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Changes to amino acid composition of bloodmeal after chemical oxidation

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Bovine-derived bloodmeal can be decoloured using peracetic acid, extruded and injection moulded into a yellow translucent bioplastic. This plastic has different properties to extruded and injection moulded bloodmeal, in that it does not require sodium sulfite or urea to be extrudable. The effect of oxidation on the physical and chemical characteristics of the proteins during bloodmeal decolouring with PAA was studied by assessing changes in the amino acid profile. Polymer interactions, hydrophilicity and the destruction of cysteine crosslinks were measured indirectly by assessing protein solubility, molecular weight distribution and by synchrotron FT-IR analysis. Increasing peracetic acid concentration resulted in an increased loss of iron due to destruction of the porphyrin groups, increased solubility due to destruction or conversion of aromatic amino acids into hydrophilic groups, destruction of lysine, reduced protein content due to increased salt content in the final product, and a larger amount of smaller protein peptides but with a similar average molecular weight to bloodmeal. Amino acid analysis showed an increase in cysteine content in the product, FTIR of the sulfur groups revealed that these were heavily oxidised, such that some would be unable to participate in disulfide bonds thereby increasing protein solubility.

Introduction

A semi-transparent bioplastic has been produced from bloodmeal by decolouring it using commercial peracetic acid (PAA) followed by extrusion.[1-3] Decolouring bloodmeal using 3 – 5 wt% PAA has been found to result in the lowering of the glass transition temperature from ~225 °C to ~35 – 45 °C[2] and also to require different processing aids for extrusion. In contrast to thermoplastic prepared from untreated bloodmeal, once decoloured, bloodmeal no longer required sodium sulfite reduction of the cysteine crosslinks in order to be extruded.[2]

Peracetic acid, which is an equilibrium mixture of peracetic acid, hydrogen peroxide and acetic acid, has been used extensively since the early 1940s due to growing concerns over the environmental impact of chlorine. It is used as a bleaching agent for textiles, as a disinfectant,[4-7] as well as in wastewater treatment. It does not form harmful disinfection by-products and decomposes safely when discharged into the environment.[8, 9]

Common oxidants such as hydrogen peroxide are unable to degrade all of the haem present in bloodmeal due to the various oxidation states of haem-iron in haemoglobin.[3, 10] However, peracetic acid adequately removed the colour from bloodmeal, with each component of the PAA solution performing a specific function in the overall decolouring and deodorising mechanisms.[10]

The presence of several reactive species and the formation of free radicals, resulted in oxidative damage to the proteins as the radicals underwent auto-reduction reactions.[11] Consequently, treating proteins with oxidising agents leads to undesired side effects, including modification of amino acid residues, protein hydrolysis or chain cleavage and protein aggregation.[7, 12] The type of damage and the extent of modification varied depending on the reduction potential and concentration of the oxidant, as well as the presence of other species which exhibited anti-oxidant or free radical scavenging properties.

Bleaching wool with performic or peracetic acid selectively oxidised cystine (disulfide crosslinks), methionine and tryptophan side chains with minimal significant degradation of the other amino-acids.[13] However, a later study indicated that performic acid also degraded serine, threonine, tyrosine, phenylalanine and histidine, while PAA oxidation resulted in the conversion of all cystine to cysteic acid, with only small losses of tyrosine, phenylalanine and histidine.[14] Oxidation of cystine bonds also occurs during hydrogen peroxide bleaching of human hair (keratin is highly crosslinked as a result of disulfide bonds between cysteine residues). As a result, oxidation of hair and wool by hydrogen peroxide or peracetic acid led to a loss in tensile strength proportional to the number of cystine residues degraded.[15-17] Oxidation of bovine serum albumin and aldolase proteins with PAA was found to induce chain fragmentation.[18]

Oxidation of amino acids in proteins is dependent on oxidant strength, concentration, pH and a range of other chemical

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conditions. The most recent investigation into the effect of PAA oxidation on dairy proteins, indicated that the amount of carbonyl groups increased while thiol (SH) groups decreased; tryptophan and methionine were most vulnerable to oxidation while lysine was not affected.[7] Neither peptide cleavage, nor protein aggregation occurred to any significant extent.[7] This is probably as a result of the protective effect of short chain carboxylic acids (or their salts) on proteins exposed to hydrogen peroxide, which has been attributed to their action as chelating agents (inhibiting Fenton-type reactions) or as scavengers of reactive oxygen species and free radicals;[19, 20] both of the latter cause protein hydrolysis. This concurs with peracetic acid decolouring of bloodmeal, in which the presence of acetic acid (or sulfuric acid) inhibited the reactivity of hydrogen peroxide.[10]

Helices, sheets, turns and coils which define the secondary structure of the protein are modified by oxidation.[21, 22] Amino acids show a propensity to form particular types of secondary structures, with most amino acids showing preference for only one type (Table 1),[23] although, the periodicity and positioning of polar and non-polar residues in the amino acid sequence is known to have a greater influence on a peptide's final secondary structure.[24] Selective oxidation of certain amino acids may influence the formation of specific secondary structures by changing the ability to form H bonds between C=O and NH groups.

The sensitivity of particular amino acid residues to oxidation by oxidants has been shown to vary between proteins and results from their position and interaction within the protein structure.[25]

Oxidation of amino acids has been shown to cause increases in local flexibility or rigidity in the protein chain, leading to an alteration in its secondary structure,[21, 26] which directly influences its physical and material properties.[27-33]

Protein oxidation may therefore adversely affect the properties and processing characteristics of proteinous bioplastics. Minor modification to amino acid residues may result in less contribution to stabilising interactions, but excessive changes may result in decreased mechanical properties such as strength, stiffness and elongation. Furthermore, protein hydrolysis or fragmentation could result in poor consolidation due to reduced chain entanglement, and along with degradation of potential crosslinking sites (through the destruction of cysteine, tyrosine and lysine residues) could result in a material with poor mechanical properties.[2]

This paper describes the effect of oxidation during bloodmeal decolouring using PAA on the physical and chemical characteristics of the proteins manifested by changes in the amino acid profile. Polymer interactions and hydrophilicity were measured indirectly by assessing protein solubility and molecular weight distribution, while the destruction of cysteine crosslinks was measured via synchrotron FT-IR analysis.

Experimental

Materials

Bovine bloodmeal was obtained from Wallace Corporation Ltd, New Zealand and sieved to utilise particles under 710 µm. Peracetic acid, an equilibrium mixture of peracetic acid, hydrogen peroxide, acetic acid and water (Proxitane Sanitiz - 5%) was purchased from Solvay Interox Pty Ltd Auckland, New Zealand and diluted with distilled water to the appropriate concentration for decolouring (Table 2). Hydrogen peroxide (30 wt% EMSURE, ISO) was purchased from Merck, Auckland, New Zealand. Analytical grade glacial acetic acid, sodium hydroxide, sodium dodecyl sulfate, sodium chloride, monosodium phosphate and disodium phosphate were purchased from ThermoFisher Scientific, Auckland, New Zealand.

Bloodmeal Decolouring

Bloodmeal was decoloured by adding 450 g of a 1 – 5 wt% peracetic acid solution, or 26 wt% hydrogen peroxide (with or without 6.6 wt% acetic acid) to 150 g bloodmeal and reacting the mixture under constant agitation for 10 min in a Kenwood mixer (KM080) to ensure homogenous decolouring.[37] This mixture was diluted with distilled water (300 g) and immediately neutralised with 1 molL^{-1} sodium hydroxide and filtered. The decoloured bloodmeal was oven dried overnight (75 ^{o}C) and passed through an IKA® MF 10 Basic Microfine Grinder (Sigma-Aldrich, Auckland, New Zealand) fitted with a 1 mm sieve plate at 3000 rpm, with a residence time of less than 10 seconds.

Yield, protein recovery and moisture content

A mass balance before and after the decolouring process (Figure 1) was used to determine yield and recovery, according to Equation 1 and 2.

$$\text{Yield} = \frac{\text{DBM}_S \times (1 - \text{MC}_{\text{out}})}{\text{BM}_S \times (1 - \text{MC}_{\text{in}})} \quad (1)$$

$$\text{Protein recovery} = \frac{\text{M(Protein)}_{\text{out}}}{\text{M(Protein)}_{\text{in}}} \quad (2)$$

Table 1. Propensity of amino acids for α-helical, β-sheet or β-strands and other secondary structures types.[23, 34-36] Other secondary structure types include turns, bends, 3-helix and random coils.

Helical	Sheet or strand	Other
Alanine	Isoleucine	Glycine
Leucine	Phenylalanine	Proline
Methionine	Tryptophan	Asparagine
Glutamine	Valine	Aspartic acid
Glutamic acid	Tyrosine	Serine
Arginine	Threonine	Histidine
Lysine	Cysteine	

Table 2. Dilution scheme and composition of solutions of equilibrium peracetic acid used for decolouring bloodmeal.

Solution	Peracetic acid (wt%)	Hydrogen peroxide (wt %)	Acetic acid (wt %)	Water (wt %)
1 wt% PAA	1	5.2	2.2	91.6
2 wt% PAA	2	10.4	4.4	83.2
3 wt% PAA	3	15.6	6.6	74.8
4 wt% PAA	4	20.8	8.8	66.4
5 wt% PAA	5	26	11	58.0

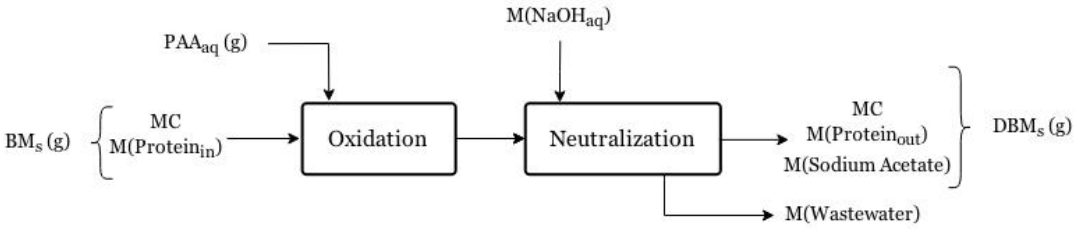

Figure 1. Schematic of the decolouring process, showing all streams in and out. This was used to determine the yield of solids and protein recovery upon decolouring.

Where BM_s is the mass of bloodmeal (g), DBM_s is decoloured bloodmeal mass, MC_{in} and MC_{out} are the moisture content of the solids in and out (wt%) and PC_{in} and PC_{out} are the protein content of the solids in and out on a dry basis (wt%). $M(protein_{in})$ and $M(protein_{out})$ represents the solid mass of protein into and out of the decolouring process, where $M(protein_{in})$ is equal to BM_s x (1 - MC_{in}) x PC_{in} and $M(protein_{out})$ is equal to DBM_s x (1 - MC_{out}) x PC_{out}.

Moisture content and thermogravimetric analysis The moisture content obtained for decoloured samples was determined by oven drying of the material (1.00 g) at 100 ^{o}C for 12 hours. This moisture content was used for calculating protein recovery, soluble fraction and crude nitrogen.
Mass loss of freeze-dried DBM samples (~10 mg) in a 0.9 g ceramic crucible was recorded during heating in dry air at 10 ^{o}Cmin^{-1} to 800 ^{o}C in a Texas Instruments SDT 2960 analyser. Moisture content was determined from the cumulative mass loss up to 120 ^{o}C. This moisture content was used to adjust results for amino acid analysis and ICP-MS.

Soluble protein fraction

The soluble fraction of decoloured bloodmeal was determined by boiling a sample (1.00 g) in 2 wt% SDS solution (200 mL) under reflux for 5 min. Samples were centrifuged at 4200 rpm (relative centrifugal force of 4102 x g) for 10 min using a Sigma 6-15 Centrifuge fitted with a Sigma swing-out rotor (Nr. 11150) and four Sigma buckets (Nr. 13520) (John Morris Scientific, Auckland, New Zealand). The supernatant was decanted through a filter and any solids retained. The insoluble pellet was washed three times using ~45 mL distilled water (centrifuging and decanting each sequential wash and filtering the supernatant to retain any solids). Washed samples and recovered solids (from each washing stage combined) were oven dried at 100 ^{o}C overnight. The soluble protein fraction was calculated using Equation 3.

$$\text{Soluble Protein Fraction} = 1 - \frac{\text{Total recovered solids}}{\text{Solids}_{in} \times (1 - \text{MC}_{in}) \times \text{PC}_{in}} \quad (3)$$

Where $Solids_{in}$ is the mass of solids initially present, MC_{in} is the moisture content of those solids and PC_{in} is the protein content of the solids.

Molecular Weight Distribution

The soluble fraction of decoloured bloodmeal was obtained using the method described above, however due to solubility limitations, bloodmeal required additional concentration by boiling for a further 10 minutes. Each sample was injected (50 µL) into a Superdex 200 gel filtration column (GE Healthcare) connected to an Akta Explorer 100 FPLC system (GE Healthcare). The column was calibrated using standards from high and low molecular weight gel filtration calibration kits comprised of aprotinin, ribonuclease A, ferritin and thyroglobulin (GE Healthcare Ltd., Auckland, New Zealand), along with bovine serum albumin fraction V, β-lactoglobulin, cytochrome C and vitamin B12 (Sigma-Aldrich, Auckland, New Zealand), see Electronic Supplementary Information. Two column volumes of 0.02 molL^{-1} phosphate running buffer (pH 7, 0.1 wt% SDS, 0.1 molL^{-1} NaCl) was applied at a flow rate of 0.5 mLmin^{-1}. Protein concentration was measured at 215 nm using an inline ultraviolet detector. The volume-average molecular weight ($\bar{M}_v$) was calculated using Equation 4, where V_i is equal to the fractional elution volume of molecules with molecular weight M_i.

$$\bar{M}_v = \frac{\sum_i^\infty V_i.M_i}{\sum_i^\infty V_i} \quad (4)$$

Crude protein (total nitrogen)

Total nitrogen was analysed independantly at the Waikato Stable Isotope Unit (University of Waikato, New Zealand) using an Elementar Isoprime 100 analyser, with a precision of ± 1 %. Each sample type was prepared once, combusted and the resulting gases separated by gas chromatography and analysed using continuous-flow mass spectrometry. All samples were referenced to a urea standard traceable to atmospheric nitrogen. Crude protein content was determined by multiplying the total weight of nitrogen present in each sample on a dry basis by a factor of 6.25, which has been deemed an accurate Jones' factor for conversion of total nitrogen in bloodmeals.[38]

Amino acid analysis

Amino acid analysis was carried out independently by an accredited laboratory (AgResearch Ltd., Palmerston North, New Zealand), via ion-exchange chromatography using post-column derivatisation with ninhydrin. Digestion of the sample to liberate amino acids was carried out according to AOAC 1990 Standard Methods 982.39 (acid hydrolysis), 988.15 (ba

Table 3. Peak assignment in the amide III region[42] and cystine oxidation products.[43-45] Each oxidation product has been given a roman numeral for ease of reference.

Name and number	Structure	Functional group	Wavenumber (cm^{-1})
Amide III region	CHNH	vCN, N-H bending	1330 – 1180
Cystine dioxide (IV, V, VI)	Cys-SO_2-S-Cys	Cys-SO_2-S-Cys	1135 – 1120
Cystine monoxide (III)	Cys-SO-S-Cys	Cys-SO-S-Cys	1087 – 1072
Cysteine sulfonic acid (IX, cysteic acid)	Cys-SO_3H	v_s.S=O sulfonate	1050 – 1036
Cysteine-*S*-sulfonate (X)	Cys-S-SO_3^-	v_sSS=O thiosulfonate	1020 – 1005

hydrolysis to quantify tryptophan) and 985.28 (to quantify cysteine and methionine).[39] Amino acids were quantified on the basis of known amounts of standards and their retention times. Each freeze dried sample was prepared once, and analysed six times for each digestion method. The percentage relative standard deviation (%RSD) reported for each amino acid was obtained over 500 observations. Raw data is given in the Electronic Supplementary Information.

Elemental iron and sulfur analysis

Bloodmeal and decoloured bloodmeal (0.25 g) were digested in 50 mL centrifuge tubes containing 65 % nitric acid (4 mL) and 30 % hydrogen peroxide (2 mL). The tubes were capped and left overnight before heating to 80 °C for 1 hour, obtaining total dissolution. The cooled digest was made up to 200 mL using 17.9 MΩ MilliQ deionised water, and filtered using a 0.45 µm Millipore syringe filter. Analysis was carried out in triplicate on each sample.

Inductively coupled plasma mass spectrometry was carried out independently at the Waikato Mass Spectrometry Facility (University of Waikato, New Zealand) and was used to measure iron and other selected alkali, alkaline earth, heavy and volatile metals of each digest using a Perkin Elmer Elan DRC II ICP-MS equipped with an ASX-320 auto-sampler. Calibration was carried out with five increasing dilutions of ICP multi-element standard solution XXI and IV and 10000 µg/mL SCP Science standards (sulfur, sodium, potassium, calcium and iron) diluted to within a working range (similar to sample concentration). Total sulfur was analysed in dynamic reaction cell (DRC) mode with oxygen used as the reacting gas, measuring the SO+ ion at m/z 49. Similarly, five calibration standards were prepared from a 1000 ppm stock solution of sulfur. Deionised water of 17.9 MΩ resistance was used for all preparation of blanks, standards and for quality assurance.

Synchrotron FT-IR microscopy

Spatially resolved FT-IR experiments were undertaken on the infrared micro-spectroscopy beam line at the Australian Synchrotron, Victoria, Australia. Individual particles of bloodmeal and decoloured bloodmeal were compressed in a diamond cell and then transferred to a barium fluoride slide. This was placed in a Linkam temperature controlled stage connected to a Bruker Hyperion 3000 with an MCT collector and XY stage. The stage was set to 24 °C and purged with nitrogen gas. A grid containing ~130 points was mapped on each particle using a 10 x 10 µm spot size. Thirty-two spectra were collected in transmission mode with a resolution of 4 cm^{-1} between 3900 and 700 cm^{-1} and averaged using Opus 7.2 software (Bruker Optik GmbH 2013) according to Bier et al.[40, 41]

Data and Statistical Analysis Data was filtered for a minimum area under the amide III region to exclude points mapped outside particles from the analysis. Integration of the absorbance bands expected for cystine oxidation (cysteine monoxide, dioxide, thiosulfate and sulfonic acid) was carried out using an Opus type B integral (OPUS 7.2) and divided by the total amide III area from 1330 – 1180 cm^{-1} (Table 3).

Results and Discussion

The primary purpose for bleaching bloodmeal is to degrade the haem chromogen by cleaving some of the conjugated methylene bridges in the haem porphyrin, forming yellow and colourless products known as propentdyopents.[46] The destruction of the ferric haem species results in an overall loss of iron (Figure 2) as free ferric ions are highly soluble in an acidic environment and are subsequently lost during the neutralisation and filtering stages of decolouring.

Using 1 – 2 wt% PAA resulted in minimal decolouring,[10] most likely due to insufficient degradation of the haem species present, accompanied by minimal loss of iron (Figure 2). At least 3 wt% PAA is required for adequate decolouring, and improved haem degradation is evidenced from a significant loss of iron. At 5 wt% PAA a gel-like mixture formed, trapping free iron, which can be removed by washing with distilled water to give an iron content of 1.04 mg/g BM.

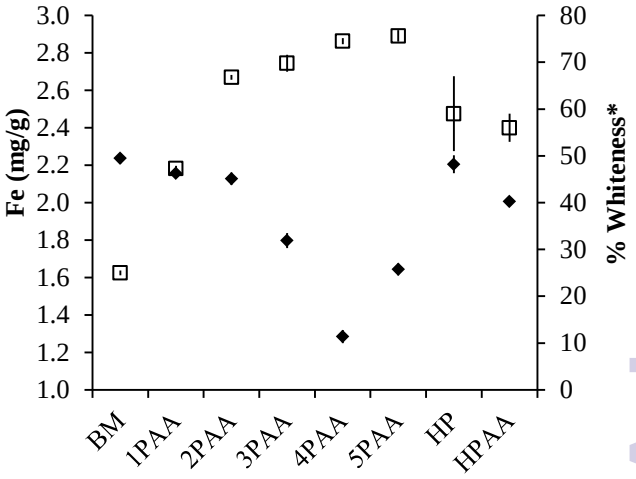

Figure 2. Concentration of iron (♦) in mg/g of bloodmeal and decoloured bloodmeal treated with 1 – 5 wt% PAA or 26 wt% HP with and without 6.6 wt% acetic acid. Washing DBM treated with 5 wt% PAA gives an iron content of 1.04 mg/g (not shown on graph). Not

Percentage whiteness[10] (▫) is given for each treatment, in this case HP solutions are 30 wt%.

By contrast, decolouring with HP led to no loss of iron, supporting the observation that HP is not an effective oxidant for decolouring bloodmeal.[10] Acetic acid increased iron solubility and is often used to strip iron from solubilised haemin during the preparation of protoporphyin from red blood cells,[47] despite lower HP consumption and an accompanying lower decolouring efficacy.[10]

Both PAA and HP are strong oxidants known to form various reactive oxygen and free radical species. In addition to decolouring, these highly reactive oxidising species are likely to result in oxidative damage to the protein, including modification of amino acid residues, protein hydrolysis, cleavage of covalent crosslinks and protein aggregation.[7] Such modifications to the BM proteins will influence the yield from the bleaching reaction as well as the physical properties of proteins thus obtained.

Yield and protein solubility

Bloodmeal is known to contain 80 – 100 wt% protein, as well as 1 – 2 wt% lipids.[48-57] The total nitrogen assay estimated the protein content of bloodmeal to be approximately 100 wt% protein (1 wt% error), and this was reduced to 89 wt% protein after decolouring (Figure 3).

Low concentrations of PAA led to very low total mass loss in contrast to the use of HP alone (Table 4). Mass loss (or gain) could either be from loss of protein (due to dissolution, or experimental error) or by precipitation of sodium acetate during neutralisation. Considering mass loss observed, salt precipitation (which would lead to an increase in mass) must be accompanied by a compensatory loss of solids, probably protein. The loss of protein was probably caused by dissolution and fragmentation, however, evidence is conflicting regarding the occurrence of chain scission.[7, 18] In contrast, use of HP alone led to significant loss of protein, which was somewhat inhibited by acetic acid. Acetic acid is known to act as an antioxidant in hydrogen peroxide,[20] preventing the formation of hydroxyl radicals which are purported to cause protein hydrolysis.[58]

The increase in protein solubility undergoes a step change around 3 – 4 wt% PAA (Figure 3), and corresponds with the previously observed rates of change for the consumption of oxidants and bleaching efficacy (percentage whiteness).[10] This is an indication that the change in observed solubility is due to the extent of oxidation (molecular weight, structure and chemical interactions within and between the proteins) and not due to the salt content resulting from neutralisation. Increased protein solubility in aqueous buffers is often correlated with a reduction in cysteine crosslinks, increased carbonyl content (and hydrophilicity) as well as hydrolysis/peptide cleavage.[7]

Table 4. Yield and protein recovery (dry mass basis) during decolouring using 1-5 wt% peracetic acid (PAA) or 26 wt% HP with and without 6.6 wt% acetic acid.

	1PAA	2PAA	3PAA	4PAA	5PAA	HP	HPAA
Yield (wt%)	98.8	101.7	95.5	97.6	98.8	78.8	92.7
Protein recovery (wt%)	97.6	96.8	90.2	87.8	87.7	75.4	84.5

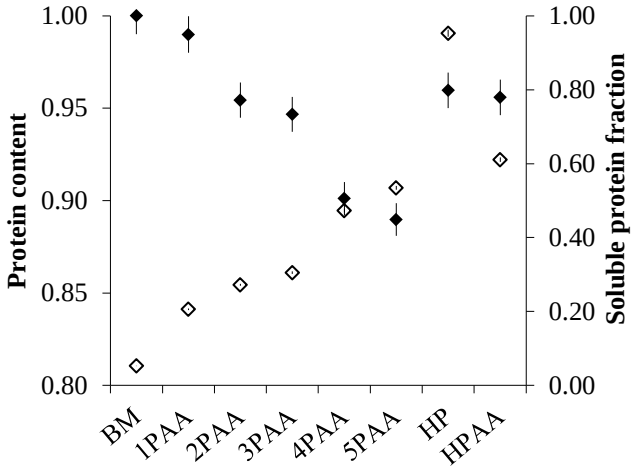

Figure 3. Fractional protein content (♦) and soluble protein fraction (◇) of bloodmeal and decoloured bloodmeal treated with 1 – 5 wt% PAA or 26 wt% HP with and without 6.6 wt% acetic acid.

Molecular weight distribution

Two large peaks at 14 mL and 16 mL (~ 67 kDa and 34 kDa) were detected in the FPLC chromatogram of the soluble fraction of bloodmeal (Figure 4A). These peaks represent the tetrameric and dimeric forms of haemoglobin, which are known to be in equilibrium in blood.[59, 60] After oxidation, a new peak emerged at 18.5 mL, corresponding to a molecular weight of ~8.8 kDa. This could be due to hydrolysis or chain cleavage, as observed by others for BSA or aldolase exposed to PAA.[18] Treatment with high levels of oxidant (5 wt% PAA, H[illegible] and HP with acetic acid) also resulted in a loss of resolution, which also indicated fragmentation of protein aggregates present in BM.

However, molecular weight analysis is limited to soluble proteins, and the insoluble fraction could have a completely different average molecular weight and/or distribution. For bloodmeal, the soluble fraction would probably be the smaller size fraction as the larger aggregated proteins formed during the bloodmeal manufacturing would remain insoluble.

The volume-average molecular weight ($\bar{M}_v$), was determined between 9 and 19 mL, excluding the void volume, SDS micelles and salts. The soluble fraction of bloodmeal had a volume-average molecular weight of ~45 kDa. Oxidation led to greater dissolution of protein, for which for samples between 1 – 4 wt% PAA led to a greater dissolution of larger molecules reflected in an increase in $\bar{M}_v$ (Figure 5). Oxidation using 5 wt% PAA resulted in a significant quantity of shorter chains, despite not having a significantly greater quantity of total dissolved solids.

The protective effect of acetic acid during oxidation using H[illegible] was observed in Figure 4B. Alone, hydrogen peroxide led to a lower average molecular weight (and 87 % soluble fraction) but in the presence of ~ 6.6 wt% acetic acid, a higher average molecular weight was observed (Figure 5) and a smaller proportion of lower molecular weight material (Figure 4B).

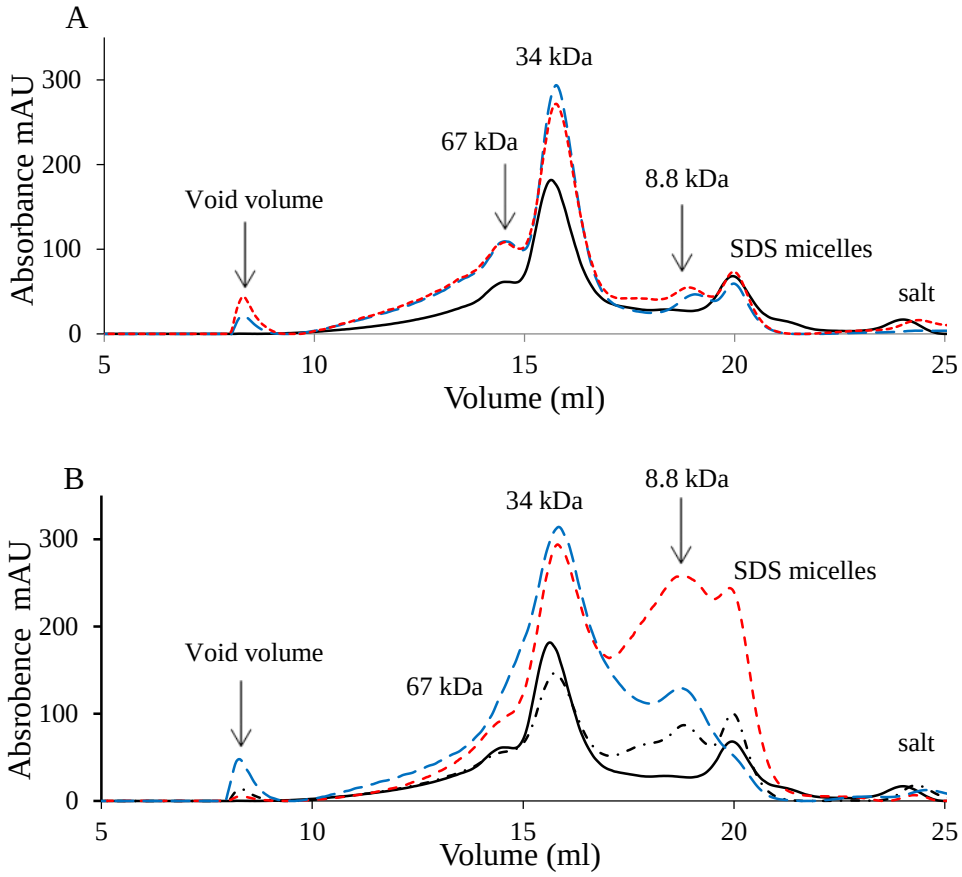

Figure 4. A: FPLC chromatogram showing the elution profiles of bloodmeal (——) and decoloured bloodmeal after treatment with 2 (– – – –) and 4 wt% PAA (– – – –), used to determine average molecular weight. Detection at 215 nm. B: FPLC chromatogram obtained from bloodmeal, BM (——) and bloodmeal decoloured with 5 wt% PAA (– – – –) 26 wt% hydrogen peroxide (– – – –) and hydrogen peroxide with acetic acid ( ) solutions, used to determine average molecular weight. Detection at 215 nm, peak assignment was predicted from the elution volumes of molecular weight markers.

Alternatively, the presence of acetic acid during decolouring with HP may lead to a larger observed conformation (and lower elution volume) due to volumetric swelling of the proteins.

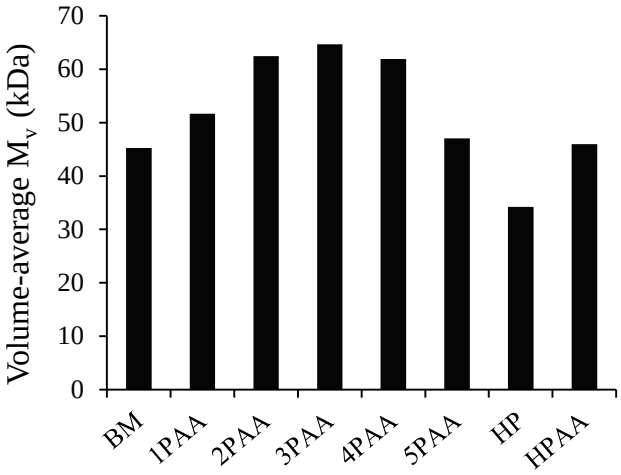

Figure 5. Volume-average molecular weights (kDa) of bloodmeal (BM) after decolouring with 1-5 wt% PAA, 26 wt% HP and 26 wt% HP containing 6 wt% acetic acid.

At 1 - 2 wt% PAA perhaps there is insufficient oxidant present to significantly change the properties of bloodmeal, which maintains low solubility, similar protein content, high yield and similar molecular weight distribution.

As the concentration of PAA was increased, a larger proportion of high molecular weight proteins become solubilised, and the formation of lower mass peptides becomes more apparent. Although the presence of acetic acid acts to inhibit extensive degradation, its protective effect is overcome at 5 wt% PAA, and fragmentation produces a significant quantity of lower molecular weight peptides (Figure 4B and 5). The increased solubility of DBM proteins between 2 – 4 wt% PAA must be attributed to changes in the amino acid profile and the stabilising interactions they are involved in, not solely the molecular weight distribution as minimal fragmentation occurred.

Primary structure

Oxidative decolouring of bloodmeal led to a shift in amino acid composition that supports a more hydrophilic material, including an increasing quantity of unidentified species (Figure 6A), which includes both salts and unidentifiable amino acids. For treatments using 1 – 4 wt% PAA, the identifiable species corresponds with the salt content of the material (within the error limitations of the experiment) suggesting that any change in amino acid composition is due to the dissolution of modified amino acids, peptides or proteins, (as all of the protein present is accounted for by detectable amino acids). However, treatment with 5 wt% PAA and HP led to the formation of unidentifiable species in excess of the salt content, indicating modification of the amino acids had occurred, and that they remained within the recovered protein. Overall there is a reduction in aromatic amino acids and lysine with an accordingly greater charged and non-polar content. As oxidation conditions become more stringent many of the amino acids show some decline, however tryptophan, histidine and lysine show losses even under milder oxidation conditions (Figure 6B).

Aromatic amino acids

Peracetic acid and hydrogen peroxide are both strong oxidants, which at pH 1 (as encountered during decolouring) are both undissociated and act as strong electrophiles, prone to attacking electron dense sites (i.e. those with low oxidation potential) such as the carbon methane bridges in haem, as well as sulfur and the aromatic amino acids phenylalanine, tryptophan, and tyrosine and primary and secondary amine containing amino acids lysine and histidine.[61, 62]

The selectivity of free radical attack on side chains is affected by the presence of functional groups which can stabilise the resulting radical. For example, hydrogen abstraction occurs preferentially at positions adjacent to electron delocalising (stabilising) groups such as the hydroxy groups (in Ser and Thr), carboxyl and amide functions (in Asp, Glu, Asn, Gln), and the guanidine group in Arg.[63] Comparatively, the protonated amine function on the Lys side chain has a similar effect to the amine group on the α-carbon, resulting in hydrogen abstraction at positions remote from both (from the C-4 and C-5 positions).[63] However, for aromatic, heterocyclic and sulfur containing amino acids (Phe, Tyr, Trp, His, Met and Cys), addition reactions generally take place over hydrogen atom abstraction from the methylene groups. This is due to the addition reactions occurring faster, as no bond breaking occurs in the transition state and the adduct species is stabilised by electron delocalisation about the ring or to the sulfur group.[63]

In light of this, it is interesting to note that phenylalanine content increased slightly with increasing PAA concentration, while hydrogen peroxide treatment led to a reduction (Figure 6B). This conflicts with other studies which indicate a loss of phenylalanine through oxidation with peracetic acid, however, under these conditions perhaps the presence of acetic acid inhibits hydroxyl radical formation, preventing oxidation of phenylalanine.

Histidine and tyrosine were both reduced by up to 40 % and all tryptophan was degraded beyond 3 wt% PAA, consistent with observations of oxidation of dairy proteins.[10] Selective oxidation of the imidazole ring of histidine in the presence of other amino acids has been demonstrated by metal-mediated ascorbic acid oxidation;[64] the product of this oxidation was the imidazolone, which would not significantly change molecular weight but would not be detected as a known amino acid. This type of selectivity is attributed to the generation of reactive oxygen species at specific metal-binding sites, such as that of histidine. From here, the highly reactive oxidants attack labile residues nearby rather than diffusing into the bulk medium.[65]

During peroxide reactions with haem, oxoferryl (Fe^{4+}=O) intermediate species are formed, generating a radical cation, typically located on the π-system of haem. The π-radical is rapidly transferred to a nearby amino acid residue, which has a low oxidation potential, such as tryptophan or tyrosine.[66] From there, it is transformed into a variety of oxidation products. For PAA and HP, these radicals are typically formed on tyrosine, tryptophan or cysteine residues.[66-73] Eventually, the free radical is deprotonated into a neutral radical[11] or terminated when it attacks an electron rich site. In the case of bovine cytochrome *c* oxidase, tyrosinyl and tryptophanyl radicals are formed when hydrogen peroxide binds to the heme unit, followed by migration from the binuclear centre, selectively oxidising surface tryptophan residues to hydroxytryptophan.[74]

Such a reduction in the quantity of non-polar aromatic groups is a result of electrophilic addition reactions (such as hydroxylation and carbonylation)[75] resulting in less hydrophobic interactions.

Polar and charged amino acids

Minimal change to the quantity of polar amino acids (threonine and serine) was observed at low PAA concentration, although an overall relative increase of ~ 7 wt% serine was observed at high concentration (Figure 6C). Hydrogen peroxide caused a noticeable increase (a relative increase of ~10 %) in the concentration of serine, but made very little difference to threonine. The increase in content is likely an artefact of reducing the content of other amino acids. By contrast, the overall relative proportion of charged amino acids increased, mostly from the large increase in aspartic acid, and to a lesser extent, glutamic acid (Figure 6D). It is thought that the increase in aspartic acid may be caused by its formation during histidine oxidation.[76] Arginine content did not appear to change after HP or PAA treatment, which is unusual given that the guanidine group should serve to stabilise the formation of intermediate radicals.

Overall DBM contains almost the same amount of hydrophobic amino acids, (sum of glycine, alanine, valine, leucine, isoleucine, proline and methionine) as bloodmeal. However, it contains significantly less of the aromatic amino acids tyrosine, and histidine, but with little change in phenylalanine and notably containing no tryptophan. There is also a relative increase in polar and charged amino acids which are expected to be more hydrophilic (threonine, serine, aspartic acid, glutamic acid and arginine).

Lysine is an anomaly, in that it would ordinarily be grouped with hydrophilic amino acids, but it undergoes such extensive degradation with PAA treatment (up to 80 % relative to the initial concentration in BM) and HP (up to 40 % relative to the initial concentration in BM) that it should be considered alone.

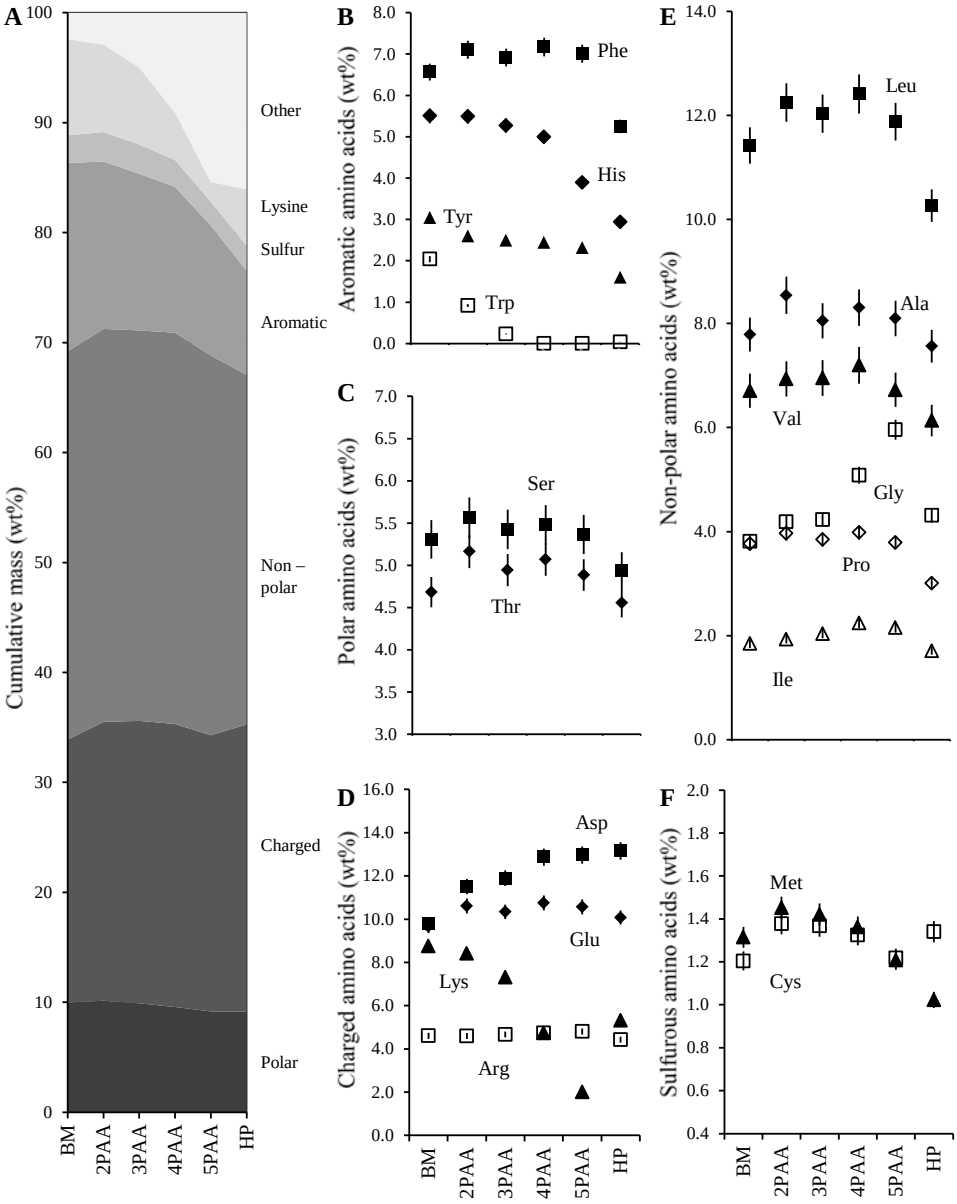

Figure 6. A: Amino acid composition of bloodmeal and decoloured bloodmeal expressed as a cumulative mass percentage. Weight percentage of B: Aromatic amino acids phenylalanine (Phe), histidine (His), tyrosine (Tyr) and tryptophan (Trp) C: Polar amino acids serine (Ser) and threonine (Thr) D: Charged amino acids aspartic acid (Asp), glutamic acid (Glu), lysine (Lys) and arginine (Arg) E: Non-polar amino acids leucine (Leu), alanine (Ala), valine (Val), glycine (Gly), proline (Pro) and isoleucine (Ile) F: Sulfurous amino acids methionine (Met) and cysteine (Cys) present in bloodmeal before and after decolouring, expressed as a percentage of crude protein. Error bars are the percentage relative standard deviation for the detection of each amino acid.

Bloodmeal is probably best known for its high lysine content compared with other protein meals, and is capable of forming covalent bonds with alanine to form lysinoalanine when exposed to heat.

Both lysine and histidine are known to undergo metal catalysed oxidation forming aminoadipic semi-aldehyde and 2-oxo-hisitidine respectively.[62] The greater reduction in lysine content observed with PAA as compared to HP is unexpected, as acetic acid is theorised to chelate metal ions which may catalyse lysine oxidation. However, while acetic acid may be capable of chelating all freely dissolved iron present, it may be unable to chelate iron trapped or loosely bound within chains of the protein, allowing the iron to facilitate oxidation much closer to the vulnerable sites. Alternatively, it may simply be due to PAA's higher reduction potential (1.81 V) compared to HP (1.76 V) or due to its greater radical longevity allowing a greater portion of lysine to be accessed.

Non-polar amino acids

Glycine, alanine, valine, leucine, isoleucine and proline are considered non-polar, but only glycine underwent a relative increase above 3 wt% PAA or to a lesser extent with hydrogen peroxide (Figure 6E). Isoleucine increased about 20 % (from 1.9 to 2.3 wt%), with a step change around 3 wt% PAA, while hydrogen peroxide had little effect. PAA had no effect on proline (within the error limits), but decreased by 10 % using HP.

Alanine and valine content did not have a clear trend, but increased slightly at high PAA content (Figure 6E). On the other hand, hydrogen peroxide treatment increased the alanine content to 8.7 wt% (~ 9% relative increase). Leucine content increased linearly with PAA concentration (from 11.7 – 12.6 wt%), but remained unaffected by hydrogen peroxide oxidation. It is most likely that the apparent increase in alanine after oxidation with HP is due to a shift in composition caused by the massive loss of aromatic amino acids and dissolution of proteins during decolouring.

Cysteine and methionine

Interestingly, there is a relative increase in sulfur containing amino acids (per gram of protein) during decolouring (Figure 6F). This suggests that the mass lost during decolouring involves peptides or proteins which contain lower concentrations of sulfur containing amino acids, compared to the overall average (i.e. contain less disulfide bridges), resulting in a higher concentration in the remaining insoluble DBM.

However, the HPLC method used to determine the amino acid profile is unable to distinguish between cystine, cysteine or cysteic acid (as formed upon exposure to peracetic acid), as the analysis of this amino acid is achieved after performic acid oxidation in order to convert all species to cysteic acid. In fact it is likely that despite being richer in sulfur containing components, the material may contain absolutely no cystine or cysteine crosslinks, as they were probably all converted to cysteic acid during decolouring. This is supported by the fact that DBM treated with 4 wt% PAA no longer requires the addition of sodium sulfite as a reducing agent to cleave disulfide bridges when it is used as a feedstock for thermoplastic processing.[2]

Cystine degradation

Some of the intermediate and final products of cystine oxidation are observed in FTIR between 1150 and 1000 cm^{-1}. Cleavage of the disulfide bond occurs through two major pathways: S-S scission or C-S scission.[77] In both cases, the main end-product is cysteine sulfonic acid (IX), more commonly referred to as cysteic acid (Figure 7). The oxidation of cystine groups responsible for the crosslinks in protein results in the formation of cysteine monoxide (Cys-SO-S-Cys, II), cysteine dioxide (Cys-SO_2-S-Cys and Cys-SO-SO-Cys, III and IV) and cysteine sulfonic acid (Cys-SO_3H, IX) as well as cysteine-*S*-sulfonate (Cys-SO_3M, X).

The four new peaks, 1150 – 1000 cm^{-1}, are easily observed after treatment (Figure 8A). Each has been assigned to oxidation products of cysteine and cysteine, and are labelled as indicated above. Treating bloodmeal with any level of PAA greater than 2 wt% PAA led to subtle changes in the FTIR spectra (Figure 8 A), generating four new peaks. These peaks increased in intensity and area with increasing PAA concentration. [43-45]

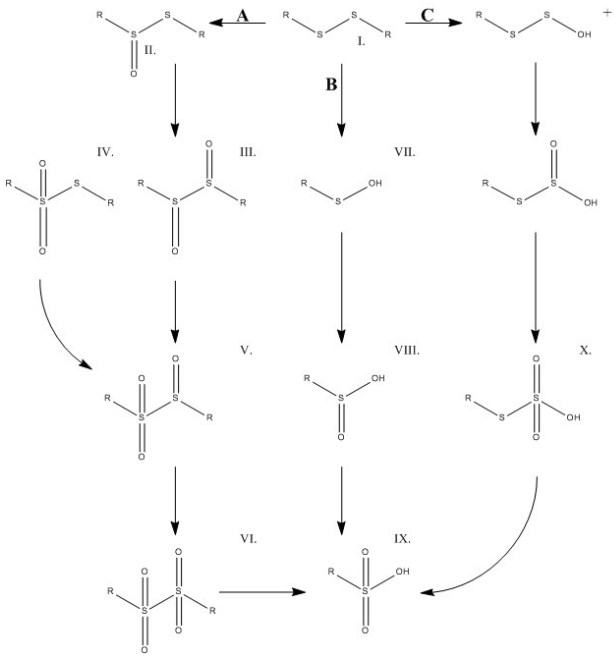

Figure 7. Reaction scheme for the oxidation of cysteine. Pathways A and B occur via S–S scission and pathway C through C–S scission. Compound I: Cystine, II: cystine monoxide, III, cystine dioxide, IV: cystine-*S*,*S*-dioxide, V: cystine trioxide, VI: cystine tetraoxide, VII: cysteine sulfenic acid, VIII: cysteine sulfinic acid, IX: cysteine sulfonic acid (cysteic acid), X: cysteine-*S*-sulfonic acid. Reproduced from ref. 77, Copyright (1966), with permission from Elsevier.

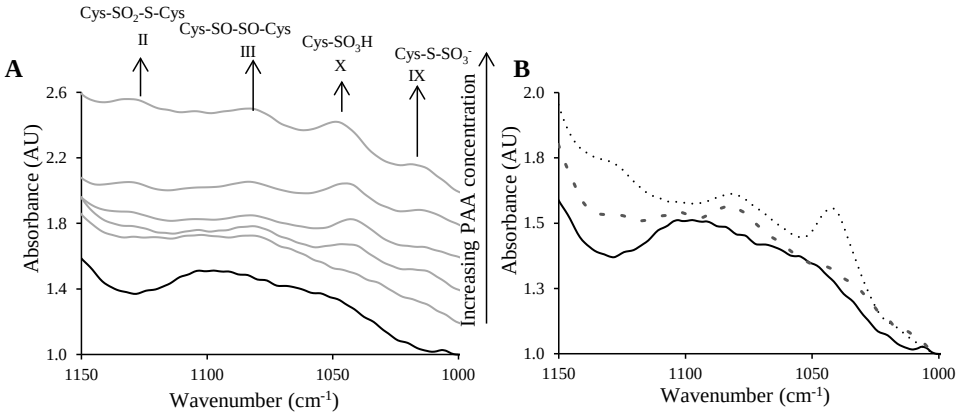

Figure 8. A: Average of 10 FTIR spectra of bloodmeal (——) and 1-5 wt% PAA decoloured bloodmeal (——), normalised at 1000 cm^{-1} and stacked for clarity. B: Average of 10 FTIR spectra of HP decoloured bloodmeal (- - - -) and HP with acetic acid decoloured bloodmeal (— — — —), normalised at 1000 cm^{-1} (solid line is bloodmeal). Cysteine oxidation products are also assigned

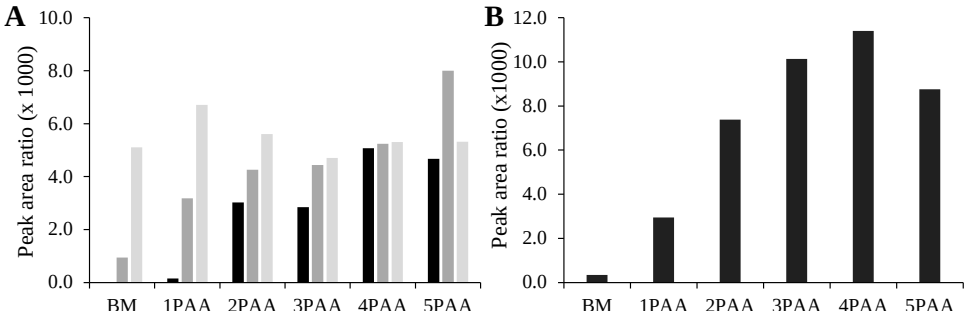

Figure 9. A: Peak area ratio of selected peaks to that of the total amide III region. Cystine dioxide ■ (Cys-SO_2-S-Cys), cysteine-*S*-sulfonate ■ (Cys-S-SO_3^-) and cystine monoxide ■ (Cys-SO-S-Cys). B: Peak area ratio of cysteic acid ■ (Cys-SO_3H) to total amide III region.

The peak at 1017 cm^{-1} appeared after oxidation with PAA only, and could be assigned to cysteine-*S*-sulfonate (R-S-SO_3^-). This compound has been observed around 1020 cm^{-1} in the FTIR spectra of other proteins after photooxidation or sulfitolysis[78-80] and is known to be formed during alkaline oxidation using permanganate or hydrogen peroxide,[81] as well as hypochlorite and persulfate.[77, 82]

Although it has not been reported to occur in wool exposed to hydrogen peroxide or peracetic acid,[82] it has been suggested to occur in wool exposed to boiling HP,[83] and upon bleaching hair with HP.[44] The generation of cysteine-*S*-sulfonic acid appears to be particularly pH sensitive, and its formation in acidic media may be quickly followed by its interaction with other products.[77] Its presence in bloodmeal decoloured with peracetic acid is evidence that cystine cleavage occurs via C-S scission (Pathway C in Figure 7).

Sodium acetate is known to have two peaks occurring at ~ 1017 cm^{-1} and 1050 cm^{-1}, which would also be expected to increase with increasing PAA treatment due to the increase in acetic acid present which would require neutralisation. However, their contribution to the overall spectra has been rejected on the basis that almost no difference is seen in this region in bloodmeal decoloured with HP containing acetic acid, which too would contain sodium acetate salt (Figure 8B). This indicates that the source of these new peaks is most likely due to oxidation reactions rather than the formation of acetate salt, leaving the designation of the peak at 1017 cm^{-1} to that of cysteine-*S*-sulfonate.

The corresponding area for each peak identified was averaged over the entire area scanned (spatial map) and plotted against the concentration of PAA used (Figure 9A). While both the cysteine-*S*-sulfonate and cystine dioxide peaks increased with PAA concentration, cystine monoxide appeared to stay almost constant. This indicates that some level of cysteine monoxide is present in BM prior to decolouring, which despite its instability, may be an artefact of the oxidising conditions during the manufacture of bloodmeal.

The ν_sS=O sulfonate absorbance band at 1044 cm^{-1} owing to cysteic acid, had the strongest signal increase, and therefore an increase in its area (relative to the amide III area) could be good marker of oxidative damage to the protein (Figure 9B). The strong relative increase would confirm previous observations regarding the effect of PAA on proteins.

Treatment with hydrogen peroxide led to a significantly lower quantity of cystine dioxide formation, with a much higher formation of cysteic acid (the expected end product). Further, the presence of acetic acid appeared to have inhibited the formation of both these oxidation products, perhaps through an anti-oxidant pathway, suppressing the formation of more reactive oxygen species such as the hydroxyl radical.

Overall the composition of decoloured bloodmeal shifted toward a material with improved hydrophilicity, with a higher proportion of charged and non-polar amino acids and a reduced quantity of aromatic amino acids. Decolouring bloodmeal led to an increase in random structures,[37] consistent with an increase in amino acids with a propensity to form turns and coils (glycine, proline, aspartic acid and serine, despite a reduction in histidine). The remaining ordered structures contained a relatively larger proportion of beta sheets compared to alpha helices, most likely caused by the large reduction in lysine and other helix-forming amino acids (leucine, methionine and glutamic acid undergo minimal changes). By contrast, a reduction in the total quantity of sheet forming amino acids (tryptophan, isoleucine, phenylalanine, valine, tyrosine, threonine and cysteine) was observed, but did not result in a material with fewer β-sheets.[37] This highlights the difficulties of using amino acid composition alone to predict secondary structure.

Conclusion

Solutions containing more than 3 wt% PAA are required for adequately decolouring bloodmeal, as observed by loss of iron. HP and HP containing acetic acid were found to result in only superficial losses of iron during decolouring confirming HP's inability to degrade all of the haem chromogens responsible for the colour of bloodmeal.

HP and PAA cause changes to the physical and chemical properties of bloodmeal proteins. PAA treatment prevented excessive protein loss (higher yield) compared to decolouring with hydrogen peroxide, and this is likely a result of the combination of oxidation mechanisms and the presence of acetate salt enhancing protein precipitation. However, the lower protein content, most noticeable after 4 – 5 wt% PAA, was due to the dilution with acetate salts.

Oxidation also resulted in an increase in the solubility of the proteins, particularly for those treated with hydrogen peroxide. All treatments resulted in a greater dissolution of protein, and were also responsible for the appearance of a peptide fragment with an approximate mass of 8.8 kDa. Using concentrations of 1 – 4 wt% PAA gave rise to soluble proteins similar in composition to that of bloodmeal. However, treatment with 5 wt% PAA or HP led to significant fragmentation of the protein chains, inhibited to some extent by the presence of acetic acid.

The protective effect of acetic acid on proteins was observed during HP/AA decolouring, resulting in less fragmentation than treatment with HP alone. This has been attributed to the antioxidant properties of acetic acid allowing chelation of free iron which would otherwise facilitate the formation of hydroxyl radical species (to cause protein hydrolysis) through Fenton type reactions. It is expected that acetic acid performs a similar role in PAA solutions.

Due to the minor contribution fragmentation is thought to have in this reaction; the overall increase in hydrophilicity of the proteins upon decolouring is most probably a result of changes to the amino acid residues of which they are comprised. This is most evident from a shift in composition toward more polar and charged amino acids, through the degradation of aromatic residues and also lysine. The most telling feature explaining the improved hydrophilicity is the formation of cysteic acid and cysteine-*S*-sulfonate, indicating that at least to some extent, crosslinking has been overcome. The cleavage of cysteine crosslinks and degradation of lysine may limit the number of new crosslinks which can form during thermoplastic processing.

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Notes and references

‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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