed of chains of amino acid residues, yielding a peptide backbone from which the side chains of the various amino acids protrude. These side chains are a critical site of protein damage. Residues with side chains containing sulfur (methionine and cysteine) or aromatic groups (tryptophan, tyrosine, phenylalanine, histidine and proline) are the most reactive and are very susceptible to oxidative damage (Davies 2005; Dyer et al. 2006a; Millington 2006b).

Factors that damage wool therefore result in chemical modifications to the residue side chains, resulting from degradation of the original amino acid residue within the protein chain. The loss of reactive residues and the formation of modifications have accordingly been observed in damaged wool (Inglis & Lennox 1963; Asquith & Brooke 1968; Asquith & Rivett 1969; Goddinger et al. 1994; Smith et al. 1994; Smith 1995; Dyer et al. 2006a; Dyer et al. 2006b). These changes, including the breakage of crosslinks related to strength and stability such as disulfides, the formation of new crosslinks such as lanthionine in different positions while the wool is under mechanical stress, and the formation of coloured products such as kynurenine, impact negatively on key wool quality attributes such as strength and whiteness (Asquith & Otterburn 1970; Bringans et al. 2006).

2.3.1 Causes of wool degradation

Wool, from the sheep’s back to the finished product, is exposed to a number of factors that may induce protein damage. These include indoor and outdoor light; elevated temperatures; oxidants
and reductants; acids and bases; and mechanical stress. Many of these conditions induce oxidative protein modification, largely through the initial generation of reactive oxygen species.

ROS are reactive nucleophiles, and include the hydroxyl radical, singlet oxygen, superoxide, peroxides and hypochlorous acid. They can be produced in wool after residues and other chromophores excited by light (such as tryptophan and its derivatives, or fluorescent whitening agents) react with molecular oxygen (Schäfer et al. 1997; Millington & Kirschenbaum 2002; Millington & Maurdev 2004; Igarashi et al. 2007), and their production can be catalysed by the presence of trace amounts of metal ions such as may be found in wool (Hartley & Inglis 1968; Davies 2004). These ROS, along with direct UVA absorption by tryptophan, cause wool yellowing, fading, breakage and embrittlement in wool exposed to light [3,5,22-25].

2.3.2 A history of investigation

Early wool research demonstrated a link between the depletion of certain amino acid residues in wool polypeptide chains and wool damage. These changes were described in efforts to determine the effect of factors such as light and processing on wool chemistry. The residues depleted were expected to form new compounds that would contribute to unwanted fibre properties such as yellowness (via chromophore formation) and brittleness (through crosslinking alterations) (Inglis & Lennox 1963).

![Scheme 2.1.](image)

The reduction in strength observed in damaged wool, which results from alkali treatments such as in reductive dyeing, was expected to be associated with the loss of cystine crosslinks, which play a significant role in determining the mechanical properties of the wool fibre. Louw (1960) observed a decrease in cystine and a corresponding increase in its oxidation product, cysteic acid, in weathered wool (Louw 1960), while Thorsen et al. detected oxidised cystine in ozone-treated
damage to cystine crosslinks by quantification (via acid hydrolysis derivatisation and colorimetric
analysis) of another cystine degradation product, dehydroalanine, possibly via β-elimination,
Scheme 2.1 (Cookson & Harrigan 1992).

Secondary reaction of dehydroalanine with cysteine or lysine residues can form new lanthionine
and lysinoalanine crosslinks in wool, Scheme 2.2 (Cookson & Harrigan 1992). Cystine crosslinks
were also observed to break and form cysteine oxidation products after mechanical wool
disruption, such as cystine dioxide, cystine monoxide and cysteic acid (Zhang et al. 2008).

\[
\text{cysteine} + \text{dehydroalanine} \rightarrow \text{lanthionine}
\]

Scheme 2.2

Other isopeptide bridges formed during wool damage include Tyr-Tyr (dityrosine) crosslinks. Raven et al. (1971) detected dityrosine in wool hydrolysates, using amino acid analysis (AAA). Its
presence in irradiated wool was confirmed by Röper & Finnimore in 1985 using high performance
liquid chromatography (HPLC) and AAA. Stewart et al. (1997) used reverse-phase (RP) HPLC to
observe an increase in dityrosine after oxidation and irradiation.

Amino acid analysis of hydrolysed wool digests and free amino acids identified correlations
between photoyellowing and the degradation of aromatic UV-absorbing tryptophan (Asquith &
Rivett 1971), tyrosine (Asquith & Rivett 1969) and phenylalanine residues (Asquith & Brooke
1968; Goddinger et al. 1994). Free solutions of tryptophan were found to generate coloured
photoproducts such as NFK, kynurenine and oxindolylalanine upon irradiation (Schäfer et al.
1997), which were considered likely contributors to wool yellowing.

Smith & Melhuish (1985) first detected NFK fluorescence in UVA-excited wool, supporting
observations made in 1976 by Nicholls & Pailthorpe. Then, Schafer et al. directly detected NFK in
detected both NFK and its derivative, kynurenic acid, in enzymatically hydrolysed proteins from yellow wool using thin layer chromatography (TLC) comparison with standards. Smith et al. (1994) used TLC and fast atom bombardment mass spectrometry to identify saturated and oxidised β-carbolines in weathered wool. Carbolines could be formed by condensation of tryptophan with α-keto acids. Based on the reactions of free tryptophan in solution, the formation of hydroxylated tryptophan derivatives and hydroxykynurenine in wool was postulated by Davidson (1996). In a breakthrough in 2006, Dyer et al. identified a wide range of oxidative modifications, including amino acid residues consistent with hydroxytryptophan and hydroxykynurenine in irradiated wool (Dyer et al. 2006a; Dyer et al. 2006b).

This recent work, employing highly sensitive high performance liquid chromatography separation and ESI-MS/MS, significantly progressed the identification of primary level degradation in wool. A number of photo-induced modifications were identified directly within the primary structure of wool proteins (Dyer et al. 2005; Bringans et al. 2006; Dyer et al. 2006b). These included eleven derived from tryptophan (hydroxytryptophan, NFK, hydroxyformylkynurenine, kynurenic acid, hydroxykynurenine, carbolines, tryptophandiones and nitrotryptophan) and three derived from tyrosine or phenylalanine (dopa, dityrosine and hydroxy-dityrosine). These modifications were located within the primary structure of proteins utilising bioinformatic analysis of known wool protein sequence data (Dyer et al. 2006b). These advances were possible due to the ultrahigh detection sensitivity and molecular specificity of mass spectrometry (Dass 2001).

2.3.3 Quantifying primary level wool damage

Following identification of the chemical species formed during wool damage, the natural progression of investigation is to quantify the formation of the modifications. Numerous tests for wool damage exploited these chemical changes in a holistic fashion, for example: alkali, acid, and urea-bisulfite solubility tests detect the increased solubility after disulfide cross-links are disrupted after oxidation (Harris & Smith 1936). Amino acid analyses by various means (Houff & Beaumont 1956; Köpke & Nilssen 1957; Milligan & Holt 1980; Steenken & Zahn 1984; Schäfer 1997) allowed the formation of residue modifications to be observed, but did not allow localisation to specific wool proteins and components.

Because tandem MS permits the mass and sequence of each amino acid residue to be determined within proteins, residue degradation may be directly localised to individual proteins, permitting a
much higher level of understanding. Methods to profile the degradation of amino acids within peptides using MS have recently been developed using model peptides and ESI-MS/MS. This resulted in the verification of numerous photomodifications noted previously in wool, and in the quantitative profiling of the degradation of susceptible tryptophan and tyrosine residues, and the formation of their degradation products (Grosvenor 2009; Grosvenor et al. 2009). An example of this profiling is illustrated in Figure 2.2.

Applied to peptides extracted from wool, this type of approach to wool damage analysis is expected to rapidly progress our understanding of the processes and chemical changes caused by the damaging factors to which wool is exposed.

![Figure 2.2](image)

**Figure 2.2** The degradation of peptides containing tryptophan (LLWLR) in solution with UVB irradiation, as assessed by (a) label-free relative ion abundance and (b) iTRAQ-assisted MS-based quantitation. Error bars represent ± SEM.

### 2.3.4 Summary

Wool science and its supporting industry have benefited greatly from investigations during the last century into the chemistry of the degradation of wool, which have yielded important insights regarding the causes of phenomena such as phototendering and photoyellowing. The last ten years have seen exciting developments in this field, with advanced HPLC separation techniques and ESI-MS/MS analysis allowing the identification of low abundance, yet important, modifications to wool chemistry.

Protocols permitting the specific profiling of primary level degradation will provide a tool to compare treatment and processing variations with their effects on chemical markers of damage. This, along with other advances in analytical protein chemistry, promises to be an aid in maintaining and improving the desirable characteristics of the natural wool fibre as a quality fibre for use in textiles and other applications.
2.4 Research approach

The aims of this research were to find ways to characterise and quantify damage-induced modifications in amino acid residues within peptides and wool-derived proteins. The suitability of MS for such tasks demanded its use in this project.

Some challenges were expected from the outset: Post-translational modifications, including degradative modifications, are, by their very nature, likely to be present at low actual and relative abundance. This is so because, even if the entire complement of degradable amino acid residues is modified, there will be multiple products formed, so that each product is less abundant than the parent. In actuality, it is unlikely that the entire parent compound would be degraded under most conditions. In addition, the most susceptible aromatic residues (such as tryptophan), which are a subject of this study, are not usually present at high relative abundance in proteins.

A further complication is presented by the wool proteins themselves: The constituent proteins are wool generally display very poor solubility, partly due to high levels of cross-links (Koehn et al. 2010). This makes them difficult to extract and characterise; this problem is exacerbated in damaged wool, which may have higher levels of cross-linking (Kearns & Maclaren 1979). For these reasons, to date, no robust quantitation techniques have been developed for fibrous protein residue modification.

This research project applied sensitive newly developed proteomic-based quantification techniques together with in-house knowledge of wool chemistry and protein oxidation to the MS-based identification of residue-level peptide and wool protein residue modifications. This enabled the reliable characterisation and tracking of specific residue modifications in wool proteins arising from damage protocols that reflect processing conditions used in the wool industry.
3 Methodology

3.1 Introduction

The methodologies required to achieve the outcomes of this project can be broken down into three primary areas: Sample preparation, treatment, and data extraction.

Sample preparation includes pre-treatment preparation of wool, the solubilisation of its constituent proteins, and the fractionation of those components. This component of the methodology was only applicable to those experiments featuring peptides derived from wool, as the synthetic model peptides required no sample preparation prior to treatment. Treatment steps involve the exposure of peptide and protein solutions to conditions expected to trigger polypeptide degradation, in order to produce samples suitable for examination by mass spectrometry. Data extraction steps comprise the preparation of treated samples for analysis by mass spectrometry, and the analysis of the resultant data sets.

3.2 Sample preparation steps

3.2.1 Peptide design

Model peptides were designed to provide a simple system in which to observe tyrosine and tryptophan degradation. The peptides, LLYLR and LLWLR, contained the central aromatic residues, tyrosine and tryptophan, flanked by relatively oxidatively stable leucine residues. The arginine residue provided comparability to tryptic peptides and assisted good analyte ionisation. The peptides were synthesised and provided by The Biopeptide Company (San Diego, CA). Upon analysis, the samples were shown to contain several partially synthesised truncations; LLW/Y, LLW/Y, LY/WLR, and W/YLR (Figure 3.1).
Figure 3.1  Mass spectrum (ESI-MS, 250-800 m/z) of the synthetic peptide mixture. Peaks corresponding to singly-charged LLYLR, LLWLR, LLY, LLW, YLR, WRL, LYL and LWLR, and doubly-charged LLYLR and LLYLR are labelled.

3.2.2  Wool sample preparation

Scouring, or washing, is required to remove the wool wax (lanolin), suint and other contaminants that coat the fibres of untreated wool (known as greasy wool). In industry, this is the first processing step, and it is necessary to allow further processing such as spinning and dyeing. Modern industrial scouring normally makes use of non-ionic surfactants in water (Teasdale 1991). Scouring is also required for experimental samples, firstly to mimic industry and standards, and secondly to remove contaminants and grease that might interfere with subsequent analysis.

The scouring protocol used in these experiments was developed for laboratory usage at WRONZ. It makes use of a non-ionic detergent, Teric GN9 (Orica Ltd, Auckland, NZ) in water at 60°C and 40°C, followed by rinses in water and organic solvents. Scouring performed at laboratory scale also removes most vegetable matter.

To enable optimal access of extraction agents to the wool proteins, scoured and dried samples were powdered by grinding over liquid nitrogen.
3.2.3 Extraction and fractionation of IFPs

Wool proteins have a high cystine content, which contributes to their insolubility. To extract and separate soluble wool protein fractions, an extraction agent was employed that increases protein solubility. Sodium tetrathionate was utilised as the active agent in oxidative sulfitolysis, a process in which cystine (RS-SR) was reduced to cysteine (RSH) and subsequently oxidised to S-sulfocysteine (RS-SO$_3^-$). Once the crosslinks are broken and the cysteine –SH groups are capped, or blocked, with SO$_3^-$, the wool proteins are much more soluble and can be further manipulated for fractionation steps (Thomas et al. 1986). The fractionation steps were an adaptation of a protocol presented in 1986, a thesis, and in-house reports (Thomas et al. 1986; Paton 2005). The final protocol represented a novel combination of these ideas.

This approach yielded enriched fractions of other classes of wool proteins (principally keratin-associated proteins: high glycine tyrosine proteins, high sulfur proteins and ultrahigh sulfur proteins) in addition to an enriched IFP fraction (see Figure 3.2). For a condensed schematic of the process used to specifically obtain an enriched IFP fraction, see Figure 6.1. The protocol for the entire fractionation process is given below:

Scoured, dried and hand-ground wool was extracted at 10 mg/ml in sulfitolysis solution [0.1 M Tris, 0.2 M sodium sulfite, 0.1 M sodium tetraborate, 8 M urea, pH 9.5 (HCl)] with vigorous agitation for 24 hours at room temperature. The extract was sieved through 125 µm mesh. The gelatinous residue was agitated in 250 ml room temperature water for 15 minutes and the filtrate passed through 125 µm mesh and combined with the original extract. The residue was washed twice more in the same manner, and the filtrates combined (SN1). The remaining solids were retained for gravimetric analysis. The filtrate was dialysed against water (Snakeskin Pleated Dialysis Tubing, Rockford, IL) and then centrifuged (Avanti J-30I centrifuge, JA-30.50 rotor, Beckman Coulter, Brea, CA) at 14000 rpm and the supernatant (SN2) was carefully removed, and the precipitate (ppt 1) retained on a cellulose acetate filter after gentle washing with water. The precipitate was then freeze dried. An equal volume of 0.04 M zinc acetate (pH 6.0-6.2 with acetic acid) was added to SN2 and allowed to stand for ten minutes. Centrifugation (JA-30.50 rotor) at 14000 rpm, allowed the separation of a precipitate (ppt 2) and a supernatant (SN3). The supernatant was dialysed against water, and then freeze-dried. The precipitate was combined with 0.02 M sodium citrate, dissolved, dialysed against water and centrifuged at 12000 rpm at -16.250 rotor. The supernatant (SN4) was carefully
removed, and the precipitate (ppt 3) gently washed over a cellulose acetate filter and then freeze-dried. Two volumes of acetone and a 20% volume of saturated ammonium sulfate were added to SN4, and stirred for two hours at room temperature. The slurry was centrifuged at 14000 rpm at 10°C (JA-30.50 rotor) to recover the supernatant (SN5), which was dialysed against water and freeze-dried, and the gelatinous precipitate (ppt4), which was dissolved in 0.04 M sodium borate, dialysed against water, and freeze-dried.

Figure 3.2  Schematic of fractionation scheme demonstrating the separation of fractions enriched in specific wool protein classes. SN = supernatant; ppt = precipitate; UHSP = ultra high sulfur proteins; HSP = high sulfur proteins; HGTP = high glycine tyrosine proteins; IFP = intermediate filaments.
See Figure 3.3 for an electrophoretic separation of some of these fractions, demonstrating the enrichment of particular classes of wool proteins. Note: The extraction of the IFP fraction is presented again in simplified form in Chapter 6, but the supernatants and precipitates are labelled differently.

**Figure 3.3** One dimensional gel of partially fractionated sulfitolysis-extracted proteins, showing enrichment of IFPs and keratin-associated proteins (KAPs), including the high-glycine tyrosine proteins (HGTPs) and high sulfur proteins (HSPs).

### 3.2.4 SDS-PAGE

One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the effectiveness of extractions (an example is given in Figure 3.3). This was performed according to standard procedures used at AgResearch based upon those developed at WRONZ, or on manufacturer’s recommendations. Specifically, 0.067 mg samples in 10 µl sample buffer (dissolved and denatured by boiling in 6 M urea, 0.062 M Tris, 6% β-mercaptoethanol, 10% glycerol, 0.001% Bromophenol Blue and 2% SDS) were loaded into 1.0 mm 12+2 comb Criterion Precast 10-20% Tris-HCl Gels (BioRad laboratories, Hercules, CA) and run at 200 V, 2.0 A using a PP200 BioRad power pack and Tris-glycine SDS (Criterion) buffer. Gels were fixed overnight in 50% methanol, 2% orthophosphoric acid, washed three times in cold water, impregnated with 34%
methanol, 17% ammonium sulfate, 2% orthophosphoric acid, and stained using 0.7% colloidal Coomassie Blue G250. Rinses with water removed excess stain, before image capture.

### 3.2.5 Solubilisation

Immediately prior to treatment, peptide and protein samples (stored as solids) were solubilised. Commercially prepared synthetic peptides were dissolved in ultrapure water at 2 mg/ml. Freeze-dried IFP fractions were dissolved completely in 0.05 M sodium tetraborate, pH 8.5 (after attempts to dissolve in water proved unsuccessful) at 5.3 mg/ml. This buffer was chosen as it was used to solubilise the IFP fraction during the preparative steps, and because it is not thought to interfere with subsequent mass spectrometric analysis.

### 3.3 Treatment steps

Treatments designed to cause measurable damage in sample peptides or proteins required a balancing act – degradation needed to be at a sufficient level to enable detection, characterisation, and the observation of damage patterns, while being mild enough to avoid total sample destruction and exceedingly complex and complete degradation patterns that would be impossible to interpret. It was also important to avoid sample loss, to enable quantitative (and fair qualitative) comparison. A variety of intensities, lengths and styles of treatment were therefore attempted before the selection of optimised protocols as presented below.

#### 3.3.1 UV irradiation

The irradiation conditions utilised in these experiments made use of low heat emission UVA and UVB LZC narrow bandwidth fluorescent lamps in a vented photo-irradiator (LZC4-14, LuzChem, Ontario, Canada). These lamp properties ensured controlled treatments; temperatures inside the treatment chamber were maintained within a range of temperature, eliminating the confounding effects of elevated temperature on protein degradation and reaction rates. Thin, UV-transparent quartz tubes were used to allow uniform exposure of the peptide samples in solution to UV light. The short times and moderate temperatures required for irradiation treatment made loss of solvent by evaporation only a minor issue, and this was prevented by sealing of the tubes with UV-transparent polyethylene film. The bulk of emission from the UVB lamps fell into the 280-360 nm range (partially extending into the UVA range) and 315-400 nm for the UVA lamps (see Figures 3.4 and 3.5). These narrow ranges allow the effects of irradiation at particular bands of UV to be determined.
3.3.2 Heating

Elevating aqueous samples to high temperatures is likely to result in evaporation. For quantitative MS analysis and for comparable treatments, it is critical that each sample should remain at constant concentration. This is particularly important when treating proteins, where dehydration and reconstitution steps should ideally be kept to a minimum, to prevent aggregation and losses in solubility.

A number of approaches were trialled that would enable small sample volumes to be heated at 90°C for hours or days, without resulting in evaporation. Many small tube systems demonstrated inconsistent seal integrity over the required time period, with losses from individual sample tubes due to perishing seals, lids, or lids that yielded under pressure. A successful system was eventually developed using 1 ml Reacti-Vials (Thermo Fisher Scientific, Fair Lawn, NJ); thick-walled small tubes made of borosilicate-strengthened glass, with a conical interior, allowing complete sample recovery (Figure 3.6).
Interestingly, hydrothermal exposure periods of 0-192 hours were used for assessing hydrothermal damage in peptides, as these periods produced a good balance of considerable degradation and product formation, without total destruction of parent peptides, or extensive degradation into products too small and generic (such as ammonia) to provide information on degradation pathways. Experiments with keratins, however, soon showed that treatment times of these lengths resulted in samples so degraded that the resulting spectra were incomprehensible as the components bore so little resemblance to the parent proteins. Instead, much shorter treatment times (up to 8 hours) were selected to provide observable degradation and interpretable data. It seems that complex protein systems are more susceptible to the effects of hydrothermal exposure. This is most probably due to elevated ROS generation through processes such as photosensitisation (Igarashi et al. 2007), connected to the greater number of sensitive functional groups within the protein systems.

3.4 Data extraction steps

To obtain information on the effects of damage within peptides and proteins, a number of steps were required. Mass spectrometric analysis necessitated samples to be prepared in particular ways. Protein samples are complex, and were first digested into shorter peptides – this not only allows bottom-up proteomic analysis, but also enhances sample solubility. Complex peptide mixtures must be separated to reduce the number of compounds that must be analysed simultaneously by the mass spectrometer – this was achieved using reverse phase HPLC. Isobaric
quantitative analysis involved the labelling of individual samples with distinct iTRAQ markers, before sample pooling, enabling quantitative comparisons during MS/MS fragmentation.

3.4.1 Digestion

Bottom-up MS analysis of proteins usually requires digestion into oligopeptides (this step was not required to analyse the effects of treatment on synthetic peptides). Wool-derived proteins were first reduced with tris(2-carboxyethyl)phosphine (TCEP), to expose free sulfhydryl groups, and then alkylated with methyl methanethiosulfonate (MMTS) or acrylamide. MMTS converts sulfhydryl groups to methylthio groups (Cys–S–CH3), while acrylamide alkylation yields a thioether derivative, Cys–S–β-propionamide. These reagents prevent the reformation of crosslinks, improving the accessibility of the protein to enzymatic digestion. Digestion was performed using sequencing-grade trypsin (Promega VS111, Sydney, Australia), a standard enzyme used for digestion in proteomics. Trypsin cleaves at the carboxyl side of lysine (K) and arginine (R) residues, yielding peptides terminating in K or R. The amine groups on these peptides render them readily ionisable for MS.

3.4.2 Isobaric labelling

To enable quantitation in MS/MS, isobaric labelling was carried out using the commercially available 4-plex iTRAQ reagents. iTRAQ labelling was performed based on the manufacturer’s recommendations, with variations according to sample type. For model peptides, 40 µl aliquots were evaporated to dryness at 30°C (Centrivap centrifugal concentrator, Labconco, Kansas City, MI) and suspended in 30 µl 0.5 M triethylammonium bicarbonate, pH 8.5. The 114, 115, 116 and 117 iTRAQ reagent vials were thawed and combined with 70 µl HPLC-grade ethanol. After agitation, 75 µl of the ethanol/label mixtures was added to each 30 µl sample (with labels randomly assigned to samples). The labelled samples were pooled after incubation at room temperature for one hour.

Wool protein samples were labelled with iTRAQ reagents following reduction, alkylation (with acrylamide or MMTS), and tryptic digestion. This procedure resulted in an approximately six-fold sample dilution (maximum starting point 5.3 mg/ml). Samples were further diluted by the addition of 15 µl 1 M TEAB to 20 µl. The protocol was a close adaptation of the manufacturer’s protocol: High grade ethanol (70 µl) was added to thawed 4-plex iTRAQ reagents (ABSciex). The iTRAQ reagents (114, 115, 116 and 117) were randomly assigned to samples, and 75 µl from each reagent was transferred to the individual sample vials. Following vortexing and room temperature
incubation for at least one hour, equal volumes of each sample were combined to generate a pooled iTRAQ-labelled sample. The pooled samples were used for all subsequent analyses.

3.4.3 MS analysis

3.4.3.1 ESI-MS/MS

Model peptide experiments were analysed using direct infusion electrospray ionisation tandem mass spectrometry (ESI-MS/MS). This ionisation method introduces the samples to the mass spectrometer as a liquid. The instrument used was a hybrid quadrupole time-of-flight mass spectrometer (QSTAR Pulsar-i, ABSciex). Due to the simplicity of the model system and the absence of insoluble salts, minimal clean-up was required for iTRAQ-labelled samples only. To remove contaminants utilised during iTRAQ labelling that might interfere with ESI-MS/MS analysis, samples were first diluted 5:7 in water, bound onto a pre-washed (50 % acetonitrile, 1% formic acid) and equilibrated (0.1% formic acid) C18 matrix StageTip (Proxeon Biosystems, Denmark), washed twice with 1% formic acid, and eluted in 50% acetonitrile, 1% formic acid. Duplicate samples were loaded into metal-coated borosilicate capillary needles (medium NanoES spray capillaries, Proxeon) after dilution in 50% acetonitrile, 0.1% formic acid. MS scans were performed at 1 kV capillary voltage between 150-1000 m/z, allowing singly and doubly charged ions from parent and modified peptides to be detected. These scans were run for long periods of ten minutes, allowing the acquisition of accurate MS peak areas for label-free quantitation. Masses seen to appear only in treated samples, and masses that corresponded to putative modifications were selected for MS/MS fragmentation.

Collision-induced fragmentation was performed using nitrogen gas. Collision energies were adjusted manually to enhance fragmentation, permitting de novo sequencing. Data was transformed using Analyst QS v1.1 service pack 7 (ABSciex). Sequencing through manual analysis of immonium ions and overlapping y-, b- and a- fragment ion series allowed the characterisation of modifications; mass changes were localised to specific residues within the peptides. Examples demonstrating the ion series used for peptide sequencing can be seen in Figures 4.1, 5.2, 6.3 and 7.1.

During MS/MS analysis of peptides labelled with iTRAQ reagents, special attention was given to acquiring reporter ion signals. After sufficient fragmentation to enable qualitative characterisation was performed, the MS/MS range was restricted to 100-200 m/z, enhancing the acquisition of report ion signals, and improving iTRAQ reporter ion quantitation.
3.4.3.2 MALDI

Wool protein samples were analysed using MALDI-MS with an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). This ionisation method introduces the samples to the mass spectrometer in a solid form, co-crystallised within a specialised matrix designed to transfer laser energy to the analytes (in this case, α-cyano-4-hydroxycinnamic acid, CHCA). Prior to analysis wool samples had been converted to peptides using enzymatic tryptic digestion. This yielded complex samples containing chemicals incompatible with mass spectrometry. The sample was fractionated, and interfering chemicals eliminated using RP-HPLC. Separation was performed after 1 in 50 dilution into 3% acetonitrile, 0.1% trifluoroacetic acid on a Proxeon Easy nLC (Bruker). Fractions were automatically spotted onto MALDI plates and co-crystallised with CHCA dissolved in a mixture of acetone, ethanol, and ammonium dihydrogen orthophosphate. Solid fractions enable automation of the Ultraflex to acquire MS data of all spots, and subsequent return to perform MS/MS on the masses that pass the search criteria. Using specialised software, MS/MS data was searched against databases of known ovine proteins to provide matches and information on damage-induced modifications. During analysis of samples labelled with iTRAQ reagents, the analytical software was modified to search for peptides modified with iTRAQ and to report the abundance of reporter ions.

3.4.4 Data analysis

Candidate photomodified peptides were initially identified through ESI-MS analysis. Selected ions were then fragmented and sequenced in ESI-MS/MS, using γ-, α- and b-series ions to confirm and locate the modification within the peptide sequence. Ions confirmed by MS/MS as corresponding to oxidatively modified peptides were selected for MS-based profiling. WLR and YLR peptides were observed as [M+H]⁺ peaks, and the larger LLWLR and LLYLR peaks were observed as [M+2H]²⁺ peaks. The relative abundance of peptides over the treatment time course was evaluated by analysis of the ESI-MS ion peak area. To minimise the signal intensity variation from run to run that has been observed to occur even under stringent run conditions, MS peak area data was collected over 20 minutes for each run. Internal spectral normalisation was performed by dividing all peak areas within each MS run by a weighting factor specific to that run. This inverse weighting factor was generated by summing the peak areas of all ions known to be derived from the model peptides (LLWLR + LLWLR+O + LLWLR+2O + ...+ YLR + YLR+O + ... etc.). This enabled runs with higher signal intensity to be directly compared with those of slightly lower signal intensity. Comparisons of relative abundance were restricted to modifications within
individual peptides, e.g. within LLWLR, to account for possible peptide-specific differences in sensitivity in ESI-MS. For ease of presentation, relative abundance scales have been arbitrarily adjusted so that at zero time, the unmodified peptide has a relative abundance of 1.0 (Ehrenshaft et al. 2009).

3.5 Summary

The methodologies selected for this work comprised a collection of preparative chemistry steps, separation techniques, treatment approaches, and sophisticated mass spectrometric technologies. Methods were selected based on the suitability of known approaches for each step, and adapted accordingly if necessary.

By combining this selection of new and established methodologies on this project, novel approaches to the quantification of degradation products of model peptides and intermediate filament proteins (IFPs) were developed during the course of this research. This enabled the generation of novel data regarding the effects of oxidative processes (UV irradiation and hydrothermal exposure) on peptides and proteins at the molecular level.
4 Profiling of residue-level photo-oxidative damage in peptides

4.1 Abstract

Protein and peptide oxidation is a key feature in the progression of a variety of disease states and in the poor performance of protein-based products. The present work demonstrates a mass spectrometry-based approach to profiling degradation at the amino acid residue level. Synthetic peptides containing the photosensitive residues, tryptophan and tyrosine, were used as models for protein-bound residue photodegradation. ESI-MS/MS was utilised to characterise and provide relative quantitative information on the formation of photoproducts localised to specific residues, including the characterisation of low abundance photomodifications not previously reported, including W+4O modification, hydroxy-bis-tryptophandione and topaquinone. Other photoproducts observed were consistent with the formation of tyrosine-derived dihydroxyphenylalanine (dopa), trihydroxyphenylalanine, dopa-quinone and nitrotyrosine, and tryptophan-derived hydroxytryptophan, dihydroxytryptophan/N-formylkynurenine, kynurenine, hydroxyformylkynurenine, tryptophandiones, tetrahydro-β-carboline and nitrotryptophan. This approach combined product identification and abundance tracking to generate a photodegradation profile of the model system. The profile of products formed yields information on formative mechanisms. Profiling of product formation offers new routes to identify damage markers for use in tracking and controlling oxidative damage to polypeptides.

4.2 Introduction

Oxidative damage to proteins and peptides correlates to an extensive range of degenerative conditions (Fu et al. 1998; Shacter 2000; Linton et al. 2001; Lee et al. 2004). Photo-oxidative damage occurs when proteins are exposed to UV radiation, typically triggering the generation of reactive oxygen species (ROS) in the presence of oxygen (Girotti & Giacomoni 2007), and is of particular importance in biological systems such as skin (Kato et al. 1992; Girotti & Giacomoni 2007), hair and eyes (Davies & Truscott 2001; Parker et al. 2004), as well as in protein-based end-products of commercial significance, such as wool (Davidson 1996), silk, milk (Dalsgaard et al. 2007) and meat (Møller et al. 2002). This damage can be characterised at the protein primary level via the analysis of residue modifications.
Typically, the most photosensitive residues are aromatic and sulfur-containing amino acids (Berlett & Stadtman 1997). With respect to photomodifications leading to discoloration in proteins, tryptophan and tyrosine photoproducts have been observed to be the chief contributors, with a cascade of oxidation products forming progressively more coloured chromophores (Dyer et al. 2005; Bringans et al. 2006; Dyer et al. 2006a; Dyer et al. 2006b).

An important aspect of polypeptide photodegradation involves the contribution of photosensitisers, which produce ROS via Type I (radical species generation via energy transfer to a neighbouring substrate) or Type II (singlet oxygen generation via energy transfer to molecular oxygen) reactions. Residues such as tryptophan and its photoproduct, kynurenine (Grossweiner 1984; Parker et al. 2004; Igarashi et al. 2007; Mizdrak et al. 2008), yield ROS in the presence of UV light, contributing to the further degradation of residues sensitive to oxidative degradation. The effects of photo-oxidative protein damage therefore extend beyond modifications to residues that absorb in the UV region, making the photodegradative protein profile particularly complex.

When considering the biological or commercial significance of residue degradation, the relative abundance of specific photoproducts is of particular importance. Profiling and tracking key residue-level photoproducts within peptides and proteins provides a means to understand and control photodegradative mechanisms (Davies et al. 1999; Gracanin et al. 2009). Both an enhanced understanding of protein photodegradative mechanisms through the characterisation of low abundance photomodifications, and also the development of protocols to examine the pattern of these modifications over the course of exposure, are important goals in protein research.

This chapter reports the characterisation of tryptophan and tyrosine residue modifications resulting from UVB and UVA irradiation of model peptides. Model peptide systems are ideal for characterising peptide-bound amino acid behaviour without the complexity of protein analysis (Kato et al. 1992; Kotiaho et al. 2000; Metz et al. 2004). The development and application of an MS-based approach to establishing relative modification abundance patterns is also outlined. This MS-based profiling has allowed observation of the progressive degradation of parent peptides and the corresponding formation of photomodifications through the changes in their relative abundance over the course of irradiation.

It is anticipated that linking of this new residue-level information to damage observed at higher orders of protein and cellular structure will facilitate powerful new strategies for understanding and controlling protein damage. This work represents important progress towards the robust
evaluation of oxidative damage levels through tracking of specific photoproducts and profiling the relative contribution of these to protein damage.

4.3 Methodology

4.3.1 Materials

Synthetic peptides, Leu-Leu-Tyr-Leu-Arg-OH (LLYLR) and Leu-Leu-Trp-Leu-Arg-OH (LLWLR) were obtained as lyophilised powdered hydrochlorides from The Biopeptide Company (San Diego, CA). MS analysis confirmed the presence of these peptides, along with the truncations WLR and YLR. ChromAR® LC-grade water and LC-grade acetonitrile from J.T. Baker (Phillipsburg, NJ); Univar formic acid from Ajax Finechem (Auckland, NZ); and NanoES spray capillaries from Proxeon Biosystems.

4.3.2 Irradiation

Triplicate aqueous tyrosine- and tryptophan-containing peptide solutions were prepared by the addition of LC-grade water to the lyophilised powders at 2 mg/ml (yielding solutions of pH 3.0-3.2). These were combined and placed at room temperature in UV-transparent quartz test tubes with gentle agitation on a carousel in a vented photo-irradiator (LZC4-14, LuzChem) lit from above and three sides with low heat emission UVA and UVB LZC narrow bandwidth fluorescent lamps. The sample tubes were sealed with UV-transparent polyethylene film to prevent evaporation and the sample depth was approximately 1 cm. The spectral distribution peaks ranged over approximately 281-360 nm for the UVB (partially extending into the UVA range) and 316-400 nm for the UVA lamps, with a maximum intensity of 52670 mW.m⁻² (Luzchem 2004a; 2004b). Irradiation was performed over 12 hours (UVB) and 24 hours (UVA). No pH change was observed after irradiation. Sample aliquots were removed and stored in the dark at -85°C at zero time and at various time-points throughout the treatment.

4.3.3 Mass spectrometry

Sample aliquots of aqueous irradiated peptide solutions were prepared for ESI-MS by dilution to 10% or 20% in 50% acetonitrile, 0.1% formic acid. Mass spectrometric analysis was performed in duplicate for each subsample on a tandem quadrupole time-of-flight (Qq-TOF) mass spectrometer (QSTAR Pulsar-i, ABlsciex), utilising direct infusion nanospray delivery of samples. Ions with m/z ratios corresponding to photo-oxidised peptides were subjected to collision-induced dissociation,
utilising nitrogen gas, with collision energies optimised for peptide fragmentation. Characterisation was performed manually through analysis of immonium ions and overlapping y, b and a fragment ion series with Analyst QS v1.1 service pack 7 (ABSciex).

4.3.4 Data analysis

Candidate photomodified peptides were initially identified through ESI-MS analysis. Selected ions were then fragmented and sequenced in ESI-MS/MS, using y-, a- and b-series ions to confirm and locate the modification within the peptide sequence. Ions confirmed by MS/MS as corresponding to oxidatively modified peptides were selected for MS-based profiling. WLR and YLR peptides were observed as [M+H]⁺ peaks, and the larger LLWLR and LLYLR peaks were observed as [M+2H]²⁺ peaks. The relative abundance of peptides over the treatment time course was evaluated by analysis of the ESI-MS ion peak area. To minimise the signal intensity variation from run to run that has been observed to occur even under stringent run conditions, MS peak area data was collected over 20 minutes for each run. Internal spectral normalisation was performed by dividing all peak areas within each MS run by a weighting factor specific to that run. This inverse weighting factor was generated by summing the peak areas of all ions known to be derived from the model peptides (LLWLR + LLWLR+O + LLWLR+2O + ...+ YLR + YLR+O + ... etc.). This enabled runs with higher signal intensity to be directly compared with those of slightly lower signal intensity. Comparisons of relative abundance were restricted to modifications within individual peptides, e.g. within LLWLR, to account for possible peptide-specific differences in sensitivity in ESI-MS. For ease of presentation, relative abundance scales have been arbitrarily adjusted so that at zero time, the unmodified peptide has a relative abundance of 1.0 (Ehrenshaft et al. 2009).

4.4 Results

4.4.1.1 Observed photomodifications

Residue modifications were characterised through comprehensive manual evaluation of ESI-MS and MS/MS ion data. The formation of a wide range of tryptophan and tyrosine residue modifications within the model peptides was observed, as summarised in Table 4.1.
Table 4.1  Observed photomodifications to tyrosine and tryptophan residues

<table>
<thead>
<tr>
<th>Δm/z</th>
<th>Modification</th>
<th>Corresponding photoproduct(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tyrosine</td>
</tr>
<tr>
<td>+16</td>
<td>+ O</td>
<td>dopa / dienone alcohol</td>
</tr>
<tr>
<td>+32</td>
<td>+ 2O</td>
<td>topa</td>
</tr>
<tr>
<td>+14</td>
<td>+ O - 2H</td>
<td>dopa-quinone</td>
</tr>
<tr>
<td>+30</td>
<td>+ 2O - 2H</td>
<td>topa-quinone</td>
</tr>
<tr>
<td>+45</td>
<td>+ N + 2O - H</td>
<td>nitrotyrosine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tryptophan</td>
</tr>
<tr>
<td>+16</td>
<td>+ O</td>
<td>hydroxytryptophan / oxindolylalanine</td>
</tr>
<tr>
<td>+32</td>
<td>+ 2O</td>
<td>NFK / dihydroxytryptophan</td>
</tr>
<tr>
<td>+4</td>
<td>+O - C</td>
<td>kynurenine</td>
</tr>
<tr>
<td>+20</td>
<td>+ 2O - C</td>
<td>hydroxykynurenine</td>
</tr>
<tr>
<td>+48</td>
<td>+ 3O</td>
<td>hydroxyformylkynurenine</td>
</tr>
<tr>
<td>+64</td>
<td>+ 4O</td>
<td>dihydroxyformylkynurenine</td>
</tr>
<tr>
<td>+30</td>
<td>+ 2O - 2H</td>
<td>tryptophandione / dihydrodioxoindole</td>
</tr>
<tr>
<td>+28</td>
<td>+ 2O - 4H</td>
<td>β-unsaturated-2,4-bis-tryptophandione</td>
</tr>
<tr>
<td>+44</td>
<td>+ 3O - 4H</td>
<td>hydroxy-bis-tryptophandione</td>
</tr>
<tr>
<td>+12</td>
<td>+ C</td>
<td>tetrahydro-β-carboline</td>
</tr>
<tr>
<td>+45</td>
<td>+ N + 2O - H</td>
<td>nitrotryptophan</td>
</tr>
</tbody>
</table>

4.4.1.2  Tyrosine

Several modifications to tyrosine residues were characterised in model peptides by detailed analysis of MS/MS fragmentation patterns (See the Discussion section and Figure 4.6). These
modifications were observed in peptides irradiated both with UVA and UVB, but were generally sequenced more readily in UVB samples, where the relative abundance of the photoproducts was generally higher. This is attributed to greater production of ROS due to the higher energy of short wavelength UVB.

After irradiation, complementary ESI-MS/MS fragment ion series confirmed the presence of Y+O modification. Ions consistent with the formation of doubly hydroxylated tyrosine (Y+2O) were also observed. Overlapping y-, a- and b-ion series confirmed Y+14 Da (probably Y+O-2H) modification. Additionally, MS/MS fragmentation produced overlapping y-, a- and b-ion series and Y+30 Da immonium ions after both UVA and UVB irradiation. This tyrosine modification is consistent with Y+2O-2H. Ions were detected in MS at 722.4 \( m/z \) (UVB irradiation only) and were confirmed through MS/MS fragmentation to correspond to Y+45 modifications (Y+NO₂-H).

4.4.1.3  Tryptophan

UVA and UVB irradiation also yielded a range of products derived from tryptophan-containing peptides, as characterised by ESI-MS/MS (See the Discussion section and Figure 4.7).

ESI-MS and MS/MS fragmentation revealed the presence of W+16 Da (W+O) and W+32 Da (W+2O) modifications (see Figure 4.1). ESI-MS/MS spectra of these modifications are shown for LLW*LR in Figure 4.1 to demonstrate their characterisation and localisation within the peptide. Ions of \( m/z \) 704.4 were detected in MS, corresponding to M+4 Da LLW*LR peptides. MS/MS fragmentation yielded ion series confirming W+4 Da (W+O-C) modification. MS/MS analysis revealed complete y- and overlapping a- and/or b-ion series and ions consistent with W+48 Da (W+3O) immonium ions. W+64 Da ions (W+4O) were also confirmed in MS/MS, with the observation of a W+64 Da immonium ion and overlapping a-, b- and y-ion series in both LLW*LR and W*LR. W+20 Da ions (W+2O-C) were also detected in MS for LLW*LR. Fragmentation yielded complete y-ion and overlapping a- and b-ion series and W+20 immonium ions.

Ions corresponding to M+30 Da were observed in ESI-MS. On fragmentation, these yielded complete or overlapping y-, a- and b-ion series and immonium ions for W+30 Da (W+2O-2H). The presence of W+28 Da ions (consistent with W+2O-4H) was demonstrated by the detection in MS/MS of overlapping or complete y-, a- and b-ion fragmentation series. W+44 Da ions for LLW*LR were also detected in MS and confirmed in MS/MS via the observation of ions consistent with W+44 Da immonium ions and overlapping y-, a- and b-ion series. Ions consistent with W+12 Da (W+C) modification were identified in MS/MS for peptides W*LR and LLW*LR, with the observation of complete and partial y-, a- and b-ion series and W+12 Da immonium ions.
MS/MS fragment ions consistent with the formation of W+45 Da (W+N+2O-H) were detected and confirmed in MS/MS with complete y-ion series and overlapping a- and b-series ions.

Figure 4.1  ESI-MS/MS spectra of LLWLR-derived ions: a) 0 h UVB, no W modification; b) 10 h UVB, W+O modification; c) 10 h UVB, W+2O modification. The y-, b- and immonium fragment ions are highlighted.

4.4.1.4  Profiling

ESI-MS/MS permits the characterisation of residue modifications and comparisons of the abundance of closely related compounds via their relative ion peak areas. The relative abundance of modifications to a particular peptide may be compared within a single MS scan, assuming that
residue-level oxidative peptide modifications have a relatively minor effect on ionisation due to their similar physicochemical properties (Mann 1999; Lu et al. 2004).

Variations in the intensity of signal from run to run, however, make direct comparisons of abundance over separate samples challenging. To enable the MS-based comparison of relative abundance over time, each MS spectrum was normalised to all other spectra by means of the peak areas of ions derived from the model peptides (see Section 4.3.4). The sum of peptide-derived ions served as an internal standard, spread over the $m/z$ range of the target ions. This approach allowed profiling of the formation of residue modifications during exposure to UV.

Mass spectrometric evaluation of peptide samples not exposed to light showed ions with $m/z$ ratios corresponding to the unmodified peptides and trace levels of oxidised peptides. After irradiation, MS analysis demonstrated the appearance of ions consistent with photo-oxidative modification of the target residues. The most abundant modifications were consistent with the addition of one or two oxygen atoms to tryptophan residues, attributed to the formation of hydroxytryptophan (W+O) and NFK and/or dihydroxytryptophan (W+2O), respectively (see Section 4.5.2 and structures in Figure 4.7). Peptides containing tryptophan residues (Figures 4.2 and 4.3) were observed to be modified considerably more rapidly than those containing tyrosine residues (Figures 4.4 and 4.5), as evidenced by greater and more definite decreases in parent peptides and larger increases in modified products.

Tryptophan modifications in LLWLR were observed to be present at very low abundance before irradiation, and to form rapidly when exposed to UVA and UVB. The UVA time-course was performed over twice as long a time-period as the UVB time-course to facilitate production of observable modification levels. After 12 hours UVB irradiation, LLWLR and WLR W+O (hydroxytryptophan) photoproducts were observed to decrease.
Figure 4.2  Relative peak areas of A) LLWLR and B) WLR and photomodifications of these after UVA irradiation: 1) W+O; 2) W+2O; 3) W+O-C; 4) W+3O; 5) W+4O; 6) W+2O-C; 7) W+2O-2H; 8) W+2O-4H; 9) W+3O-4H; 10) W+C; 11) W+N+2O-H. Spectra peak areas are internally weighted, and the relative abundance of the peak area of the unmodified peptide at zero time is defined as 1.0. Error bars represent standard errors of the means (±SEM).
Figure 4.3  Relative peak areas of A) LLWLR and B) WLR and photomodifications of these after UVB irradiation: 1) W+O; 2) W+2O; 3) W+O-C; 4) W+3O; 5) W+4O; 6) W+2O-C; 7) W+2O-2H; 8) W+2O-4H; 9) W+3O-4H; 10) W+C; 11) W+N+2O-H. Spectra peak areas are internally weighted, and the relative abundance of the peak area of the unmodified peptide at zero time is defined as 1.0. Error bars represent standard errors of the means (±SEM).
Figure 4.4  Relative peak areas of photomodifications of A) LLYLR and B) YLR after UVA irradiation: 1) Y+O; 2) Y+2O; 3) Y+O-2H; 4) Y+2O-2H; 5) Y+N+2O-H. Spectra peak areas are internally weighted, and the relative abundance of the peak area of the unmodified peptide at zero time is defined as 1.0. Error bars represent standard errors of the means (±SEM).

Figure 4.5  Relative peak areas of photomodifications of A) LLYLR and B) YLR after UVB irradiation: 1) Y+O; 2) Y+2O; 3) Y+O-2H; 4) Y+2O-2H; 5) Y+N+2O-H. Spectra peak areas are internally weighted, and the relative abundance of the peak area of the unmodified peptide at zero time is defined as 1.0. Error bars represent standard errors of the means (±SEM).
4.5  Discussion

4.5.1  General

A mixture of short, arginine-terminated synthetic peptides (sequence LLXLR and XLR, where X = W or Y) was selected for this work. The inclusion of an arginine residue at the C-terminus was designed to provide analogy to peptides derived from tryptic digestion, while the relatively short peptide length favours the formation of ions in a low charge state (Chowdhury et al. 1990). The aromatic residues, flanked by relatively photostable leucine residues, provided a good model system for examining oxidative modifications to internal and terminal residues. A high peptide concentration of 2 mg/ml was utilised to amplify the products of photosensitisers such as kynurenine formed from tryptophan photodegradation and to simplify the characterisation and profiling of very low abundance residue modifications in ESI-MS.

Tryptophan and tyrosine residues were selected due to their susceptibility to photo-oxidative damage (Davidson 1996) and biological and commercial relevance. Tyrosine yields chromophoric photoproducts, which contribute to the degeneration of proteins in commercially important materials such as textiles (Goddinger et al. 1994; Bringans et al. 2006; Dyer et al. 2006a; Dyer et al. 2006b) and in biological tissues such as lenses (Wells-Knecht et al. 1993; Linton et al. 2001; Parker et al. 2004) and atherogenic plaques (Fu et al. 1998). Tryptophan, also implicated in lens discoloration (Hains & Truscott 2007) and age-related disease (Batthyány et al. 2000) has been strongly associated with protein degradation leading to protein product performance degeneration, particularly photoyellowing (Lennox et al. 1966). Free and bound tryptophan have been found to produce a variety of products upon oxidation (Simat & Steinhart 1998; Davies 2004). The peptide solutions were irradiated with well-defined wavelength bands in the UVA and UVB ranges.

4.5.2  Characterisation

Characterisation of residue-level modifications to peptides and proteins provides insight into the agents and mechanisms of oxidative damage. This will lead to more targeted approaches to ameliorating their effects, both in commercially valuable materials and living systems. The identification of the species formed during photodegradation leads to increased understanding of the roles of even low abundance modifications in, for example, photoyellowing. Even low quantities of highly chromophoric species such as nitrotryptophans (King & Lawrence 1995;
Yamakura et al. 2005; Dyer et al. 2006b) and kynurenines (Tokuyama et al. 1967; Bringans et al. 2006) may contribute extensively to observable colour changes.

MS/MS analysis of tryptophan- and tyrosine-containing peptides after irradiation resulted in the characterisation of a wide range of residue modifications. Their structures and proposed pathways of formation are represented in Figures 4.6 and 4.7. Tyrosine and tryptophan photoproducts characterised by MS/MS implicate the hydroxyl radical as the predominant ROS contributing to UV-induced residue modification in this model peptide system. Singlet oxygen and a reactive nitrogen species, probably peroxynitrite, were also implicated as contributing to photo-oxidative damage.

Y+O residue modification is consistent with the formation of dihydroxyphenylalanine (dopa) (Goddinger et al. 1994; Parker et al. 2004; Mizdrak et al. 2008), or a C-1 alcohol. Dopa, along with dityrosine, is a characteristic marker of hydroxyl radical-related damage (Dean et al. 1993; Gieseg et al. 1993; Nappi et al. 1995; Wright et al. 2002). Singlet oxygen-mediated oxidation, on the other hand, may form a C-1 hydroperoxide from tyrosine residues, which decomposes to the corresponding dienone alcohol (also Y+O) (Davies 2003; Davies 2004). The oxidative products of this alcohol are not well characterised. In free or N-terminal tyrosine, Y+O may also correspond to the singlet-oxygen mediated 3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indol-2-carboxylic acid (HOHICA) (Davies 2003; Davies 2004). Both hydroxyl radicals and singlet oxygen can be formed in biological systems during irradiation (Guptasarma et al. 1992). The characterisation of Y+2O modification, consistent with the photoformation of hydroxylated dopa, trihydroxyphenylalanine (topa) (Dean et al. 1993; Ogata 2007), provides evidence for the involvement of the hydroxyl radical. Further products consistent with the initial formation of dopa were characterised: Y+14 modification, most likely Y+O-2H, is consistent with the formation of a dopa-derived quinone, which can be formed by hydroxyl radical-mediated nucleophilic attack on the phenol ring (Dean et al. 1993; Gieseg et al. 1993; Nappi et al. 1995); and Y+30 topaquinone (Y+2O-2H), probably a hydroxylation product of dopaquinone or the product of a hydroxyl radical-mediated nucleophilic addition to topa (Nappi et al. 1995). Free topa can be easily oxidised to topaquinone (Rosenberg et al. 1991; Rescigno et al. 1998), which is a known cofactor in enzymes such as amine oxidases (Wilce et al. 1997), and is involved in the melanin biosynthesis pathway (Canovas et al. 1982), but this is the first time its photoformation in peptides has been described.

With respect to protein photoyellowing, it is of note that topa oxidation products are an orange-red colour (Rosenberg 1991), and may therefore contribute towards photoyellowing. These
secondary and tertiary oxidation products of dopa lend support for the role of the hydroxyl radical in the formation of photomodifications in peptides, particularly for tyrosine.

W+O modification is consistent with the hydroxylation of tryptophan to form hydroxytryptophan (Finley et al. 1998; Simat & Steinhart 1998; Dyer et al. 2006a; Dyer et al. 2006b) (or its isomer, oxindolylalanine (Van de Weert et al. 1998)), the formation of which has been observed in free amino acids, peptides and proteins, and also suggests the involvement of hydroxyl radicals (Maskos et al. 1992). Tryptophan modification of +2O is attributed to two major oxidative products in peptides; N-formylkynurenine (NFK) (Balasubramanian et al. 1990; Guptasarma et al. 1992; Simat & Steinhart 1998; Kotiaho et al. 2000; Linton et al. 2001; Davies 2004) and dihydroxytryptophan (Domingues et al. 2003; Yamakura et al. 2005). Dihydroxytryptophan formation is consistent with the hydroxylation of hydroxytryptophan (Domingues et al. 2003). NFK may be formed though cleavage of the pyrrole ring via the hydroxyl radical (Maskos et al. 1992; Domingues et al. 2003; Dyer et al. 2006b) or singlet oxygen-mediated (Gracanin et al. 2009) formation (and subsequent decomposition) of a C3-position tryptophan hydroperoxide or a dioxetane at the C2-C3 bond (Gracanin et al. 2009). It is probable that the W+2O products detected in these model peptides represent a mixture of dihydroxytryptophan and NFK. MS/MS characterisation of a W+4 product that probably corresponds to W+2O-CO is consistent with the presence of kynurenine, which is formed by the hydrolysis of NFK (Finley et al. 1998; Davies & Truscott 2001).

The MS/MS characterisation of W+48 Da and W+64 Da modifications, respectively, indicates the addition of three and four oxygen atoms. The addition of three oxygen atoms implicates the formation of the coloured photoproduct, hydroxyformylkynurenine (Nakagawa et al. 1985; Bienvenut et al. 2002). The work by Nakagawa et al. featured methylene blue-sensitised oxidation, which supports the involvement of singlet oxygen, but methylene blue has also been demonstrated to generate hydroxyl radicals (Buettner et al. 1984). The addition of four oxygen atoms to a single tryptophan residue may represent dihydroxyformylkynurenine, probably formed from hydroxyformylkynurenine. The formation of a W+4O oxidation product has not been reported previously. A further possible derivative of hydroxyformylkynurenine was characterised in the form of a W+2O Da modification, consistent with W+2O-C (hydroxykynurenine), formed via the loss of a carboxyl group from hydroxyformylkynurenine or hydroxylation of kynurenine (Schäfer et al. 1997; Bienvenut et al. 2002).

Support for the inclusion of dihydroxytryptophan in the assignment of the W+2O modification is provided by the characterisation of W+30 Da and W+28 Da modifications. W+30 Da modification
(probably W+O-2H) is consistent with the formation of a tryptophandione (Dyer et al. 2006b), probably a tryptophylquinone (Ozaki et al. 2001; Hyun & Davidson 2002) or its isomer, 2,6-dihydro-2,6-dioindole (Hara et al. 2001; Pfister et al. 2005). Tryptophandiones may be formed via hydroxytryptophan oxidation (Wu & Dryhurst 1996; Hara et al. 2001; Klarskov et al. 2003). Further reduction of this product may lead to the formation of W+2O-4H, β-unsaturated-2,4-bis-tryptophandione; this mass change was previously noted by our group in irradiated wool proteins (Dyer et al. 2006b). This product may itself be hydroxylated to form a hydroxy-bis-tryptophandione, explaining the observation of W+44 Da (W+3O-4H) products. These observations provide confirmation in a simple model system of the tryptophandione and β-unsaturated-2,4-bis-tryptophandione products previously detected in photoyellowed wool proteins (Dyer et al. 2006b).

Tryptophan has been reported, under certain conditions, to undergo cyclisation via condensation with α-keto acids (notably formed during protein irradiation (Meybeck & Meybeck)), to form yellow (fluorescent) β-carbolines (Dillon et al. 1976; Schäfer et al. 1997), such as tetrahydro-β-carboline (Lippke et al. 1983), characterised by a +C mass addition. Ions consistent with this modification were identified by ESI-MS/MS in this study, providing supporting evidence for the contribution of carbolines to the protein photo-oxidation profile.

The identification of X+45 Da modifications on the tryptophan- and tyrosine-containing peptides, consistent with W+NO2-H (nitrotryptophan) and Y+NO2-H (nitrotyrosine) formation, suggests the formation and attack of a reactive nitrogen species (RNS) such as peroxynitrite. Protein-bound nitrotryptophan and nitrotyrosine have previously been observed to form in the presence of potent RNS (Ischiropoulos & Al-Mehdi 1995; Alvarez et al. 1996; Yamakura et al. 2001; Abello et al. 2009). The observation of both nitrotyrosine and nitrotryptophan is of considerable interest, as this provides strong supporting evidence for the contribution of nitration to protein photo-oxidative degradation, as suggested in recent studies on collagen and keratin photoyellowing (Dyer et al. 2006b; Dyer et al. 2009). The mechanism of RNS formation requires further studies and clarification; however, RNS could conceivably be formed via ammonia, which is released during UV degradation of peptides and amino acids (Inglis & Lennox 1963; Reubsaet et al. 1998). UV irradiation of ammonia in solution results in the formation of nitrites and nitrates (Zheng et al. 1998), along with a decrease in pH (Beckles & Diyamandoglu 2006). Peroxynitrite is a primary UV photoproduc of nitrates (Plumb & Edwards 1992). Other RNS that may cause tyrosine and tryptophan nitration include NO2⁻ radicals, nitric oxide, nitrogen dioxide and nitrous acid (Abello et al. 2009).
Figure 4.6  Proposed pathways of tyrosine degradation to photoproducts
Figure 4.7  Proposed pathways of tryptophan degradation to photoproducts.
The photoproducts characterised here in this model aqueous system have expanded our understanding of the behaviour of peptide-bound aromatic residues during UV irradiation. The variety of photoproducts formed indicates the contribution of more than one ROS, and particularly implicate hydroxyl radicals, along with singlet oxygen and peroxynitrite.

4.5.3 Profiling

MS-based abundance profiling allowed the formation of individual modifications to peptides to be monitored over time. This demonstrated the degradation of peptides containing the aromatic residues, tryptophan and tyrosine, and the progressive formation of residue modifications within those peptides. Both major and minor photomodifications to Y and W (as confirmed in MS/MS, Table 4.1) were profiled.

A primary application of modification profiling is its use in identifying appropriate peptide candidates to function as markers of the extent of oxidative damage in biological systems. A good candidate would be relatively abundant, compatible with LC-MS/MS analysis and would display sufficient sensitivity to the relevant insult, in this case UV irradiation. In complex biological systems, this candidate would also need to be reproducibly extractable from the substrate. In this model system, the major photoproducts were W+O and W+2O, and so the formation of these was the most easily tracked, with distinct trends in abundance evident over time (Figures 4.2 and 4.3, products 1 and 2).

MS/MS-based characterisation allowed the localisation of photoproducts present at very low abundance to specific residues within peptides. For using characterisation as an indicator of the contribution of a modification to the total modification profile, this is potentially both a strength and a weakness, as, while complex mixtures of photoproducts can be characterised, successful characterisation is not well correlated with the abundance of those products within the mixture. This was demonstrated by the tyrosine degradation profiles; sensitive MS/MS characterisation confirmed the presence of tyrosine photoproducts (indicating the degradation of tyrosine residues). Abundance profiling, however, shows the majority of these well-characterised tyrosine photoproducts to be present at very low abundance (evidenced by maximum product levels only 10% as abundant as unmodified parent at zero time, and by large measurement errors), to the point that most are probably unsuitable for use as quantitative damage markers using this technique (Figures 4.4 and 4.5). Further development and refinement of this quantitative approach may enable sufficient reproducibility of such low abundance photoproducts to permit their use as markers of oxidative damage.
Tryptophan modifications were much more abundant, and were observed to clearly increase in relative abundance over the course of irradiation, until (after UVB irradiation) a slight reduction in the relative abundance of the major oxidative products (W+O and W+2O) was observed (Figure 4.3). This is consistent with the oxidative degradation of these primary photoproducts to secondary and tertiary products such as kynurenine (W+O-C, product 3) and hydroxyformylkynurenine (W+3O, product 4). The higher abundance of these tryptophan photoproducts makes them ideal markers of photo-oxidative damage to these peptides. Tryptophan products were expected to be produced more abundantly than tyrosine photoproducts under irradiation, due to the higher sensitivity of tryptophan residues to irradiation and photooxidation (Igarashi et al. 2007; Kerwin & Remmele 2007).

UVB irradiation was observed to generate proportionately more advanced (secondary and tertiary) photoproducts (such as product 4: W+3O, and product 5: W+4O) than UVA, in both tryptophan- and tyrosine-containing peptides. This was particularly noticeable in the short peptide, WLR (Figures 4.2 and 4.3). It is interesting to note that location of the tryptophan residue on the C-terminal appears to promote enhanced formation of specific photoproducts, notably W+O and W+2O photoproducts. Compare Figure 4.3a (LLWLR) with Figure 4.3b (WLR).

The abundance profiling of specific modifications also sheds light on potential sequences of formation: For instance, the most abundant photomodification corresponded to W+O (hydroxytryptophan), which strongly implicates attack of the hydroxyl radical as the key mechanism of photo-oxidation in this aqueous system (Maskos et al. 1992). The next most abundant modification corresponded to the addition of two oxygen atoms to Trp, probably due to dihydroxytryptophan (formed via hydroxyl radical (Domingues et al. 2003)) and/or NFK (formed via singlet oxygen (Gracanin et al. 2009) or hydroxyl radical attack (Maskos et al. 1992; Domingues et al. 2003)).

This aqueous peptide model system allowed the development and validation of a relatively straightforward MS-based approach to profiling the formation of specific residue modifications in peptides and proteins. Co-injection of parent and product species allowed the direct comparison of ion signals, enabling informative contrasts of the abundances of products arising from each peptide. In this approach ion abundance data is presented informatively, with each data point only representing one ion, rather than as ion proportions, e.g. oxidised over unmodified species (Schey et al. 2000), so that changes in the abundance of one species do not affect the representation of another. Internal weighting was used as an alternative to the use of a single standard (Gracanin et al. 2009), where a similar but unweighted approach only allows
quantitative comparison between modified species, not across time-points (between samples). Quantitative comparisons across samples are crucial to visualising the rates of product formation and in gaining insights into the pathways of formation of specific products.

4.6 Conclusions

The characterisation and relative quantification of residue-specific photoproducts in model peptides as described here has provided valuable information on UVA and UVB-induced photomodifications of aromatic residues. The development of a label-free protocol for relative abundance tracking in peptides holds potential for studies of protein residue degradation, such as in redox proteomics, where it is expected to enable improved MS-based modification analysis.

Photomodifications to tryptophan and tyrosine were comprehensively characterised. This included the first direct identification of W+4O modification (possibly dihydroxylformylkynurenine), hydroxy-bis-tryptophandione and topaquinone within irradiated peptides. The photoformation of nitrotryptophan and nitrotyrosine was also confirmed. The characterisation of nitrated aromatics is of particular significance, as it implicates the involvement of an RNS in protein photodegradation, which may be formed through the reaction of ROS with the products of peptide breakdown. The range of products characterised also implicates the hydroxyl radical, in particular, as the key ROS responsible for protein photo-oxidation of aromatic residues in aqueous systems.

This work has developed and validated a relatively straightforward approach to the profiling of residue modification in peptides. The profiling involved visualising modifications to target residues over time in terms of both the pattern of generated species and their respective changes in relative abundance. It is envisaged that this will provide an important set of tools for establishing oxidative mechanisms and routes of damage formation. Further development of these tools will also facilitate the identification and tracking of specific markers of protein degradation in complex biological systems.
Isobaric labelling approach to the tracking and relative quantitation of peptide damage at the primary structural level

5.1 Abstract

Protein oxidative damage lies behind skin and hair degradation and the deterioration of protein-based products, such as wool and meat, in addition to a range of serious health problems. Effective strategies to ameliorate degenerative processes require detailed fundamental knowledge of the chemistry at the molecular level, including specific residue-level products and their relative abundance. Here a new means of tracking damage-induced side-chain modification in peptides is presented using a novel application for isobaric label quantification. Following exposure to elevated temperature, UVA and UVB radiation, tryptophan and tyrosine damage products in synthetic peptides were characterised and tracked using ESI-MS/MS and iTRAQ labelling-based relative quantification. An in-depth degradation profile of these peptides was generated, enabling the formation of even low abundance single residue-level modifications to be sensitively monitored. The development of this novel approach to profiling and tracking residue-level protein damage offers significant potential for application in the development and validation of protein protection treatments.

5.2 Introduction

The degradation of amino acid residues within proteins is a critical concern across a broad spectrum of substrates. Protein damage is an important component in the development of a range of diseases, such as cataracts (Hains & Truscott 2007), atherosclerosis (Fu et al. 1998) and ageing (Linton et al. 2001), and in the processing- and age-related deterioration of protein-based materials such as hair (Nogueira et al. 2006), skin (Trautinger 2007), wool (Davidson 1996), silk (Leaver & Ramsay 1969), milk (Dalsgaard et al. 2007) and meat (Møller et al. 2002). Accordingly, many of the residue modifications associated with protein damage have been qualitatively characterised using techniques such as Raman spectroscopy (Osticioli et al. 2008) and MS (Ronsein et al. 2009). Nevertheless, at present, holistic assays (such as the detection of carbonyl groups (Levine et al. 1994; Cabisco et al. 2000), assays for specific oxidation products (Heinecke
et al. 1993; Shacter 2000), or physical changes in the substrate (Reubsaet et al. 1998; Yuen et al. 2007)), which suffer from a lack of specificity and an inability to localise damage to specific proteins (Halliwell & Whiteman 2004), remain the predominant methods for quantifying protein damage. Residue-level changes, which can impart significant changes in protein functionality, can be induced by exposure to heat (Asquith & Otterburn 1970), light (Kerwin & Remmele 2007), cellular sensitisers (Linton et al. 2001), oxidants (Criado et al. 2008), and acids (Davies 2005), and are therefore important in both intra- and extra-cellular systems. The impact of residue-level damage on biological and product systems and the expanding field of redox proteomics demand accurate and sensitive tools for profiling and tracking damage at the molecular level.

This chapter presents a novel utilisation of isobaric labelling for tracking protein damage, which utilises the accuracy of mass spectrometry (MS) for qualitative and quantitative characterisation of residue damage. Isobaric labelling has been developed for the mass spectrometric comparative quantitation of proteins (or of cellular post-translational modifications such as phosphorylation), via the labelling and pooling of peptides or proteins with isomer-balanced chemicals so that differentially-labelled peptides have the same mass and chemical composition. Isomer differences are revealed during MS/MS fragmentation, allowing relative quantitation between samples (Ross et al. 2004). The primary isobaric labelling system currently employed is iTRAQ labelling (Aggarwal et al. 2005), which enables labelling of up to eight samples with isobaric tags; other systems reported include tandem mass tags (Thompson et al. 2003) and isobaric peptide termini labelling (Koehler et al. 2009). In shotgun proteomics (Aebersold & Mann 2003) the relative abundance of several peptides, as determined by the peak areas of the iTRAQ reporter ions, is used to determine the abundance of their parent proteins (Aggarwal et al. 2006). Protein quantitation thus uses quantitative data from several peptides to make an estimation of protein abundance. This chapter details a novel additional application for stable isotope labelling: the quantitation of low abundance degradative modifications in individual peptides. This approach requires sufficiently accurate quantitative information to be obtained from a single peptide variant to allow observation of the differences between samples, necessitating a modified approach to quantitative analysis. The ability of isobaric peptide labelling to deliver sufficiently accurate quantitation of specific peptide modifications is evaluated.

In contrast to holistic approaches to protein damage quantitation, this approach provides information on the specific chemistry of the residue modification, its location in a specific residue within a peptide sequence, and its rate of formation. This enables the generation of detailed
degradation profiles, which may provide information on the chemistry of modification, including the formative mechanisms and pathways of formation.

This chapter describes the application of isobaric quantitation to tracking the low-abundance residue-level damage inflicted on short synthetic peptides exposed to high temperatures, UVA and UVB irradiation.

5.3 Methodology

5.3.1 Materials

HPLC-grade ethanol was obtained from Thermo Fisher Scientific; ChromAR® LC-grade water and LC-grade acetonitrile from J.T. Baker; Univar formic acid from Ajax Finechem; and NanoES spray capillaries from Proxeon Biosystems (Odense, Denmark). Synthetic peptides, Leu-Leu-Tyr-Leu-Arg-OH (LLYLR) and Leu-Leu-Trp-Leu-Arg-OH (LLWLR) were obtained as lyophilised hydrochloride powders from The Biopeptide Company. MS analysis confirmed the presence of these peptides, along with the truncations LLW and LLY. iTRAQ reagents were obtained from ABSciex.

5.3.2 Peptide exposure

Aqueous tyrosine- and tryptophan-containing peptide solutions were prepared by addition of HPLC-grade water to the lyophilised powders at 2 mg/ml, pH 3.5 (not adjusted). Samples exposed to UVA or UVB were transferred to UV-transparent quartz test tubes sealed with UV-transparent polyethylene film at an approximate sample depth of 1 cm and placed in a photo-irradiator (LZC4-14, LuzChem) with UVA or UVB LZC narrow bandwidth lamps. The spectral distribution peaks ranged over approximately 281-360 nm for the UVB and 316-400 nm for the UVA lamps, with a maximum intensity of 52670 mW.m⁻².nm⁻¹ (Luzchem 2004a; 2004b). Irradiation was performed over 12 hours (UVB) and 24 hours (UVA). Samples exposed to heat were transferred to sealed narrow glass tubes and heated at 90°C over 192 hours. Sample aliquots were removed and stored in the dark at -85°C at zero time and at various time-points throughout the treatment.

5.3.3 Isobaric labelling

Aqueous sample aliquots of 40 µl were evaporated to dryness at 30°C in a Centrivap centrifugal concentrator (Labconco) and reconstituted in 30 µl 0.5 M triethylammonium bicarbonate, pH 8.5. Labelling with iTRAQ reagents was performed essentially as per the manufacturer’s protocol: 70 µl
MS-grade ethanol was added to each iTRAQ reagent vial (114, 115, 116 and 117), combined, and added to the 30 µl sample aliquot. Aliquots from each sample were checked by MS for complete labelling, after which the samples were pooled. Following labelling, three aliquots from each sample were de-salted and purified by 5:7 dilution in water before processing through C18 StageTips (Proxeon Biosystems) and elution into 50% acetonitrile, 1% formic acid.

5.3.4 Mass spectrometry

ESI-MS analysis was performed on a tandem quadrupole time-of-flight (Qq-TOF) mass spectrometer (QSTAR Pulsar-i; ABSciex) utilising direct infusion nanospray delivery of aqueous samples. Collision-induced dissociation (CID) utilised nitrogen gas, with collision energies manually adjusted for optimal fragmentation. The QSTAR was operated with Analyst QS service pack 7 software (ABSciex). Sequencing was performed manually through analysis of overlapping fragment ion series. Duplicate MS scans were taken for each subsample from 200-900 m/z. MS/MS was performed on randomised order peak-lists of masses of modified and unmodified peptides to generate iTRAQ reporter ion data. Pooled iTRAQ samples were used throughout these experiments.

5.3.5 Data analysis

A combination of MS and MS/MS data was used to track the formation of modifications in the model peptides: the MS/MS data revealed the relative abundance of any given peptide between samples (via the peak areas of reporter ions), allowing the change in the abundance of that peptide over the time course to be observed. In MS, the peak areas corresponding to the \( m/z \) of peptides labelled only at the N-terminus were recorded, allowing estimates of the relative abundance of each peptide species to the others present at each time point. iTRAQ labels were not detected on tyrosine residues within these peptides. The inter-species comparisons were based on an assumption that modified peptides can be expected to ionise similarly in MS, due to their possession of very similar chemical and physical characteristics to their parent peptides (Mann 1999; Lu et al. 2004). These estimations of species abundance were used to provide species-specific weighting factors for the iTRAQ reporter ion data. These inter-species comparisons (MS) are less reliable than iTRAQ-generated intra-species quantitation (MS/MS), but nevertheless provide a valuable opportunity for estimating the relative abundances of the degraded species. This generated a peptide degradation profile for the samples over the time course, with increases or decreases in peptide abundance evident from MS/MS data, and the
relative abundance of particular species evident from the MS data. For ease of presentation, relative abundance scales have been arbitrarily adjusted so that at zero time, the unmodified peptide has a relative abundance of 1.0 (Ehrenshaft et al. 2009).

5.4 Results and discussion

Modifications to tyrosine and tryptophan residues previously observed and characterised in model peptides (Chapter 4, Grosvenor et al. 2009) were identified by their m/z ratios and targeted in MS/MS to generate iTRAQ reporter ion ratios. Monitored tryptophan and tyrosine degradation products are represented in Table 5.1 (Dyer et al. 2006b; Grosvenor et al. 2009). The structures are given in Figure 5.1.

The formation of sixteen degradation products (with a wide range of relative abundance) in four peptides was tracked following exposure to UVA, UVB and heat. Refer to Figure 5.1 for the structures of these products. MS scans provided information on which modifications within parent peptides were most abundant, while MS/MS fragmentation released isobaric reporter ions that demonstrated the changes in abundance (rate of formation/degradation) of individual modifications over exposure. An MS scan of a pooled, treated sample is shown in Figure 5.2, providing an example of the peak area differences that can be used to estimate the species-to-species relative abundance and normalise isobaric quantitative information. The inset demonstrates the degradation of LLW during heat treatment, as represented by decreases in the relative abundance of the respective iTRAQ reporter ions. Isobaric labelling enables much more accurate quantification than is possible using MS peak area analysis alone – as samples are pooled, experimental variation is greatly reduced, allowing the comparison of stoichiometric reporter ions within a single MS/MS scan (Ross et al. 2004). By combining this information with MS-derived information regarding the contribution of the specific modification to the total ion count, a degradation product profile can be visualised, showing the change in abundance of multiple degradation products over the treatment. The depletion of parent peptides may also be visualised using isobaric reporter ion ratios (Figure 5.3). Depletion data provide a measure of the extent of degradation within a sample.
<table>
<thead>
<tr>
<th>Mass change</th>
<th>Modification</th>
<th>Proposed Product(s)</th>
<th>Numbering for Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>+16 Da</td>
<td>W+O</td>
<td>hydroxytryptophan</td>
<td>Product 1</td>
</tr>
<tr>
<td>+32 Da</td>
<td>W+2O</td>
<td>dihydroxytryptophan/ N-formylkynurenine</td>
<td>Product 2</td>
</tr>
<tr>
<td>+14 Da</td>
<td>W+O-C</td>
<td>kynurenine</td>
<td>Product 3</td>
</tr>
<tr>
<td>+48 Da</td>
<td>W+3O</td>
<td>hydroxyformylkynurenine</td>
<td>Product 4</td>
</tr>
<tr>
<td>+64 Da</td>
<td>W+4O</td>
<td>dihydroxyformylkynurenine</td>
<td>Product 5</td>
</tr>
<tr>
<td>+20 Da</td>
<td>W+2O-C</td>
<td>hydroxykynurenine</td>
<td>Product 6</td>
</tr>
<tr>
<td>+30 Da</td>
<td>W+2O-2H</td>
<td>tryptophandione</td>
<td>Product 7</td>
</tr>
<tr>
<td>+28 Da</td>
<td>W+2O-4H</td>
<td>β-unsaturated tryptophandione</td>
<td>Product 8</td>
</tr>
<tr>
<td>+44 Da</td>
<td>W+3O-4H</td>
<td>hydroxy-bis-tryptophandione</td>
<td>Product 9</td>
</tr>
<tr>
<td>+12 Da</td>
<td>W+C</td>
<td>tetrahydro-β-carboline</td>
<td>Product 10</td>
</tr>
<tr>
<td>+45 Da</td>
<td>W+N+2O-H</td>
<td>nitrotryptophan</td>
<td>Product 11</td>
</tr>
<tr>
<td>+16 Da</td>
<td>Y+O</td>
<td>diphenylalanine (dopa)</td>
<td>Product 12</td>
</tr>
<tr>
<td>+32 Da</td>
<td>Y+2O</td>
<td>triphenylalanine (topa)</td>
<td>Product 13</td>
</tr>
<tr>
<td>+14 Da</td>
<td>Y+O-2H</td>
<td>dopa-quinone</td>
<td>Product 14</td>
</tr>
<tr>
<td>+30 Da</td>
<td>Y+2O-2H</td>
<td>topa-quinone</td>
<td>Product 15</td>
</tr>
<tr>
<td>+45 Da</td>
<td>Y+N+2O-H</td>
<td>nitrotyrosine</td>
<td>Product 16</td>
</tr>
</tbody>
</table>
Figure 5.1  Derivatives of tryptophan and tyrosine, with modification and product number.
**Figure 5.2**  Portion of an MS scan of a pooled iTRAQ labelled mixture of heat treated and untreated peptides LLWLR, LLW, LLYL and LLY. MS relative abundance is indicated by an arbitrary scale. Inset: MS/MS data showing the detection of iTRAQ reporter ions for singly charged LLW. Reporter 114 corresponded to untreated sample, 115 to 48 h heat-treated sample, 116 – 96 h, 117 – 192 h.

**Figure 5.3**  Depletion of parent peptide, LLW, after exposure to UVB, UVA and 90°C heat for up to 8, 24 and 192 hours, respectively. Changes in abundance were determined by iTRAQ reporter ion abundance. Relative abundance represents the peak area relative to unmodified LLW at 0 h, defined as 100. Error bars represent ±SEM (note: error bars on the 0-192 h heat columns are present, but small).
Figure 5.3 demonstrates the depletion of the peptide, LLW, following exposure to UVB and UVA radiation, and to hydrothermal insult. The observed decay in this peptide and in others was tracked using isobaric reporter ion MS/MS and MS scan data. The precision of measurement is indicated by small standard errors of the mean (error bars), while the rapid degradation observed after UVB irradiation is consistent with the susceptibility of tryptophan residues to the ROS generated by short wavelength irradiation (Igarashi et al. 2007). The profiles for the degradation products of four peptides exposed to three damage protocols are represented in Figures 5.4 to 5.6.

**Figure 5.4** Tryptophan- and tyrosine-derived modifications in LLW*, LLW*LR, LLY* and LLY*LR following UVB exposure. Changes in the abundance of modified species were determined by isobaric reporter ion abundance, while the relative abundance of the modified species was determined by MS scan. Relative abundance represents the peak area relative to the unmodified peptides, LLW, LLWLR, LLY or LLYLR, at 0 h, defined as 100. Error bars represent ±SEM.
Figure 5.5  Tryptophan- and tyrosine-derived modifications in LLW*, LLW*LR, LLY* and LLY*LR following UVA exposure. Changes in the abundance of modified species were determined by isobaric reporter ion abundance, while the relative abundance of the modified species was determined by MS scan. Relative abundance represents the peak area relative to the unmodified peptides, LLW, LLWLR, LLY or LLYLR, at 0 h, defined as 100. Error bars represent ±SEM.
Figure 5.6  Tryptophan- and tyrosine-derived modifications in LLW*, LLW*LR, LLY* and LLY*LR following hydrothermal insult at 90°C. Changes in the abundance of modified species were determined by isobaric reporter ion abundance, while the relative abundance of the modified species was determined by MS scan. Relative abundance represents the peak area relative to the unmodified peptides, LLW, LLWLR, LLY or LLYLR, at 0 h, defined as 100. Error bars represent ±SEM.

This approach allowed the progression of multiple peptide products to be monitored with high sensitivity, and subtle differences between samples to be identified. Up to eleven tryptophan residue modifications and five tyrosine residue modifications (see Chapter 4 for a description of characterisation and formation) were observed to be formed from the synthetic peptides, LLWLR, LLW, LLYLR and LLY after radiation or hydrothermal insult. The contribution of each modified species to the degradation of each peptide can be assessed by its relative abundance, while its change in abundance (rate of formation) can be seen over the time course.

Tracking enabled the subtle differentiation of degradation patterns between internalised or C-terminal tryptophan residues (Figures 5.4 through 5.6), with advanced oxidation products, such as W+2O-2H, W+2O-4H and W+C (8, 9 and 10) in tryptophan and Y+2O-2H (15) in tyrosine seen to contribute more significantly toward degradation profiles in peptides with terminal aromatic
residues than in those with internalised residues, compared to primary products such as W+O, W+2O, and W+O-C (1, 2 and 3) in tryptophan and W+O (12) in tyrosine. Note that residue location within peptides is expected to affect reactivity, with factors such as steric hindrance and electron density affecting reaction with nucleophiles (Broo et al. 1998; Davies 2003).

Table 5.2 Abundance of selected LLWLR products after exposure to UVB radiation relative to unmodified LLWLR at 0 hours (defined as 100). Values in brackets represent the SEM expressed as a percentage of the relative abundance value

<table>
<thead>
<tr>
<th>LLWLR</th>
<th>0 h UVB</th>
<th>2 h UVB</th>
<th>4 h UVB</th>
<th>8 h UVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan [W]</td>
<td>100 (8%)</td>
<td>71 (3%)</td>
<td>50 (4%)</td>
<td>25 (28%)</td>
</tr>
<tr>
<td>Product 1 [+O]</td>
<td>0.47 (29%)</td>
<td>3.43 (7%)</td>
<td>5.92 (0.3%)</td>
<td>4.74 (3%)</td>
</tr>
<tr>
<td>Product 2 [+2O]</td>
<td>0.38 (23%)</td>
<td>1.80 (5%)</td>
<td>3.31 (5%)</td>
<td>3.80 (5%)</td>
</tr>
<tr>
<td>Product 3 [+O-C]</td>
<td>0.84 (11%)</td>
<td>1.09 (6%)</td>
<td>1.43 (1%)</td>
<td>1.75 (7%)</td>
</tr>
<tr>
<td>Product 4 [+3O]</td>
<td>0.12 (10%)</td>
<td>0.27 (7%)</td>
<td>0.43 (2%)</td>
<td>0.56 (2%)</td>
</tr>
<tr>
<td>Product 5 [+4O]</td>
<td>0.05 (44%)</td>
<td>0.06 (7%)</td>
<td>0.09 (10%)</td>
<td>0.11 (10%)</td>
</tr>
<tr>
<td>Product 6 [+2O-C]</td>
<td>0.09 (37%)</td>
<td>0.09 (14%)</td>
<td>0.12 (10%)</td>
<td>0.12 (19%)</td>
</tr>
<tr>
<td>Product 7 [+2O-2H]</td>
<td>0.08 (10%)</td>
<td>0.40 (8%)</td>
<td>0.59 (4%)</td>
<td>0.76 (1%)</td>
</tr>
<tr>
<td>Product 8 [+2O-4H]</td>
<td>0.08 (18%)</td>
<td>0.20 (3%)</td>
<td>0.25 (7%)</td>
<td>0.35 (1%)</td>
</tr>
<tr>
<td>Product 9 [+3O-4H]</td>
<td>0.08 (23%)</td>
<td>0.18 (2%)</td>
<td>0.31 (5%)</td>
<td>0.37 (4%)</td>
</tr>
<tr>
<td>Product 10 [+C]</td>
<td>0.05 (51%)</td>
<td>0.15 (2%)</td>
<td>0.19 (3%)</td>
<td>0.19 (10%)</td>
</tr>
<tr>
<td>Product 11 [+N+2O-H]</td>
<td>0.12 (27%)</td>
<td>0.26 (3%)</td>
<td>0.42 (4%)</td>
<td>0.57 (2%)</td>
</tr>
</tbody>
</table>
The generally less regular patterns of formation (higher error, inconsistent trend) observed for tyrosine degradation products compared to tryptophan products are consistent with the lower susceptibility of tyrosine residues than tryptophan residues to direct irradiation and to the oxidative modification during irradiation and heating (Bruskov et al. 2002a; Davies 2003). Tracking revealed the already relatively high levels of some oxidative modifications prior to some treatments (such as Y+O), allowing this to be compared to their abundance post-treatment. Oxidative modifications such as this that were observed in untreated samples are attributed to in-solution sample oxidation during storage.

The formation of even low abundance residue modifications was evident (see Figure 5.6, products 4 to 11 in LLWLR.) The sensitivity of an isobaric MS approach to tracking protein damage is indicated by the precision of measurement of even low abundance compounds (compare the low abundance of products 4-11 in Figure 5.6 with the relatively low levels of error presented in Table 5.2). Figure 5.6 demonstrates that, compared to hydroxytryptophan residue formation (W+O, product 1), the relative abundance of products 4 to 11 is very low, especially when derived from LLWLR. Table 5.2 demonstrates the successful tracking of the increasing abundance of these minor LLWLR derivatives (products 4-11) over UVB exposure. Associated errors tend to be larger relative to the measurement when products are detected at low abundance, as would be expected, but still permit the observation of their formation.

5.5 Conclusions

This novel application of isobaric labelling allowed the mass spectrometric tracking of single peptide-bound tryptophan and tyrosine modification, facilitating in-depth degradation profiling. This represents a significant variation from the conventional usage of isobaric labelling of multiple peptides to generate quantitative information on one protein. The new approach yielded information on the degradation product species formed, their contribution to the degradation products pool, and their formation during the course of insult. Low variation between replicates demonstrated the suitability of the approach for precisely tracking the formation of even very low abundance modified peptides. Subtle differences in damage profiles arising from peptides containing internalised and terminal aromatic residues, and from three damage protocols, were resolved sensitively and precisely. This application of isobaric labels confirms that sufficient sensitivity and accuracy may be attained through analysis of individual peptides to provide quantitative information on even low-abundance derivatives.
It is envisaged that further application of this new approach to damage profiling of residue modifications will permit analogous characterisation and tracking of low abundance residue modifications in a range of complex proteinaceous systems. Isobaric quantitative systems are ideal for more complex systems, as isobaric peptides for quantitative analysis are engineered to co-elute. Comparisons of inter-species abundance in more complex systems would naturally have to be limited to species that co-elute. In a complex proteomic analysis, the peptides used for protein identification are usually longer than the model peptides utilised in the present study. However, as iTRAQ labelling agents bind to peptides of all sizes as used in proteomic studies, this is not expected to impact on the effectiveness of this technique. Notably, relevant potential applications include the utilisation of residue-level damage markers to develop and validate protein protective strategies for agricultural products, or for tissues such as skin and hair. Tracking may monitor the formation of residue modifications characteristic of specific insults, such as UV exposure, or may also be utilised to follow the appearance of post-translational modifications induced by cellular means, rather than by exogenous factors. This will allow more sensitive analysis of cell-programmed and damage-induced modification of proteins, yielding more detailed information than is available from cruder, holistic techniques or from non-quantitative MS analysis. Damage profiling offers a route to sensitive evaluation of mitigation strategies developed for protein-based products such as foods and natural textiles or for susceptible cellular tissues.
6 Proteomic characterisation of hydrothermal redox damage

6.1 Abstract

Peptide and protein damage contributes to the loss of quality and value in protein-based food and textile products, as well as to the degeneration of biological tissues such as hair and skin. The effects of elevated temperature on such substrates at the molecular level are, however, relatively unknown. This chapter examines the response of peptides and proteins to hydrothermal damage using mass spectrometry and reports the location of molecular markers of hydrothermal damage within wool proteins.

The hydrothermal exposure of model peptides containing the oxidatively-sensitive residues, tryptophan and tyrosine, revealed the formation of a number of products, such as hydroxytryptophan and dihydroxyphenylalanine. A variety of degradation products were also observed in intermediate filament proteins, including deamidation, and the oxidation of histidine, tyrosine and tryptophan residues.

The products observed during hydrothermal exposure indicated the involvement of reactive oxygen species. Molecular markers were identified within a proteinaceous system to allow the evaluation of damage type or severity. These findings have important implications for the thermal processing of foods and textiles.

6.2 Introduction

In addition to their critical roles in living tissues, proteins and peptides constitute fundamental components in many bio-based products, notably foods and textiles. Protein composition and modification profiles correlate to key product properties including texture (Stanley et al. 1996), flavour (Spanier et al. 2004), strength (Hearle 2000) and water-retention (Tornberg 2005; Lund et al. 2011). During processing, both foods and textiles are often subjected to elevated temperatures, with resultant thermal modification. Thermal protein modification is known to be associated with the deterioration of flavour (Kwok & Niranjan 1995), colour (Pagliarini et al. 1990; Schwartz & Mckinnon 2000) and nutritional value (Schwass & Finley 1984), but is not well understood at the molecular level. Heat damage to proteins in biological systems is also of
concern, as localised heat generation undoubtedly generates biomolecular modification, particularly for hair and skin where temperatures outside of physiological range are most commonly encountered (Reutsch & Kamat 2004; Lin et al. 2006). These modifications may possibly also be generated at ambient or slightly higher temperatures, although at slower rates.

Underpinning the observed changes noted in heated materials due to tertiary protein changes such as aggregation and precipitation are changes in primary structure, which occur in the form of residue side-chain modification. Despite the importance of proteins to a vast range of products, there is little knowledge about these fundamental processes.

Proteins exposed to elevated temperature are known to react with sugars and longer carbohydrates via the Maillard reaction, giving rise to a range of modifications from early to late stage glycation products (Davis et al. 2001). The resulting blocked lysine residues lead to damaged foods with lower nutritive quality and digestibility (Baptista & Carvalho 2004). The Maillard reaction is not a single reaction, but rather a series of reactions giving rise to ever more complex products.

\[ \text{Glucose} \quad \text{Amino acid} \quad \text{Amadori compound} \]

Scheme 6.1 Schematic example of the initial reaction in a Maillard reaction series, in this case, between glucose and an amino acid.

A variety of holistic assays have been developed to assess the effects of heat damage to proteins. These include the indirect measurement of damage using the furosine content (Baptista & Carvalho 2004; García-Baños et al. 2004) and the detection of residue changes using amino acid analysis. Using amino acid analysis, researchers have determined the formation of lanthionine crosslinks and the break-down of disulfide crosslinks and other peptide bonds (causing brittleness), and the degradation of certain amino acid residues (forming coloured products) after exposure of wool proteins to high temperatures (Sweetman 1967; Duffield & Lewis 1985; Brack et al. 1999; Schwartze & Mckinnon 2000).
Soft ionisation techniques (electrospray ionisation, ESI, and matrix-assisted laser ionisation/desorption, MALDI) for mass spectrometry are particularly suited to the detailed analysis of changes to peptides and proteins (Hamdan & Righetti 2005), yielding significantly more information than less specific assay approaches. Tandem mass spectrometry (MS/MS) allows the modifications to individual amino acid residues within proteins to be identified (Hamdan & Righetti 2005).

This chapter presents a proteomic evaluation of hydrothermal damage in model peptides and proteins. The model peptides contain residues sensitive to oxidation, tyrosine and tryptophan, in order to characterise, profile and track oxidative damage resultant from hydrothermal insult, at the molecular level. Subsequently, it reports the detailed proteomic profiling of hydrothermal damage in wool IFPs. The products found in both the model peptides system and in proteins extracted from wool provide new insights into the mechanisms underlying hydrothermal damage. Peptide markers for hydrothermal damage were also found, with potential applications in future protein damage evaluation. It is anticipated that these molecular markers of damage will assist with the evaluation of hydrothermal modification, and will also provide tools for developing and validating damage mitigation treatments.

### 6.3 Materials and methodology

#### 6.3.1 Materials

Nonyl phenol ethoxylate (trade-name Teric GN9) was obtained from Orica Ltd. Dichloromethane, ethanol, urea, zinc acetate and acetic acid were obtained from Merck Chemicals (Darmstadt, Germany). ChromAR® LC-grade water and LC-grade acetonitrile, sodium tetraborate and hydrochloric acid from J.T. Baker; and Univar formic acid from Ajax Finechem. Tris, sodium tetrathionate sodium citrate and acetic acid were obtained from BDH (Poole, England). Triethylammonium bicarbonate and tris(2-carboxyethyl)phosphine were obtained from Sigma-Aldrich Inc (St Louis, MO). Synthetic peptides, Leu-Leu-Tyr-Leu-Arg-OH (LLYLR) and Leu-Leu-Trp-Leu-Arg-OH (LLWLR) were obtained as lyophilised powdered hydrochlorides from The Biopeptide Company. MS analysis confirmed the presence of these peptides, along with the truncations LWLR, WLR, LYLR and YLR. Sequencing grade trypsin was obtained from Promega. Acrylamide was obtained from BioRad Laboratories. α-Cyano-4-hydroxycinnamic acid was obtained from Bruker. NanoES spray capillaries were obtained from Proxeon Biosystems.
6.3.2 Hydrothermal treatment of peptides

Tryptophan and tyrosine-containing peptides were dissolved in 1 ml LC-grade water at 1 mg/ml and heated in sealed vials at 90°C for periods of up to ten days, specifically, 0, 24, 48, 72, 96, 192 and 240 hours. Samples were transferred immediately to -85°C prior to ESI-MS/MS analysis.

6.3.3 Extraction and fractionation of IFPs

De-tipped greasy Merino wool samples were laboratory-scoured by immersion and agitation in 60°C 0.15% Teric GN9 for 2 min, in 40°C 0.15% Teric GN9 for a further 2 min, in 40°C water for 2 min and in 60°C water for 2 min. The wool samples were air-dried in the dark for 3 h, then immersed and agitated for 30 s in two exchanges of dichloromethane, two exchanges of ethanol, and two exchanges of water. The scoured wool was air-dried in the dark overnight and hand-ground to a fine powder over liquid nitrogen, before desiccation under vacuum over P₂O₅. Wool proteins were extracted by vigorous shaking on a reciprocal shaker at room temperature for 24 h at 10 mg/ml in 0.1 M Tris, 0.2 M sodium sulfite, 0.1 M sodium tetrathionate, 8 M urea, pH 9.5 (HCl) (Thomas et al. 1986). This solution improves wool extractability by reducing the disulfide cross-links in wool (RS-SR) to cysteine (RSH) residues, which are subsequently oxidised to S-sulfocysteine (RS-SO₃⁻). Breaking the cross-links and incorporating charged groups into the proteins increases their solubility and makes the notoriously insoluble wool proteins more amenable to subsequent fractionation.
Figure 6.1  Summary workflow for preparation of a protein fraction enriched in IFPs. Note: For an expansion on this workflow, refer to Figure 3.1.

The supernatant (SN1) was obtained by sieving the extract through 125 µm mesh. The filtrate was combined with that resulting from two re-suspensions of the gelatinous residue in water followed by sieving. The filtrate was dialysed against water. The pellet was removed by centrifugation, and an equal volume of 0.04 M zinc acetate (pH 6.1, acetic acid) was added to the supernatant (SN2), to stand at room temperature for 10 min. The pellet (Ppt 1) was obtained by centrifugation at 14000 rpm in a J-30.50 rotor in an Avanti J-30I centrifuge (Beckman), and solubilised in 0.02 M sodium citrate. After dialysis against water, the supernatant (SN3) was obtained by centrifugation. The supernatant was added to twice its volume of 80% acetone, 20% saturated ammonium sulfate, which was stirred for two hours. The precipitate (enriched IFP fraction, Ppt 2) was isolated by centrifugation, dissolved in 0.05 M sodium tetraborate, dialysed against water, and freeze-dried on a Dura-Dry freeze-dryer (FTS Systems, Stone Ridge, NY) This is described in schematic form in Figure 6.1. For a more detailed explanation of wool protein fractionation, yielding the IFP fraction and others, please see Chapter 3, Section 3.2.2.
6.3.4 Hydrothermal treatment and digestion of wool proteins

Quantities of freeze-dried enriched IFP protein fraction were solubilised at 5.3 mg/ml by vigorous shaking in 0.05 M sodium tetraborate buffer, pH 8.5 (HCl) overnight. Centrifugation revealed no pellet. Aliquots of 750 µl were transferred into air-tight Reacti-Vials (Thermo Fisher Scientific) and placed at 90°C for 0, 2, 4 and 8 hours.

After heat exposure, 10 µl aliquots of the samples were diluted in 10 µl 1.0 M triethylammonium bicarbonate buffer, pH 8.5, followed by reduction at 56°C with 20 mM tris(2-carboxyethyl)phosphine, alkylation with 60 mM acrylamide, and overnight digestion with 0.4 µg trypsin.

6.3.5 Mass spectrometry

6.3.5.1 Peptides

Heat-treated peptide samples were diluted one thousand-fold in 50% acetonitrile, 0.5% formic acid. Samples were submitted for duplicate ESI-MS/MS analysis on a tandem quadrupole time-of-flight (Qq-TOF) mass spectrometer (QSTAR Pulsar-i, ABSciex) via direct infusion nanospray delivery. Ions with m/z ratios corresponding to masses of interest were subjected to collision-induced dissociation, utilising nitrogen gas, with collision energies optimised to permit de novo peptide sequencing. Characterisation was performed manually through analysis of immonium ions and overlapping y, b and a fragment ion series with Analyst QS v1.1 service pack 7 (ABSciex).

6.3.5.2 Keratin proteins

The protein samples were digested overnight at 37°C with sequencing grade trypsin (Promega VS111) to form peptides. The digested samples were diluted 1 in 50 in 3% acetonitrile, 0.1% trifluoroacetic acid. Digests were separated on a Proxen EasynLC (Bruker) using an in-house prepared Microsorb C18 300-5 media (Varian, Palo Alto, CA) 150 µm ID 45 cm pre-column and a 75 µm ID 18 cm analytical column. Fractions were spotted onto MTP AnchorChipTM 800/384 TF MALDI plates (Bruker) using a Proteineer fc (Bruker) fraction collector, along with a matrix consisting of (1:6:3:1 α-cyano-4-hydroxycinnamic acid saturated acetone: ethanol: acetone: 0.1 M ammonium dihydrogen orthophosphate). Automated MALDI-MS/MS analysis was performed on an UltraFlex III MALDI-TOF/TOF mass spectrometer (Bruker). Calibration was performed using Peptide Calibration Standard (Bruker) containing angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1-17, ACTH clip 18-39 and somatostatin 28. Automated MS scans were
performed in the range 900 – 2500 m/z at optimised laser intensity, and precursors were fragmented in LIFT mode. Peak lists were searched through Mascot against an in-house database via ProteinScape (Bruker) software. Search criteria were: Semi-trypsin; Ovis aries; ±100 ppm peptide mass tolerance; fixed modification – propionamide (C); variable modifications – deamidation (NQ), acetyl (N-term), carbamyl (N-term), oxidation (WYPMH), double oxidation (WYPH), kynurenine (W), +2O-2H (WY).”

6.4 Results

Six synthetic model peptides and a fraction of wool proteins (the IFPs, or structural keratins) were subjected to hydrothermal damage by in-solution exposure to elevated temperature (90°C). The synthetic peptides contained the sensitive residues, tyrosine and tryptophan, flanked by relatively un-reactive residues. The keratin proteins contained a range of residues and were extracted and partially fractionated prior to treatment.

6.4.1 Peptides

Peptides damaged by hydrothermal exposure at 90°C for periods of 24, 48, 72, 96, 144, 192 and 240 hours gradually formed a range of modified peptide products. The parent peptides, containing unmodified tyrosine and tryptophan residues, remained detectable over the treatment times trialled. ESI-MS/MS analysis of the resultant ions confirmed the formation of three tyrosine and nine tryptophan hydrothermal modifications. These were identified through peptide fragmentation and sequencing, with all modifications confirmed through full and complementary a, b and y fragment ion series. The modifications observed corresponded to the following nominal residue mass changes: Y + 16 Da (Y + O), Y + 32 Da (Y + 2O), Y + 30 Da (Y + 2O – 2H), W + 16 Da (W + O), W + 32 Da (W + 2O), W + 30 Da (W + 2O – 2H), W + 4 Da (W + O – C), W + 20 Da (W + 2O – C), W + 48 Da (W + 3O), and W + 12 (W + C). Tryptophan modifications were observed to form at a faster rate than tyrosine modifications. The majority of these modifications were observed in two or three peptide sequences. Figure 6.2 illustrates that the tryptophan and tyrosine hydrothermal modifications observed after heating were comparable to those previously observed in UV-induced protein oxidation (Chapter 4, Grosvenor et al. 2009), which were attributable to the formation of ROS.
Table 6.1  Modifications observed in LLWLR and LLYLR model peptides after 240 h hydrothermal exposure, with observed m/z ratios and fragment ions itemised.

<table>
<thead>
<tr>
<th>Mod</th>
<th>m/z</th>
<th>Observed fragment ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>W+O</td>
<td>716.466</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', y_3', )</td>
</tr>
<tr>
<td></td>
<td>716.461</td>
<td>b-series ( b_2, b_3, b_3', b_3' )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2, a_3, a_3', a_4 )</td>
</tr>
<tr>
<td>W+2O</td>
<td>732.446</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', )</td>
</tr>
<tr>
<td></td>
<td>732.441</td>
<td>b-series ( b_2, b_3, b_3' )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2, a_3 )</td>
</tr>
<tr>
<td>W+2O-2H</td>
<td>730.427</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', )</td>
</tr>
<tr>
<td></td>
<td>730.425</td>
<td>b-series ( b_2, b_3, b_3' )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2, a_3, a_3', a_4 )</td>
</tr>
<tr>
<td>W+3O</td>
<td>748.436</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', )</td>
</tr>
<tr>
<td></td>
<td>748.417</td>
<td>b-series ( b_2, b_3, b_3' )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2, a_3, a_3', a_4 )</td>
</tr>
<tr>
<td>W+O-C</td>
<td>704.446</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', )</td>
</tr>
<tr>
<td></td>
<td>704.548</td>
<td>b-series ( b_2, b_3, b_3' )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2, a_3, a_3', a_4 )</td>
</tr>
<tr>
<td>W+2O-C</td>
<td>720.441</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', )</td>
</tr>
<tr>
<td></td>
<td>720.442</td>
<td>b-series ( b_2, b_3, b_3' )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2 )</td>
</tr>
<tr>
<td>Y+O</td>
<td>693.430</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', )</td>
</tr>
<tr>
<td></td>
<td>693.428</td>
<td>b-series ( b_2, b_3, b_3' )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2, a_3, a_3', a_4 )</td>
</tr>
<tr>
<td>Y+2O</td>
<td>709.425</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', )</td>
</tr>
<tr>
<td></td>
<td>709.357</td>
<td>b-series ( b_2, b_3 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2 )</td>
</tr>
<tr>
<td>Y+2O-2H</td>
<td>707.420</td>
<td>y-series ( y_1, y_1', y_2, y_2', y_3, y_3', )</td>
</tr>
<tr>
<td></td>
<td>707.412</td>
<td>b-series ( b_2 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-series ( a_2, a_3 )</td>
</tr>
</tbody>
</table>

Note: W* or Y* indicates the corresponding modified residue; fragment ions followed by " indicate loss of ammonia from the fragment ion; fragment ions followed by º indicate loss of water from the fragment ion.
Figure 6.2  Tryptophan and tyrosine modifications confirmed in model peptides by ESI-MS/MS. Sequences in the bottom of the boxes indicate the peptides in which the modifications were located.
Table 6.2  Relative abundance of modified peptides formed (expressed as modified peak areas/unmodified peaks areas) from LLWLR and LLYLR model peptides after hydrothermal treatment, demonstrating the gradual formation of oxidative modifications.

<table>
<thead>
<tr>
<th>Modification</th>
<th>W+O</th>
<th>W+2O</th>
<th>W+2O-2H</th>
<th>W+3O</th>
<th>W+O-C</th>
<th>W+2O-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours at 90°C</td>
<td>Peak area of modified peptide/ unmodified peptide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.1%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>174</td>
<td>1.9%</td>
<td>0.4%</td>
<td>0.1%</td>
<td>0.2%</td>
<td>0.8%</td>
<td>0.1%</td>
</tr>
<tr>
<td>192</td>
<td>2.7%</td>
<td>1.3%</td>
<td>0.1%</td>
<td>0.5%</td>
<td>0.8%</td>
<td>0.1%</td>
</tr>
<tr>
<td>240</td>
<td>5.6%</td>
<td>1.2%</td>
<td>0.3%</td>
<td>0.8%</td>
<td>1.3%</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Modification</th>
<th>Y+O</th>
<th>Y+2O</th>
<th>Y+2O-2H</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2%</td>
<td>0.1%</td>
<td>0.0%</td>
</tr>
<tr>
<td>174</td>
<td>1.2%</td>
<td>0.3%</td>
<td>0.1%</td>
</tr>
<tr>
<td>192</td>
<td>2.0%</td>
<td>0.6%</td>
<td>0.1%</td>
</tr>
<tr>
<td>240</td>
<td>2.4%</td>
<td>0.5%</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

6.4.1 Proteins

To proteomically evaluate hydrothermal damage in a model protein system, and to identify molecular markers of damage, the structurally important class of IFPs were isolated from wool. Briefly, a complement of wool proteins was extracted in a solution containing sodium tetrathionate to enhance solubility. An extended fractionation protocol allowed the generation of a fraction enriched in IFPs, which was subjected to hydrothermal treatment (Figure 6.1).

While most amino acid residues are susceptible to oxidation, some residues are more sensitive to certain kinds of insult than others. In addition to the tryptophan and tyrosine oxidative modifications observed in the model peptide studies, a range of additional potential modifications were also proteomically profiled. These included oxidation and hydroxylation of methionine and histidine residues; deamidation of glutamine and asparagine residues; and carbamylation of the N-terminus or lysine, alanine and cystine residues (Hains & Truscott 2007; Guedes et al. 2009; Madian & Regnier 2010).
Figure 6.3  Spectrum showing the observation via LC-MALDI-MS/MS of a modified wool keratin peptide, AEAESWYR, where the tryptophan had degraded to kynurenine (Kyn) after exposure to 4 h hydrothermal damage. The y and b-series ions are noted, along with the immonium ions.

To identify hydrothermal damage in wool, the structurally important class of IFPs were isolated (see Section 6.3.3). Briefly, a complement of wool proteins was extracted in a solution containing sodium tetrathionate. This reagent improves wool protein extractability by reducing the disulfide cross-links in wool (RS-SR) to cysteine (RSH) residues, which are subsequently oxidised to S-sulfocysteine (RS-SO$_3$). Breaking the cross-links and incorporating charged groups into the proteins increases their solubility, improving subsequent fractionation. An extended fractionation protocol allowed the generation of a fraction enriched in IFPs, which was subjected to hydrothermal treatment (Figure 6.1).
Table 6.3  Modified peptides formed in hydrothermally damaged IFPs, suitable for use as molecular markers. Each unmodified peptide was identified in untreated IFPs, while its modified form was identified only after exposure to hydrothermal insult. Modified residues are marked in underlined bold text.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Sequence</th>
<th>Protein (s)</th>
<th>m/z</th>
<th>Sequence</th>
<th>Protein (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1293.60</td>
<td>AQYEALVETNR</td>
<td>K31, K33b, K34</td>
<td>906.43</td>
<td>FLEQONK</td>
<td>K83, K85, K86</td>
</tr>
<tr>
<td>1295.59</td>
<td>2 x deam</td>
<td></td>
<td>907.43</td>
<td>+ deam</td>
<td></td>
</tr>
<tr>
<td>1449.70</td>
<td>AQYEALVETNR</td>
<td>K31, K33b, K34</td>
<td>1136.56</td>
<td>KYEEEVALR</td>
<td>K85</td>
</tr>
<tr>
<td>1451.69</td>
<td>2 x deam</td>
<td></td>
<td>1194.57</td>
<td>+ acetyl + dopa (Y+O)</td>
<td>K85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1196.51</td>
<td>+ deam + Ox (H)</td>
<td></td>
</tr>
<tr>
<td>1109.50</td>
<td>DVEEWNIR</td>
<td>K31, K33b, K34</td>
<td>1011.43</td>
<td>AEAESWYR</td>
<td>K86, K83, K85</td>
</tr>
<tr>
<td>1125.47</td>
<td>W + O</td>
<td></td>
<td>1027.46</td>
<td>W + O</td>
<td></td>
</tr>
<tr>
<td>1141.48</td>
<td>W + 2O</td>
<td></td>
<td>1043.41</td>
<td>W + 2O</td>
<td></td>
</tr>
<tr>
<td>1113.46</td>
<td>Kyn (W+O-C)</td>
<td></td>
<td>1015.41</td>
<td>Kyn (W+O-C)</td>
<td></td>
</tr>
<tr>
<td>1157.50</td>
<td>W + 3O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1124.51</td>
<td>LESEINTYR</td>
<td>K33b, K34</td>
<td>1845.09</td>
<td>LASELNHQVEVLEGYK + Ox (H)</td>
<td>K85</td>
</tr>
<tr>
<td>1125.51</td>
<td>+ deam</td>
<td></td>
<td>1845.88</td>
<td>+ deam + Ox (H)</td>
<td></td>
</tr>
<tr>
<td>1140.51</td>
<td>dopa (Y+O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>999.50</td>
<td>LVVQDNAK</td>
<td>K31, K33b, K35</td>
<td>952.47</td>
<td>LQFFQRN</td>
<td>K83</td>
</tr>
<tr>
<td>1001.48</td>
<td>2 x deam</td>
<td></td>
<td>953.44</td>
<td>+ deam</td>
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<tr>
<td>1627.81</td>
<td>LNVEVDAAPTVDLNR</td>
<td>K31, K33b, K34</td>
<td>1845.08</td>
<td>LSSELNHQVEVLEGYK</td>
<td>K83</td>
</tr>
<tr>
<td>1627.81</td>
<td>deam</td>
<td></td>
<td>1845.88</td>
<td>+ deam</td>
<td></td>
</tr>
<tr>
<td>1626.81</td>
<td>2 x deam</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1367.63</td>
<td>ONHEEVTNLR</td>
<td>K33b</td>
<td>1401.72</td>
<td>RTKEEINLNR</td>
<td>K86, K85, K83</td>
</tr>
<tr>
<td>1368.62</td>
<td>deam</td>
<td></td>
<td>1402.67</td>
<td>+ deam</td>
<td></td>
</tr>
<tr>
<td>1369.63</td>
<td>2 x deam</td>
<td></td>
<td>1403.68</td>
<td>2 x deam</td>
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</tr>
<tr>
<td>1198.50</td>
<td>NHEEANSLR</td>
<td>K34</td>
<td>1192.60</td>
<td>TAAENFVALK</td>
<td>K75 (cytoskeletal)</td>
</tr>
<tr>
<td>1200.45</td>
<td>2 x deam</td>
<td></td>
<td>1193.59</td>
<td>+ deam</td>
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</tr>
<tr>
<td>327.60</td>
<td>SHNHEEVTOLR</td>
<td>K31</td>
<td>1245.62</td>
<td>TKEEINLNR</td>
<td>K86, K85, K83</td>
</tr>
<tr>
<td>1328.59</td>
<td>+ deam</td>
<td></td>
<td>1247.59</td>
<td>2 x deam</td>
<td></td>
</tr>
<tr>
<td>1369.64</td>
<td>+ acetyl</td>
<td></td>
<td>1288.62</td>
<td>+ acetyl + deam</td>
<td>K86, K85, K83</td>
</tr>
<tr>
<td>1386.68</td>
<td>+ acetyl + deam + Ox (H)</td>
<td>K31</td>
<td>1289.58</td>
<td>+ acetyl + 2 deam</td>
<td></td>
</tr>
</tbody>
</table>
LC-MALDI analysis of hydrothermally damaged IFPs identified over 300 modified peptides, a number of which possessed characteristics suitable for marker peptides (listed in Table 6.1). These peptides were not observed to be present in untreated wool proteins. Deamidation of asparagine (N) and glutamine (Q) residues was the most common modification observed, with hydrothermally-induced deamidative modifications observed in thirteen Type I IFPs, and eight Type II IFPs. Tryptophan, histidine and tyrosine modifications were also identified. Tryptophan oxidation was observed in the IFP peptides, DVEEWYIR, AEAESWYR (see Figure 3) and RDVEEWYIR. Tyrosine modification was confirmed in two IFP peptides, KYEEEVALR and LESEINTYR, while histidine oxidation to oxohistidine (H + O) was spotted in SNHEEENVTLR.

6.5 Discussion

The model peptide and IFP analyses provided an intriguing insight into the mechanism by which heat induces damage in polypeptide chains. The tryptophan and tyrosine modifications observed within the model peptides and proteins on hydrothermal exposure are associated with production of ROS (Grosvenor et al. 2009) as is the oxo-histidine modification noted within the IFPs. The product profile of the model peptide system, particularly, is interesting in its similarity to that observed after UV irradiation, which is mediated by ROS attack (Grosvenor et al. 2009) – notably hydroxyl radicals in the case of wet irradiation (Millington & Kirschenbaum 2002; Dyer et al. 2006b). Single and double hydroxylation of tyrosine to form dopa and topa (Y+O and Y+2O,
respectively) and, subsequently, their quinone derivatives is associated with the action of the hydroxyl radical (Dean et al. 1993; Nappi et al. 1995). Hydroxytryptophan (W+O), its W+2O derivative, dihydroxytryptophan, and its W+2O-2H dione derivative are probably formed through the action of hydroxyl radical (Maskos et al. 1992; Domingues et al. 2003; Klarskov et al. 2003). N-formylkynurenine, another compound consistent with W+2O, its hydrolysis products, kynurenine (W+O-C) and hydroxykynurenine (W+2O-C), and its oxidation product, hydroxyformylkynurenine (W+3O) may be formed by both hydroxyl radical and singlet oxygen attack (Guptasarma et al. 1992; Davies & Truscott 2001; Bienvenut et al. 2002; Gracanin et al. 2009). These results strongly indicate that heating alone is sufficient to form ROS such as hydroxyl radical and singlet oxygen in solutions containing complex biological molecules, without any contribution from other widely-acknowledged oxidative insults such as short wavelength radiation or cellular processes, which are known to produce ROS (Davies & Truscott 2001; Lee et al. 2004). These observations are consistent with the detection of ROS-induced DNA damage in systems exposed to elevated temperatures (Bruskov et al. 2002a,b). The mechanisms behind this intriguing hydrothermal generation of ROS require further fundamental studies.

The keratin proteins extracted from wool served as a model for the identification of potential damage markers in proteinaceous systems. In this case hydrothermal damage was characterised in IFPs (or keratins), a structurally significant class of proteins in wool, hair and skin. A number of considerations needed to be taken into account when selecting damage markers. An ideal damage marker for evaluating hydrothermal damage in proteins would be present in sufficient abundance in undamaged samples, and contain a damage-sensitive residue. It would also need to ionise readily under the MS ionisation conditions, and to facilitate simple detection, the ion peak would need to be free of co-eluting peptides, eliminating the need for extensive fractionation.

The peptides identified as suitable markers of damage are listed in Table 6.1. As anticipated, many of the same oxidative modifications of tryptophan and tyrosine noted in the model peptides were also seen in the keratin proteins. Higher order oxidation products, such as quinones and tryptophandiones were not identified in the IFPs. This is probably attributable to the low relative abundance of these higher order products in more complex biological samples.

The involvement of ROS in hydrothermal protein damage has important implications for the thermal processing of foods and textiles. In addition to the aggregation and precipitation associated with changes to tertiary and quaternary protein structure that result in textural changes in food (Lagrain et al. 2008), and massive proteolytic changes noted particularly in meat (Spanier et al. 2004), these results demonstrate that elevated temperatures clearly induce
primary structural changes (alterations to the polypeptide sequence by means of side-chain and terminal modifications) – which may themselves induce secondary and tertiary structural changes (Dalsgaard & Larsen 2009). The kind of modifications observed are known to have important effects on the characteristics of modified tissues and materials (Davies & Truscott 2001; Dyer et al. 2006a), and will no doubt play an important role in future investigations of the effects of processing and storage on valuable protein-based products.

It is suggested that a critical first step in the analysis of residue-level modification in commercial products should be the characterisation of potential peptide markers of damage. This was demonstrated with the observation of numerous modified peptides in IFPs after hydrothermal exposure. The identification of specific molecular-level modifications to proteins damaged by heat (or other) treatments will represent a significant improvement over holistic assay-based approaches to damage assessment, providing a valuable tool to researchers interested in the improvement of product quality. It is anticipated that a more detailed understanding of protein modification at the molecular level will lead to significant advances in protecting the quality and function of proteins in agricultural and food products.

6.6 Conclusion

Thermal damage to proteins is poorly studied in contexts outside of heat-shock responses mediated by the cell. To the best of my knowledge, this study represents the first redox proteomic evaluation of protein hydrothermal damage in a non-living system, and is certainly the first ever molecular-level characterisation of hydrothermal damage within IFPs, an important class of proteins in both agricultural and personal care applications.

Numerous oxidative modifications, including kynurenine, oxohistidine and dihydroxyphenylanine, were observed in aqueous peptides exposed to elevated temperatures. The products observed are consistent with ROS playing a significant role in hydrothermal protein modification. This has previously only been observed in the context of DNA damage (Bruskov et al. 2002a). Validation of these results at the whole protein level was provided through proteomic observation of corresponding modifications in solubilised intermediate filament proteins exposed to elevated temperature.

The new knowledge represented in this study as to the role of ROS in hydrothermal protein damage will no doubt be important both in developing effective mitigation strategies and also in furthering our understanding of the effects of cooking in protein-based foods. In combination
with the use of specific molecular markers of damage, this information facilitates molecular-level evaluation of the effects of heat treatments on agriculturally important foods and textiles.
7 Determination and validation of markers for heat-induced damage in keratins

7.1 Abstract

Protein-based tissues of cosmetic and commercial importance are frequently exposed to elevated temperatures during personal care applications, processing treatments and food preparation. Hydrothermal processes cause protein deterioration, impacting negatively on the value or condition of these materials. To investigate hydrothermal damage in proteins at the molecular level, Type I and II intermediate filament proteins (keratins) from wool were characterised using advanced quantitative techniques based on isobaric iTRAQ labelling and mass spectrometry. Twenty wool peptides were observed to consistently degrade during hydrothermal exposure. These peptides acted as molecular markers of damage – specific indicators of the extent of heat-induced damage within an extracellular protein system.

This technology will be of value in assessing the severity of damage imparted after the hydrothermal exposure of wool products and related substrates such as human hair. Molecular damage markers identified within wool and other materials will provide a route to sensitive and specific evaluation of the effects of protein deterioration, facilitating the development of targeted approaches to damage mitigation within fields as diverse as personal care, anti-ageing technologies, food science and textiles processing.

7.2 Introduction

Proteins are vulnerable to the degradative effects of oxidation, which results in reduced structural integrity and/or functionality due to chemical modifications to the protein molecular structure. In wool, oxidative modification ultimately results in lowered tensile strength and abrasion resistance, along with undesirable colour changes (France & Weatherall 1994; Millington 2006a). In other materials, protein degradation is associated with disease, lowered food quality and premature ageing (Stadtman 2006; Williams 2006; Baron et al. 2007; Lund et al. 2011). Protein oxidation occurs primarily through the action of reactive oxygen species (ROS) and their derivatives, which, in combination, react with a large variety of functional groups (Davies 2005). Despite this, protein oxidation is quite selective, with certain portions of these large molecules more susceptible to modification than others (Guedes et al. 2009). Consequently, sensitive amino
acid residues such as asparagine, glutamine, and aromatic residues are modified differently depending upon their location within the protein structure.

The effects of oxidation, in wool and in other proteins, have been primarily characterised at the level of changes in the amino acid composition, without location-based information. Our team’s recent mass spectrometry-based characterisations of damaged proteins (Dyer et al. 2006a; Dyer et al. 2006b; Dyer et al. 2009; Dyer et al. 2010) allowed the identification of damaged residues within specific proteins. We have also presented methods to quantify damage within peptides and proteins (Grosvenor et al. 2009; Dyer et al. 2010; Grosvenor et al. 2010) – see Chapters 4, 5 and 6.

High temperatures are commonly employed during wool processing. Although this is known to contribute to wool degradation, detailed information of the molecular effects of heat on wool is minimal (Bide 1992; Simpson 2002). Modification caused by heat (or hydrothermal exposure) represents a subset of oxidative damage. To identify and track markers of degradation to wool through a damaging protocol, extracted wool proteins were subjected to elevated temperature (90°C). Following hydrothermal exposure, damaged peptides within the wool proteins were tracked using commercially available isobaric labelling reagents for quantitation (iTRAQ) and reverse phase liquid chromatography (RP-LC) coupled to matrix-assisted laser desorption/ionisation tandem mass spectrometry (MALDI-MS/MS) for separation and analysis. The intermediate filament proteins (IFPs) of wool examined here display significant homology to analogous proteins in human hair, skin and nails (Lynch et al. 1986), meaning that the specific results are of relevance to all these materials, while the principle of marker identification extends to proteins of all sorts.

7.3 Materials and methods

7.3.1 Materials

Methyl methanethiosulfonate was obtained from ABSciex; sodium tetrathionate from BDH. Reacti-Vials, HPLC-grade ethanol, LC/MS-grade water and trifluoroacetic acid were all obtained from Thermo Fisher Scientific. Triethylammonium bicarbonate and tris(2-carboxyethyl)phosphine were obtained from Sigma-Aldrich Inc; LC-grade acetonitrile, sodium tetraborate and hydrochloric acid from J.T. Baker. Sequencing grade trypsin was obtained from Promega. Acrylamide was obtained from BioRad Laboratories. α-Cyano-4-hydroxycinnamic acid and ammonium phosphate buffer were obtained from Bruker Daltonics.
7.3.2  **Wool protein extraction and fractionation**

Wool proteins were extracted at pH 9.5 and crudely fractionated as described earlier (Chapter 6). Briefly, the extraction solution made use of sodium tetrathionate, a sulfitolyis reagent that reacts with cysteine residues to form sulfo-cysteine, which increases the solubility of high-sulfur proteins (Thomas *et al.* 1986). The extracted proteins were precipitated and dialysed to form an enriched fraction of IFPs, which represent the major class of proteins within wool.

7.3.3  **Hydrothermal treatment**

IFPs were dissolved in 0.05 M sodium tetraborate, pH 8.5 (HCl) at 5.3 mg/ml and heated in sealed 1 ml ReactiVials at 90°C for 0, 2, 4 and 8 hours. Samples were transferred immediately to -85°C prior to LC-MALDI-MS/MS analysis. This was performed in duplicate.

7.3.4  **Digestion and alkylation**

Treated samples were diluted with triethylammonium bicarbonate (TEAB) to give ~ 50 µg protein in 20 µl 0.5 M TEAB, 0.025 M sodium tetraborate, pH 8.0. After reduction with tris(2-carboxyethyl)phosphine at 56°C for 45 min, alkylation was performed at room temperature with either 62 mM acrylamide or 40 mM methyl methanethiosulfonate for 30 min. Digestion was performed after the addition of acetonitrile to 7% with sequencing grade trypsin at 37°C for 16 hours.

7.3.5  **Mass spectrometric characterisation**

Digested samples were labelled with 4-plex iTRAQ reagents in randomised order according to the manufacturer’s protocol. Labelled samples were pooled and separated over 80 min at a flow rate of 300 nl/min with a 3-55% B (acetonitrile/0.1% trifluoroacetic acid)using a Proxeon Easy-nLC and a Proteineer fc fraction collection robot. Samples were loaded onto a 30 mm, 100 µm ID trap column (packed in-house with Microsorb C18 300-5 media, Varian, Palo, Alto, CA) and eluted over an 18 cm, 75 µm ID in-house packed C18 analytical column. Fractions were spotted post-column onto 600 µm AnchorChip plates (Bruker) along with saturated α-cyano-4-hydroxy cinnamic acid in acetone mixed 1:10 with 6:3:1 ethanol/acetone/10 mM ammonium phosphate, 0.1% trifluoroacetic acid with a flow rate of 700 nl/min. External calibration was performed using a Peptide Calibration Standard (Bruker) containing ACTH clip [1-17] (2093.0862 Da), ACTH clip [18-39] (2465.1983 Da), angiotensin I (1296.6848 Da), angiotensin II (1046.5418 Da), bombesin (1619.8223 Da), somatostatin (3147.471 Da) and substance P (1347.7354 Da) diluted five-fold.
with matrix solution. An Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker) was used for automated sample characterisation. The settings for MS/MS and stable isotope labelling chemistry detection were adjusted for peak area recognition for iTRAQ reporter ions.

7.3.6 Data analysis

To identify the proteins from which the sample peptides were derived, data was first processed through BioTools (Bruker), and searched against Ovis aries entries in an NCBI-nr database (NCBI-nr_20091103) augmented with in-house sheep sequences using Mascot v2.2.06 (Matrix Science, London, UK). Fixed modifications were specified as either propionamide or methylthio (C), and 4-plex iTRAQ (K and the N-terminus). Variable modifications were deamidation (Q and N), single oxidation (C, H, M, Y, W and F), double oxidation (C, Y, W and F), kynurenine (W), hydroxykynurenine (W), and dopa-quinone (Y). Enzyme specificity was set to semi-trypsin, with two allowable missed cleavages. Data was compiled and analysed using ProteinScape 2.1 (Bruker) with acceptance score thresholds for proteins and peptides set at 40 and 15, respectively.

7.4 Results

Intermediate filament proteins extracted from wool were exposed to elevated temperatures in the presence of water for 0, 2, 4 and 8 hours, causing hydrothermal damage. Following protein reduction, alkylation and digestion, the resultant peptides were differentially labelled with isobaric iTRAQ reagents and analysed using RP-HPLC MALDI-MS/MS. Peptides were identified to specific proteins using automated database searching, and particular attention was paid to those peptides that decreased in abundance after hydrothermal exposure.

Mass spectrometric characterisation and quantitative evaluation using iTRAQ reporter ion abundance resulted in the identification of molecular damage markers for keratin proteins. These peptides demonstrated measurable decreases in abundance when subjected to hydrothermal insult. In some cases, low abundance degradative products of these peptides were detected. Because of the complexity and dynamic range of biological extracts, detected and identified peptides susceptible to oxidative damage vary in their suitability for use as molecular markers of damage. The peptides described below represent those with characteristics suitable for use as reliable markers: sufficient abundance/ion signal to permit automated Mascot identification, consistent decreases in parent peptide abundance over the four time-points measured, and, in some cases, increases in a known degradation product.
Over 140 peptides (some native, others modified) were identified within the IFP samples. The keratin peptides, ATAENEFVALK, ENAELESR and SNHEEEVNTLR, from the K31, K34 and K86 wool proteins, respectively, were observed using iTRAQ reporter ion ratios to degrade during hydrothermal exposure (see Table 7.1 and Figure 7.2), with concurrent formation of their deamidated products. These three peptides were identified using automated database searching with high scores of confidence (over 60). The peptide TAAENEFVALK and its deamidated product, from a cytoskeletal IFP, K75, were also observed to respectively degrade and form during exposure to heat. Consistent increases in the oxidative modification of the susceptible tyrosine
and tryptophan residues (to form AEAES[Kyn][dopa]R) were associated with the degradation of AEAESWYR (from Type II K86). Figure 7.1 illustrates the characteristic MS/MS fragmentation ions used for identification and quantitation of iTRAQ-labelled AEAESWYR in a pooled sample.

Additional peptides demonstrated consistent degradation patterns, but were not associated with consistent increases in degradation products. These are listed in the two lower sections of Table 7.1. The confidence in the peptide identifications for these markers ranged more widely than the three most certain marker peptides (based on peptide ion scores, from 16 to 90). Parent peptide degradation, as determined by measuring iTRAQ reporter ion relative abundance, is represented graphically in the right-most column.

![Figure 7.2](image_url) Decreasing relative abundance of marker peptides in wool keratins after exposure to hydrothermal insult at 90°C, as determined by iTRAQ reporter ion abundance in MALDI-MS/MS. Error bars represent ± standard deviation (SD). Initial relative abundance defined arbitrarily as 1.0. Insets: Changing relative abundance of deamidated products of ATAENEFVALK, ENAELESR, and SNHEEEVNTLR.

Many peptides that did not qualify to be classed as damage markers displayed modifications after hydrothermal damage. These included a number of single or double deamidations (noted on IFP peptides DSLENTLTEEAR, HQEKEQIK, IDPNIQVR, LESEINTYR, LLEGEEQR, LNVEVDAAPTVDLHLNVEI, LQAQNVR, LSELNSLQEVLG, LTAENVAK, LYNLGGTR, LYQEEIR, NHEEANSNR, QLERENAELESR, QNEHEQVNTLR, QNEQYQVLLDVR, QTEELNK, RLYEEEIR, SDLEANSEALIQEIDFLR, TKYTELGLR, TVNALEVELQAQHNL and YEEVEALR). Additional oxidative modifications to aromatic residues that were noted included tyrosine derivatives (dopa in
DAAYLNK, ERQNQEYRVLLDVR, QDMASLVKQY and VVTSEQQLQSYQSDLDLR, dopa-quinone on LQEVLEEGK, SKYETELGLR and YOSYFR, topa-quinone on QSYQSDDLDR and SLYNLG); tryptophan derivatives (kynurenine in DVEEWYIR and WQLYQNR, hydroxytryptophan in AEAESWYR, dihydroxytryptophan/ N-formylkynurenine in AEAESWYR and DVEEWYIR, hydroxykynurenine in FYQNQR); and a histidine derivative (hydroxyhistidine in LLKPLNLEIDHNQR).

Table 7.1 Marker peptides for molecular-level hydrothermal damage observed by MS/MS in keratin proteins from wool. Relative abundance of parent peptide as determined by the measurement of iTRAQ reporter ions (peak area) represented graphically in the right-most column.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Source protein(s)</th>
<th>Mass</th>
<th>Score*</th>
<th>Degradation product</th>
<th>Reporter ions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Excellent marker peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATAENEFVALK</td>
<td>Type II K86</td>
<td>1479.81</td>
<td>95</td>
<td>+ deam (N)</td>
<td></td>
</tr>
<tr>
<td>EMAELSR</td>
<td>Type I K33b, K34</td>
<td>1090.53</td>
<td>62</td>
<td>+ deam (N)</td>
<td></td>
</tr>
<tr>
<td>SNHEEVNTR</td>
<td>Type I K31</td>
<td>1470.71</td>
<td>87</td>
<td>+ deam (N)</td>
<td></td>
</tr>
<tr>
<td><strong>Good marker peptides</strong>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAFDCK</td>
<td>Type II K86</td>
<td>1098.63</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLEQCNK</td>
<td>Type II K86</td>
<td>1193.66</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LQFYQNR</td>
<td>Type II K86</td>
<td>1111.59</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHYDDIASR</td>
<td>Type II K86</td>
<td>1190.58</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AQQDDIASR</td>
<td>Type II K86</td>
<td>1181.57</td>
<td>65</td>
<td>+ kyn (W)</td>
<td></td>
</tr>
<tr>
<td>EEINELNR</td>
<td>Type II K86</td>
<td>1159.6</td>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEAESWYR</td>
<td>Type II K86</td>
<td>1154.54</td>
<td>55</td>
<td>+ kyn (W), + copa (Y)</td>
<td></td>
</tr>
<tr>
<td>TAAENEFVALK</td>
<td>Type II K75</td>
<td>1479.82</td>
<td>50</td>
<td>+ deam (N)</td>
<td></td>
</tr>
<tr>
<td><strong>Acceptable marker peptides</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AQYEALVETNR</td>
<td>Type I K33b, K34</td>
<td>1436.73</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLTGGFGSR</td>
<td>Type II K83</td>
<td>994.53</td>
<td>72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGDLTR</td>
<td>Type II K86</td>
<td>855.43</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISPGYSVTR</td>
<td>Type II K86</td>
<td>1122.61</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAADDFR</td>
<td>Type I K33b, K32</td>
<td>950.49</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RISGGFR</td>
<td>Type II K75</td>
<td>1022.57</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TKYETEVSLR</td>
<td>Type I K33b, K34</td>
<td>1512.83</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YEEEIRVL</td>
<td>Type II microfibrillar</td>
<td>1193.64</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YESER</td>
<td>Type I K34</td>
<td>826.39</td>
<td>22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# Highest score observed; * Degradation of parent peptide observed in both samples, degradation product observed to increase; ** Degradation of parent peptide observed in both samples, or in one sample accompanied by an increase in a degradation product; *** Degradation of parent peptides observed in one sample.
7.5 Discussion

Isobaric quantitation of hydrothermally damaged protein samples allowed the observation of consistent degradation in a number of peptides. This resulted in the identification of peptides that served as markers of hydrothermal damage. These peptides were all present in the samples in sufficient abundance to allow detection and automated identification. Each damage marker proved sensitive to hydrothermal exposure, consistently decreasing in abundance over the treatment period. While degradation products were observed for many of the damage markers, those reported here demonstrated expected increases in abundance along with treatment. These modified peptides may themselves be utilised as (inverse) markers of damage.

A comparison of this study to that presented in Chapter 6 reveals some elements in common: Both studies identified SNHEEVTNR and AQYEALVETNR from Type I IFPs and FLEQQNK, AEAESWYR and TAAENEFVALK from Type II IFPs as damage markers (even though based on different criteria – the presence of degradation products only in treated samples in the first instance, and consistent decreases in abundance, as measured by iTRAQ reporter ions, in the second instance). This provides additional support for their suitability for this role.

As evidenced by the small number of modified peptides found to be suitable for this usage in this study, the use of degradation products as molecular-level markers of damage presents a higher level of difficulty in complex samples, due to the low abundance of such peptides. Nevertheless, while making full use of the quantitative information provided using damage markers, less-abundant modifications observed within samples may provide information on the ROS or other factors contributing to the modification footprint.

7.6 Conclusions

The identification and isobaric quantification of peptide markers of hydrothermal degradation within wool proteins has provided a set of tools to assess the extent of damage inflicted on a given sample. This can be applied to validating the effectiveness of a protective treatment, or the detrimental side-effects of a potentially damaging high-temperature process. Three sequences were classified as excellent marker peptides and have a high likelihood of observation in wool samples, together with their degradation products. These peptides display measureable and consistent sensitivity to hydrothermal exposure, along with hydrothermally-induced formation of deamidated products. Those peptides classified as good and acceptable marker peptides may
prove to be reliable only in some samples, but nevertheless show good potential for use in this manner.

Although these peptides have been selected for their use in evaluating protein damage in wool, similar markers may be utilised to evaluate damage in related substrates such as human hair, skin, or nails. This study demonstrated that quantitative mass spectrometry using isobaric labels permits the acquisition of data sensitive enough to detect the treatment-induced changes in abundance of specific peptides. We anticipate that the development of targeted protective treatments for protein-based materials and tissues, or of milder processing and treatment high temperature steps for protein-products and personal care applications, will be assisted by the advanced damage tracking protocols presented here.


8 Research programme outcomes

This thesis reports a series of chapters progressing our understanding of the effects of light and heat on peptides and proteins, with especial focus on the intermediate filament proteins of wool, an agriculturally important New Zealand product. Mechanistic studies involving the exposure of synthetic model peptides first examined the degradation of simple peptides exposed to UVA, UVB or 90°C under aqueous conditions. Conclusions were drawn regarding the involvement of ROS in both light- and heat-induced polypeptide degradation, and approaches to generating degradation profiles were developed using label-free and isobaric mass spectrometric technologies.

8.1 Discussion

8.1.1 Characterisation and profiling of the degradation of model peptides

The first research goal of the programme was to thoroughly characterise the degradation of amino acid residues in model peptides using mass spectrometric techniques. By initially investigating a simplified system, using synthetic peptides, the degradation of two important contributors to protein-based chromophore formation, tryptophan and tyrosine, could be studied without the complications implicit in studies of more complex materials. This allowed robust conclusions to be drawn regarding the mechanisms of photo- and hydrothermal-induced degradation.

Mass spectrometric investigations of model peptides damaged by exposure to UV light (UVA and UVB) resulted in the comprehensive characterisation of the photoproducts of tryptophan and tyrosine (Chapter 4). The products formed and profiled pointed to the involvement of two unexpected reactive species, hydroxy radical and peroxynitrite (or a closely related species). Notably, this work directly demonstrated for the first time the formation of W+4O modification (postulated to represent dihydroxyformylkynurenine), hydroxy-bis-tryptophandione (W+3O-4H), and topa-quinone (Y+2O-2H). The observation of nitrotryptophan and nitrotyrosine within aqueous peptide solutions indicated the formation of peroxynitrite via the interaction of ROS with peptide breakdown products. The observation of these nitrated residues within irradiated proteins provides confirmation for previous reports of detections (using aqueous protein systems) within irradiated wool and collagen proteins (Dyer et al. 2006b; Dyer et al. 2009). It is hypothesised that the degradation of polypeptides to ammonia (Inglis & Lennox 1963; Reubsaet et al. 1998) provides the basis for the generation of reactive nitrogen species within these...
aqueous systems. The nitrates formed by the irradiation of ammonia may themselves be photo-oxidised to peroxynitrite (Plumb & Edwards 1992; Zheng et al. 1998; Beckles & DIYamandoglu 2006).

peptides (UV) → ammonia → nitrates and nitrites (UV) → peroxynitrite

The second research goal was to develop MS-based quantitation protocols for the observation of modifications to specific amino acid residues. A relatively straightforward mass spectrometric approach to relative quantitation was trialled in the simplified model peptide system, allowing comprehensive degradation profiles to be established (see Chapter 4). These analyses allowed the formation of individual modifications to peptides to be monitored over time and were based on the ion count of each peptide and its modification in relation to the total ion signal. This method allowed the degradation of parent peptides and the formation of the products to be presented graphically in an interpretable fashion. This approach was less accurate where the abundance of modified species was very low (this was particularly a problem for the lower abundance tyrosine modifications).

An improved quantitative profiling approach is reported in Chapter 5. Here, a commercially available isobaric label system for quantitation (iTRAQ) was used to generate degradation profiles based on the ion count of specially designed reporter ions tagged to each sample, and measured during tandem MS. The relative abundance of the reporter ions in MS/MS reflects the relative abundance of a given peptide within the sample, while MS scans were used to provide information on which modifications within parent peptides were most abundant. This improved tracking approach aided detailed degradation profiling. This application of iTRAQ differed from its conventional usage, as reporter ion information from only one peptide was required to draw quantitative information. Although using only one peptide for quantitation meant that there was less information available, the data confirmed that isobaric labels may confer information of sufficient accuracy from individual peptide modifications to provide quantitative information on even low-abundance derivatives.

8.1.2 Characterisation and profiling of the degradation of intermediate filament proteins

The third research goal was to locate and characterise protein degradation products in wool at a primary structural level. Using approaches developed in the model peptide work, the effects of hydrothermal degradation on IFPs extracted from wool were investigated (Chapter 6). Among the modified peptides (degradation products) that were sufficiently abundant and that ionised
sufficiently well in MALDI-MS to allow confident automated identification, some that were observed in hydrothermally-treated samples were not present within the control (Chapter 5), so can be utilised as markers. The degradation products included oxidative modifications to tyrosine, tryptophan and histidine residues, in addition to acetylated and deamidated products. These products were identified at specific amino acid residues (the primary structural level) within identified wool proteins, providing information on the effects of hydrothermal protein degradation at a level of detail previously unreported.

Although the effects of heat shock have been described in living organisms, degradation within such systems reflect the influences of more than simply heat, for instance the action of enzymes such as proteases and the release of cellular ROS (Parag et al. 1987; Miller et al. 2008). In contrast, this work resulted in the first report of the molecular-level effects of hydrothermal exposure on a protein system exterior to the cell, revealing the role of ROS in hydrothermally induced protein degradation itself. This was an exciting discovery, as ROS involvement in heat-induced extracellular protein degradation has not previously been reported.

The final research goal was to develop robust proteomic-based approaches to quantify protein damage in wool. Using an isobaric mass spectrometric labelling technique (iTRAQ reagents), the abundance of native and modified peptides was tracked in untreated and treated (exposed to elevated temperature) wool protein samples (Chapter 7). This resulted in the identification of a number of peptide damage markers, which may now be used to evaluate the extent of hydrothermal damage before and after processing steps or the application of treatments. This provided confirmation that the isobaric quantitative strategy provides sufficiently sensitive detection of quantitative sample-to-sample differences in individual peptides to permit tracking of a marker throughout a damage protocol.

8.1.3 Assumptions

8.1.3.1 Model peptide modifications

It has been commented that the peptide truncations and oxidation observed in ESI during the model peptide work may sometimes be due to fragmentation within ESI, rather than in the sample itself. However, in the model peptide samples, ESI-induced oxidation and cleavage is unlikely given the following considerations: (1) The reproducibility of the experimental results imply real experimental changes (2) Zero time controls included in the experiments compensate for any artefactual elevation of modifications (3) Electrolyte levels were low, minimising the
chances of ESI fragmentation (Wang et al. 2010). Capillary voltages applied (1.0 kV) were below the levels determined to be detrimental to sample integrity (Boys et al. 2009).

8.1.3.2 Molecular species-to-species comparisons

The quantitative profiling portions on this work used both label-free (Chapter 4) and iTRAQ label routes (Chapter 5) to compare the relative abundance of specific peptides between samples. Allowing for the errors implicit in each system, this is a safe assumption, as this step compares identical molecules (with identical ionisation efficiencies). In order to provide an additional level of information, however, sample-to-sample comparisons were supplemented by species-to-species comparisons. These used the ion counts of particular peptides (e.g. LLWLR and LL[Kyn]LR) to represent their relative abundance in a sample. This representation is based on the assumption that these two species are detected similarly in ESI-MS/MS. It is known that sometimes, chemical changes to peptides may alter their ionisation (Brancia et al. 2002), but the information gleaned from species-to-species comparisons is so useful that this assumption is deemed worthwhile. Moreover, although the peptides compared in this manner do differ chemically in one residue, they can still be regarded as possessing similar chemical properties due to their otherwise identical natures. Species-to-species comparisons were accordingly restricted to peptides differing by only one residue.

8.1.3.3 Sample recovery

Quantitative comparison between samples relies on either complete recovery of all samples, known recoveries of all samples, or identical recoveries for all samples. In the model peptide samples, this was accomplished by eliminating evaporation (particularly problematic for heated samples) and using a peptide concentration that was readily soluble in water. The method finally selected for optimal heat treatment involved the use of robust internal vials (Reacti-Vials) – this is detailed in the Methodology chapter, section 3.3.2. The same treatment approach was utilised for the heating of IFPs, but heating protein solutions results in another problem – aggregation and precipitation. The potential exists for treated samples to display lowered solubility and thus sample recovery than their untreated equivalents, due to increased crosslinking and aggregation. This was addressed by the selection of the most soluble component of the wool proteins, the IFPs (initial trials using alternative components – the ultrahigh sulfur proteins and the high glycine tyrosine proteins – were plagued with insolubility issues), and selecting only that fraction of the IFPs that was readily soluble in the buffer of choice. In addition, the treatment times used for hydrothermal exposure of wool proteins were greatly reduced from those used during peptide
treatment. This had the advantage of generating a less degraded (and less complex) treated sample, resulting in simpler mass spectrometric analysis, which was mild enough to eliminate problems with precipitation after treatment. No precipitates were detected after treatment and digestion, suggesting complete sample recovery. Consequently, sample-to-sample comparisons assumed equal recovery of all samples.

8.1.3.4 Product identifications

The identification of oxidative peptide and protein products in this study was based on mass spectrometric evaluation. Changes to peptides are detected by changes in their mass; by fragmenting the ions in tandem mass spectrometry, the mass of each amino acid residue within a protein can be determined, and its deviation from the unmodified mass calculated. In the model peptide work, these mass changes were restricted to the tyrosine and tryptophan residues. In a simplified system such as this, the identity of the ions detected can be deduced more readily, as the parent peptides are known. The assignment of modified residues to putative products was based on possible atomic changes that could give rise to such a mass change, and on what is known about the chemistry of tryptophan and tyrosine degradation. For instance, the observation of a residue of mass 190.08 Da (4 Da greater than tryptophan, at 186 Da) was assumed to represent a known tryptophan product, kynurenine (W + 2O – CO) (Davies & Truscott 2001). This, in turn, provided evidence as to the nature of the residue of mass 206.07 Da (20 Da greater than tryptophan), which was described as the known tryptophan product, hydroxykynurenine (W + 3O – CO) (Bienvenut et al. 2002). The identification of wool-derived peptides and their modifications was based on automated searching against a database of known wool proteins, with allowance for variable modifications based on the observations in the model peptides. Hence, there is a high level of confidence in the degradation product assignments.

8.1.4 Non-wool applications

This thesis focussed on the oxidative degradation behaviour of protein-based systems to protein damage in wool and wool products, due to the importance of these products to New Zealand. Nevertheless, the findings have value for other systems. In addition to the clear relevance of the role of specific ROS in light- and hydrothermal-induced residue damage (as demonstrated in the model peptide work) and in methods to identify molecular markers of damage in any protein system, the degradation and damage markers identified in the specific keratin proteins examined in this work will probably be applicable to related materials.
The wool IFPs studied in this thesis are type I and II IFPs (the keratinous IFPs), which are also present, with species-specific variations, in human hair, skin and nails (Schweizer et al. 2006; Gu & Coulombe 2007; Szeverenyi et al. 2008), and mammalian fur, horns and claws (Clerens et al. 2009; Fudge et al. 2009; Tombolato et al. 2010). Type I and II IFPs share significant sequence homology, particularly in the α-helical regions, with other IFPs, the type IIIs (vimentin and desmin), the type IVs (nestin, synmenin and neurofilament triplet proteins), and the type Vs (the nuclear lamins) (Fisher et al. 1986; Eriksson et al. 2009). It is anticipated, therefore, that those regions of wool IFPs that proved especially sensitive to oxidative damage (particularly the identified damage marker peptides) may be echoed in related proteins, meaning that these damage markers may be of use in assessing damage in a very wide range of substrates.

8.2 Concluding remarks

The research reported in this thesis has significantly advanced our knowledge regarding the nature of residue-level damage within peptides and proteins in response to irradiation and hydrothermal exposure. Novel insights were drawn concerning the involvement of reactive oxygen species in UV- and hydrothermal-induced polypeptide damage, and in the response of wool proteins to heat and light in a complex system.

Close mass spectrometric evaluation of peptide samples after exposure to heat and light revealed the formation of multiple tyrosine and tryptophan products. These included both known and previously unrecorded modifications. This demonstrated the involvement of ROS in the photo-oxidative and, notably, heat-induced degradation of the peptides. Specifically, products specific to the action of hydroxyl radical and a reactive nitrogen species (most probably peroxynitrite) were detected.

The label-free and isobaric labelling approaches to quantitative profiling of primary and secondary degradation products that were developed using model peptides provided an excellent foundation for application to proteinaceous samples, along with detailed information on the degradative profile of peptide-bound tyrosine and tryptophan. This provided new insight into the pathways of formation of residue modifications and into the chemical species or physical stresses that give rise to specific changes in wool proteins. The techniques demonstrated the excellent sensitivity of mass spectrometry-based techniques to small changes in the abundance of modified peptides. The protocols developed during this research will assist future fundamental wool
researchers in assessing and tracking damage and in developing novel procedures to ameliorate damage associated with wool protein degradation.

### 8.2.1 Future directions

This programme focussed on the mass spectrometric identification of protein degradation products. Conclusions were formed regarding the nature of the causative ROS on the basis of product identification and plausible routes of formation. The results presented in this thesis provide ideas for research in related disciplines. Investigations into the chemistry of wool and more general chemical oxidation could focus on definitively identifying the ROS responsible, perhaps through spin-trapping or other ROS assays, and the conclusive characterisation of protein product isomers, such as the W+2O products, $N$-formylkynurenine and dihydroxytryptophan. Such investigations will provide useful supplementary information to researchers focussed on mitigating the negative effects of oxidative protein degradation.

Regarding industrial applicability, it is anticipated that the marker peptides identified in wool IFPs will provide a molecular tool for the evaluation of degradation in whole wool samples and wool-based products. Sensitive molecular markers of oxidative damage are ideal tools to track and map the impact of processing protocols, environmental exposure, or leading edge protective treatments on wool quality. The influence of factors like bleaching, high-temperature dyeing, carbonising, and shrink-resist treatments will be able to be evaluated for the first time with highly sensitive techniques. It will also be of interest to observe how the degradation of marker peptides correlates with more traditional markers of damage, such as alkali solubility, dye uptake, or tests of strength. Research concerning human tissues containing proteins that bear similarities to those from wool, such as skin, hair and nails, that are also subjected to protein oxidation and premature ageing will no doubt also benefit from our increased understanding of the processes of structural protein degradation.

Quantitative mass spectrometry combined with the molecular damage marker approach need not be restricted to wool or intermediate filament applications. As brought out in Chapter 2, oxidative protein damage is of critical importance in many materials. The techniques developed in this programme will be of use in evaluating the effects of external damaging influences on other proteinaceous products of commercial importance. These include high-protein foods (particularly meat, in which the protein content contributes to key attributes such as texture and flavour), cosmetics, and other natural textiles such as silk. Ultimately, this approach may also benefit
research into intracellular systems, where protein oxidation contributes to a large range of disease states.
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