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ORIGINAL ARTICLE

Profiling of residue-level photo-oxidative damage in peptides

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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. Electrospray ionisation tandem mass spectrometry (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.

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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 and Giacomoni 2007), and is of particular importance in biological systems such as skin (Kato et al. 1992; Girotti and Giacomoni 2007), hair and eyes (Davies and 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 sulphur-containing amino acids (Berlett and Stadtman 1997). With respect to photomodifications leading to discolouration 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, 2006a, b; Bringans et al. 2006). 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.

We report 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). We also outline the development and application of an MS-based approach to establishing relative modification abundance patterns. 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.

We anticipate 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.

Methodology

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, USA). MS analysis confirmed the presence of these peptides, along with the truncations WLR and YLR. ChromAR® LC-grade water was obtained from Mallinckrodt Chemicals; LC-grade acetonitrile from J.T. Baker (Phillipsburg, NJ, USA); Univar formic acid from Ajax Finechem (Auckland, NZ); and NanoES spray capillaries from Proxeon Biosystems (Odense, Denmark).

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, Ontario, Canada) 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 52,670 mW m^{-2} (Luzchem 2004a, b). Irradiation was performed over 12 h (UVB) and 24 h (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.

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*, Applied Biosystems, Foster City, CA, USA), 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 (Applied Biosystems).

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 $[M + H]^{1+}$ 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 min 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).

Table 1 Observed photomodifications to tyrosine and tryptophan residues

$\Delta m/z$	Modification	Corresponding photoproduct(s)
Tyrosine		
+16	+O	Dopa/dienone alcohol
+32	+2O	Topa
+14	+O–2H	Dopa-quinone
+30	+2O–2H	Dopa-quinone
+45	+N+2O–H	Nitrotyrosine
Tryptophan		
+16	+O	Hydroxytryptophan/oxindolylalanine
+32	+2O	NFK/dihydroxytryptophan
+4	+O–C	Kynurenine
+20	+2O–C	Hydroxykynurenine
+48	+3O	Hydroxyformylkynurenine
+64	+4O	Dihydroxyformylkynurenine
+30	+2O–2H	Tryptophandione/dihydrodioxoindole
+28	+2O–4H	β-Unsaturated-2,4-bis-tryptophandione
+44	+3O–4H	Hydroxy-bis-tryptophandione
+12	+C	Tetrahydro-β-carboline
+45	+N+2O–H	Nitrotryptophan

Results

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 1.

Tyrosine

Several modifications to tyrosine residues were characterised in model peptides by detailed analysis of MS/MS fragmentation patterns (See the "Discussion" section and Fig. 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.

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 + NO_2–H).

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 Fig. 7).

ESI-MS and MS/MS fragmentation revealed the presence of W + 16 Da (W + O) and W + 32 Da (W + 2O) modifications. ESI-MS/MS spectra of these modifications are shown for LLW*LR in Fig. 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.

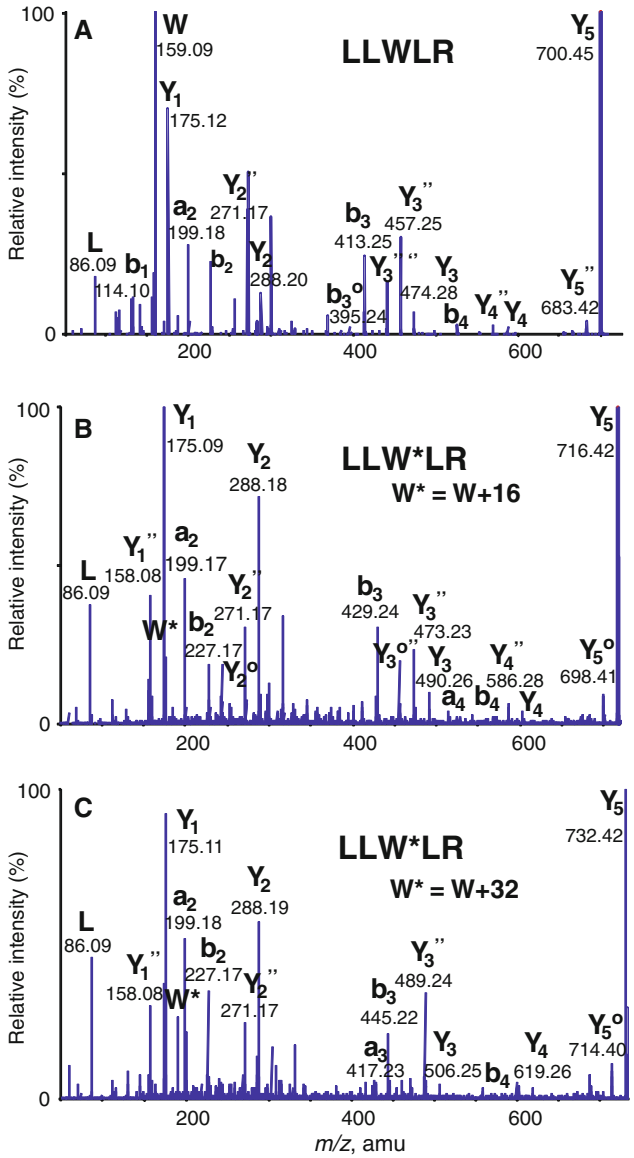

Fig. 1 ESI-MS/MS spectra of *LLWLR*-derived ions: **a** 0 h UVB, no W modification; **b** 10 h UVB, W + O modification; **c** 10 h UVB, W + 2O modification. The y-, b- and immonium fragment ions are annotated

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 the "Methodology" section, "Data analysis"). 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 *N*-formylkynurenine (NFK)/dihydroxytryptophan (W + 2O), respectively (see the "Discussion" section, "Characterisation"). Peptides containing tryptophan residues (Figs. 2, 3) were observed to be modified considerably more rapidly than those containing tyrosine residues (Figs. 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

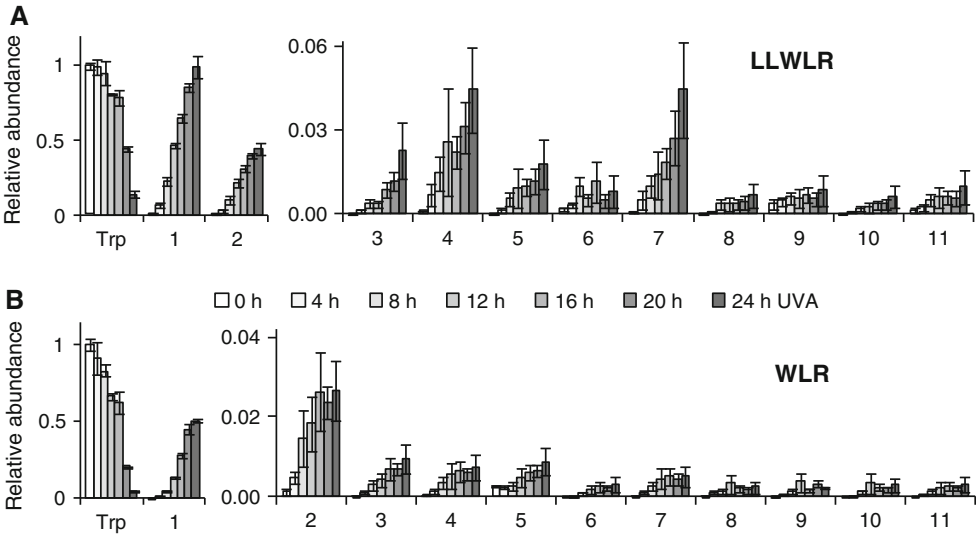

Fig. 2 Relative peak areas of **a** *LLWLR* and **b** *WLR* and photomodifications of these after *UVA* irradiation: *1* W + O; *2* W + 2O; *3* W + O–C; *4* W + 3O; *5* W + 4O; *6* W + 2O–C; *7* W + 2O–2H; *8* W + 2O–4H; 9 W + 3O–4H; *10* W + C; *11* W + N + 2O–H. Spectra peak areas are internally weighted, 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 mean (±SEM)

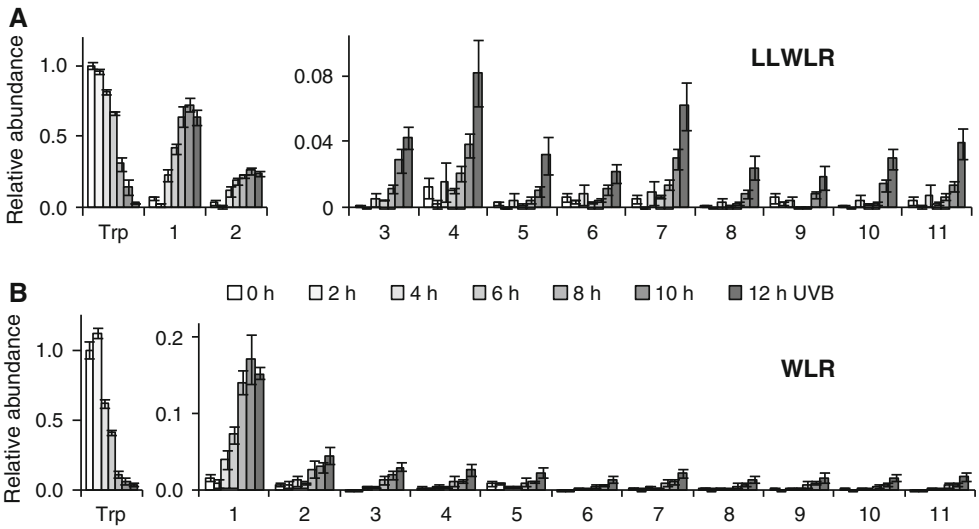

Fig. 3 Relative peak areas of **a** *LLWLR* and **b** *WLR* and photomodifications of these after *UVB* irradiation: *1* W + O; *2* W + 2O; *3* W + O–C; *4* W + 3O; *5* W + 4O; *6* W + 2O–C; *7* W + 2O–2H; *8* W + 2O–4H; *9* W + 3O–4H; *10* W + C; *11* W + N + 2O–H. Spectra peak areas are internally 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 mean (±SEM)

observable modification levels. After 12 h UVB irradiation, LLWLR and WLR W + O (hydroxytryptophan) photoproducts were observed to decrease.

Discussion

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 an ideal 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 textiles (Goddinger et al. 1994; Bringans et al. 2006; Dyer et al. 2006a, b) and 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 discolouration (Hains and Truscott 2007) and age-related disease (Batthyány et al. 2000), has been strongly associated with protein degradation leading to protein product performance degeneration,

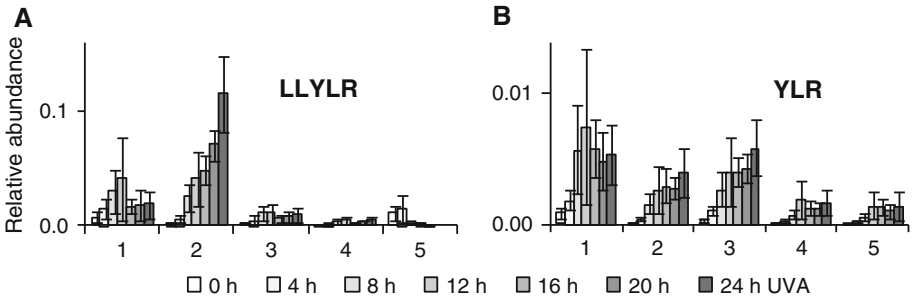

Fig. 4 Relative peak areas of **a** *LLYLR* and **b** *YLR* and photomodifications of these after *UVA* irradiation: *1* Y + O; *2* Y + 2O; *3* Y + O–2H; *4* Y + 2O–2H; *5* Y + N + 2O–H. Spectra peak areas are internally weighted, and the relative abundance of the peak area of the unmodified peptide at zero time is defined as 1.0. *Error bars* represent standard errors of the mean (±SEM)

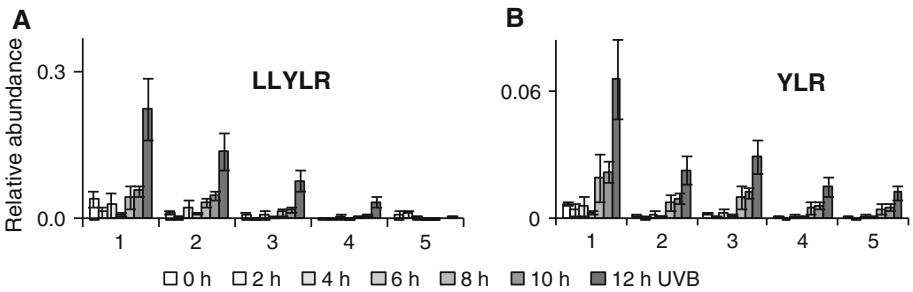

Fig. 5 Relative peak areas of **a** *LLYLR* and **b** *YLR* and photomodifications of these after *UVB* irradiation: *1* Y + O; *2* Y + 2O; *3* Y + O–2H; *4* Y + 2O–2H; *5* Y + N + 2O–H. Spectra peak areas are internally weighted, and the relative abundance of the peak area of the unmodified peptide at zero time is defined as 1.0. *Error bars* represent standard errors of the mean (±SEM)

particularly photoyellowing (Lennox et al. 1966). Free and bound tryptophan have been found to produce a variety of products upon oxidation (Simat and Steinhart 1998; Davies 2004). The peptide solutions were irradiated with tight wavelength bands in the UVA and UVB ranges.

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. Identifying 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 and Lawrence 1995; Yamakura et al. 2005; Dyer et al. 2006a, b) 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 Figs. 6 and 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 (RNS), probably peroxynitrite, were also implicated as contributing to photooxidative 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, 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, 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

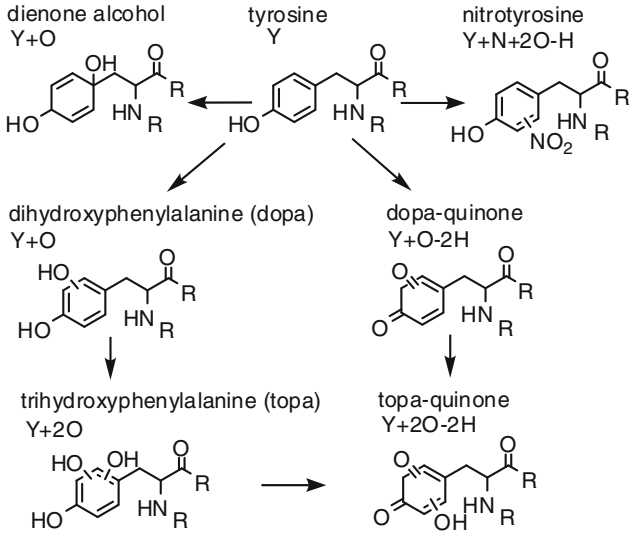

Fig. 6 Proposed pathways of tyrosine degradation to photoproducts (Dyer et al. 2006b; Ogata 2007; Mizdrak et al. 2008)

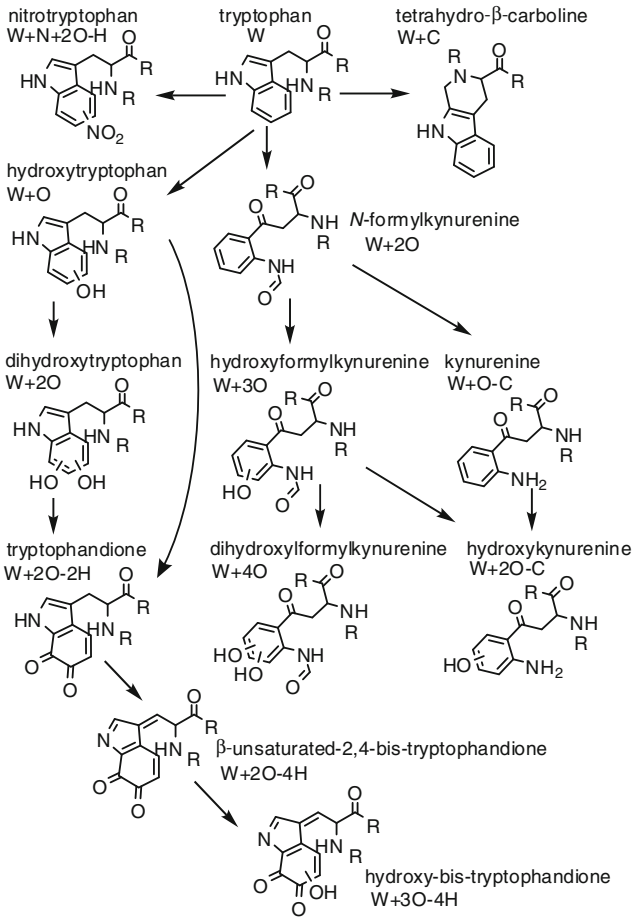

Fig. 7 Proposed pathways of tryptophan degradation to photoproducts (Davies and Truscott 2001; Dyer et al. 2006a, b)

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 (Rescigno et al. 1998; Rosenberg et al. 1991), 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 et al. 1991). 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 and Steinhart 1998; Dyer et al. 2006a, b) [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 and 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 and Truscott 2001).

The MS/MS characterisation of W + 48 and W + 64 Da modifications, respectively, indicates the addition of three oxygen atoms, which implicates the formation of the coloured photoproduct, hydroxyformylkynurenine (Nakagawa et al. 1985; Bienvenut et al. 2002), and of four oxygen atoms to a single tryptophan residue,

which may represent dihydroxyformylkynurenine, probably formed from hydroxyformylkynurenine. The formation of a W + 4O oxidation product has not been specifically reported previously. A further possible derivative of hydroxyformylkynurenine was characterised in the form of a W + 20 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 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 and Davidson 2002) or its isomer, 2,6-dihydro-2,6-dioxindole (Hara et al. 2001; Pfister et al. 2005). Tryptophandiones may be formed via hydroxytryptophan oxidation (Wu and 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 and Meybeck 1965)], to form yellow 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 protein photo-oxidation pathways.

The identification of X + 45 Da modifications on the tryptophan- and tyrosine-containing peptides, consistent with W + NO_2–H (nitrotryptophan) and Y + NO_2–H (nitrotyrosine) formation, suggests the formation and attack of a RNS, such as peroxynitrite. Protein-bound nitrotryptophan and nitrotyrosine have previously been observed to form in the presence of potent RNS (Abello et al. 2009; Alvarez et al. 1996; Ischiropoulos and Al-Mehdi 1995; Yamakura et al. 2001). 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, 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 and 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 and Diyamandoglu 2006). Peroxynitrite is a primary UV photoproduct of nitrates (Plumb and Edwards 1992). Other RNS that may cause tyrosine and tryptophan nitration include NO_2• radicals, nitric oxide, nitrogen dioxide and nitrous acid (Abello et al. 2009).

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 indicate the contribution of more than one ROS, and particularly implicate hydroxyl radicals, along with singlet oxygen and peroxynitrite.

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 1) were profiled.

A primary application of modification profiling is its use in identifying appropriate peptide candidates to function as oxidative damage markers 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 (Figs. 2, 3, products 1 and 2).

MS/MS-based characterisation allowed the localisation of photoproducts present at very low abundance to specific residues within peptides. 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), whereas

abundance profiling shows the majority of 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 (Figs. 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 (Fig. 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 oxidation (Igarashi et al. 2007; Kerwin and Remmele 2007).

UVB irradiation was observed to generate proportionately more minor (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 (Figs. 2, 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 Fig. 3a (LLWLR) with Fig. 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, as an alternative to the use of a single standard (Gracanin et al. 2009), allowed the comparison of relative abundance across consecutive samples (compare Ehrenshaft et al. 2009, where a similar but unweighted approach only allows quantitative comparison between modified species, not across time-points). 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.

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 dihydroxyformylkynurenine), 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.

We have developed and validated a relatively straightforward approach to the profiling of residue modification in peptides. This profiling involved visualizing modifications to target residues over time in terms of both the pattern of generated species and their respective changes in relative abundance. We envisage 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.

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Glossary

ESI-MS	electrospray ionisation mass spectrometry
MS/MS	tandem mass spectrometry
NFK	*N*-formylkynurenine
ROS	reactive oxygen species
RNS	reactive nitrogen species

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