STRUCTURAL CHARACTERISATION OF PRE-PROCESSED THERMOPLASTIC PROTEIN DERIVED FROM BLOODMEAL

J. M. Bier[1], C. J. R. Verbeek[1] and M. C. Lay[1]

[1]School of Engineering
University of Waikato:
Private Bag 3105
Hamilton 3240
Jmb101@waikato.ac.nz

ABSTRACT

Additives are required to convert bloodmeal powder into an extrudable thermoplastic protein-based bioplastic. These include a protein denaturant, a surfactant, a reducing agent and plasticisers. The objective of this work was to assess the structural changes induced in bloodmeal by these additives prior to extrusion. Structure was investigated using Fourier transform infrared (FT-IR) spectroscopy, wide angle X-ray scattering (WAXS) and synchrotron light based FT-IR microspectroscopy.

FT-IR results suggested the additives reduced α-helical content. The shape of the amide I region (1600 – 1700 cm^{-1}, representing carbonyl group stretching in the protein backbone) is known to depend on protein secondary structures. Bloodmeal showed a broad, convoluted peak in this region, with a maximum in the range 1648 – 1658 cm^{-1}, associated with α-helices. With processing additives, a dip was seen in the α-helix region, with twin peaks emerging either side of it. Urea, one of the additives, also absorbs in the amide I region and may also contribute to a change in its shape. Analysis of the amide 3 region supported a reduction in the ratio of α helices to β sheets. Further support of structural changes was shown by WAXS. The additives decreased the sharpness of peaks corresponding to 4.8 Å and 10 Å, thought to represent intra-helix spacing and inter-helix packing respectively.

FT-IR microspectroscopy at the Australian Synchrotron enabled spatial variations in secondary structure to be explored using peaks in the amide 3 region. Spatial distribution of secondary structure was detected in bloodmeal and thermoplastically modified bloodmeal prior to extrusion (PPM-TEG). Bloodmeal showed domain separation on the approximate order of 10 μm, whilst PPM-TEG appeared to have larger phases and overall reduced α-helical content, relative to beta sheets.

INTRODUCTION

Thermoplastic modification of inedible, low-value agricultural by-products such as the proteins in bloodmeal represents an opportunity for New Zealand to produce its own bioplastic resins domestically, without competing with global food supply. Raw blood from the meat processing industry contains approximately 80% water, 18% protein and 2% fats and minerals and, for environmental and economic reasons, is typically dried into bloodmeal powder. Addition of protein denaturants, surfactants and crosslink reducing agents to bloodmeal modifies it to form an extrudable thermoplastic (Verbeek and van den Berg, 2010a). The objective of this work was to assess the structural changes induced in bloodmeal by these additives prior to extrusion. Changes to protein secondary structure and the presence of ordered regions were investigated using wide angle X-ray

scattering (WAXS), globar-source FT-IR spectroscopy and synchrotron light-based FT-IR microspectroscopy.

Protein-based plastics can absorb water, be brittle and have low strength in comparison to conventional plastics (Verbeek and Bier, 2011). Understanding how protein secondary structures such as α-helices and β-sheets are transformed and distributed by processing may enable the manipulation of these structures to improve resultant material properties. It has previously been pointed out that thermoplastic processing of proteins generally, but not exclusively, favours increases in β-sheet content (Verbeek and van den Berg, 2010b). For zein proteins, samples with increased proportions of α-helices have been shown to produce better blown films than those with high relative β-sheet content (Oliviero et al., 2010), indicating that manipulating the presence and distribution of secondary structures prior to extrusion may improve processability and properties.

FT-IR is an established technique for characterising protein structure, commonly making use of absorbance in the amide 1 region (1600–1700 cm^{-1}), which is mostly due to vibration of C=O bonds (Jackson and Mantsch, 1995). Stronger hydrogen bonding involving this group reduces electron density within the C=O bond causing absorbance at lower wave numbers (Jackson and Mantsch, 1995). Protein secondary structures are stabilised by hydrogen bonds leading to characteristic absorbances for different secondary structures. For example, 1620–1640 cm^{-1} for beta sheets (Yu et al., 2004), 1648–1670 cm^{-1} for helices and 1675–1695 cm^{-1} for anti-parallel beta sheets (Jackson and Mantsch, 1995). Determination of absolute secondary structure content with FT-IR relies on assumptions that are not necessarily valid for proteins (Yu, 2006), however a strength of the technique is exploring relative differences in secondary structure induced by external factors (Jackson and Mantsch, 1995).

Spatial variations in secondary structure may mimic micro-crystalline regions in synthetic polymers, which can increase some mechanical properties such as toughness. Although regular FT-IR can be used to determine secondary structure, the resolution is not high enough to distinguish between different regions within the same material. Synchrotron light is highly collimated and typically 100–1000 times brighter than conventional globar sources used for FT-IR. This enables the exploration of microstructures of biologically derived samples at ultra-spatial resolutions with high signal to noise ratios (Yu et al., 2004). Synchrotron based FT-IR microscopy has been shown to spatially resolve protein secondary structures in plant-based samples and feather proteins (Yu et al., 2004, Yu, 2006) and also examined phase separation in blends of gelatin and maltodextrin (De Giacomo et al., 2008).

The objective of this study was to determine structural changes in bloodmeal after thermoplastic modification. Globar-source FT-IR as well as synchrotron-based FT-IR microscopy was used to assess average composition and spatial variation of secondary structures respectively.

METHODS

Materials and sample preparation

Thermoplastic protein (PPM-TEG) was produced from bloodmeal (BM) using a patented process (Verbeek et al., 2007). 20 g urea (Ballance Ag-Nutrients), 6 g sodium dodecyl sulphate (SDS) (Merck) and 6g sodium sulphite (SS) (Ajax Finechem) were dissolved in 80 g distilled water at 50°C. The solution was mixed with 200 g sieved

bloodmeal (Wallace Corporation) in a high-speed mixer for 10 minutes. 40 g triethylene glycol (TEG) (Merck) was added after the first 6 minutes of blending. The resultant mixture was dried over two nights in a Freezone® 2.5 Litre Benchtop freeze-dryer (Labconco Corporation, Kansas City) set to auto mode (Collector temperature -50°C, vacuum <11 Pa).

Analysis

Fourier transform infrared (FT-IR) spectroscopy

Samples were prepared from freeze-dried material in potassium bromide (KBr) pellets (1 mg sample per 100 mg salt). FT-IR spectra were recorded using a Perkin Elmer spectrophotometer performing 16 scans from 4000 to 400 cm^{-1} at 4 cm^{-1} resolution. Before each sample, a background scan was conducted and automatically subtracted from the sample spectra.

Wide angle x-ray scattering (WAXS)

Powder x-ray scattering was recorded with using a Phillips system running X'Pert Data Collector vs. 2.0b and XPERT-MPD vs. 2.7 control software. A PW3373/00 Cu LFF DK233995 x-ray tube was used at a voltage of 40 kV and current of 40mA, which provided x-rays with a wavelength of 1.54 Å. Samples were scanned from $2\theta = 2°$ to $2\theta = 60°$ at 0.020° steps.

Synchrotron light based FT-IR micro-spectroscopy.

Spatially resolved FT-IR experiments were undertaken on the infrared microspectroscopy beamline at the Australian Synchrotron, Victoria, Australia. Fine particles were chosen and flattened between diamond cells for mounting into a Bruker Hyperion 3000 with an MCT collector and XY stage. For each point on the grid (Fig. 1), 32 spectra were collected with a resolution of 4 cm^{-1} between 3900 and 700 cm^{-1} and averaged using Opus 6.5 software (Bruker Optik GmbH 2009). Imaging of α-helix to β-sheet intensity ratios in the amide 3 region was determined using the Opus 6.5 software after baseline correction. The peak between 1295 and 1330 cm^{-1} was taken as representing α-helices and the peak between 1220 and 1245 cm^{-1} was taken as representing β-sheets (Cai and Singh, 1999).

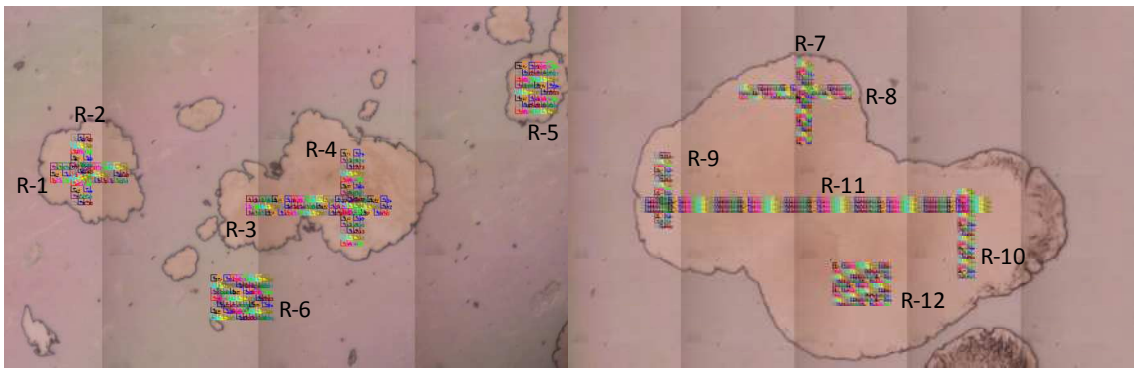

Fig. 1: Video image showing grid maps for which spectra were collected for Bloodmeal (left) and PPM-TEG (right).

RESULTS AND DISCUSSION

FT-IR spectroscopy

Fig. 2 shows representative FT-IR spectra collected from KBr discs. In the amide 1 region, bloodmeal showed a distinct peak at 1654 cm^{-1}, within the region normally associated with alpha helices (Jackson and Mantsch, 1995). For PPM-TEG, peaks at either side of this were present (1632 and 1665 cm^{-1}). Urea is used as one of the additives and also absorbs in the amide 1 spectral region. Scans of urea alone showed peaks at 1604, 1629 and 1684 cm^{-1}, which could be contributing to a perceived increase in beta structures. To correct for this, freeze-dried bloodmeal and urea were dry mixed at the same ratio as in PPM-TEG. It was assumed that without water there would be no reaction between these and hence no structural rearrangements. The resulting plot is also shown in Fig. 2. Unfortunately, in the amide 1 region the spectra looked very similar to PPM-TEG and showed that on visual inspection alone it is unclear if structural rearrangements were occurring or if urea was simply interfering.

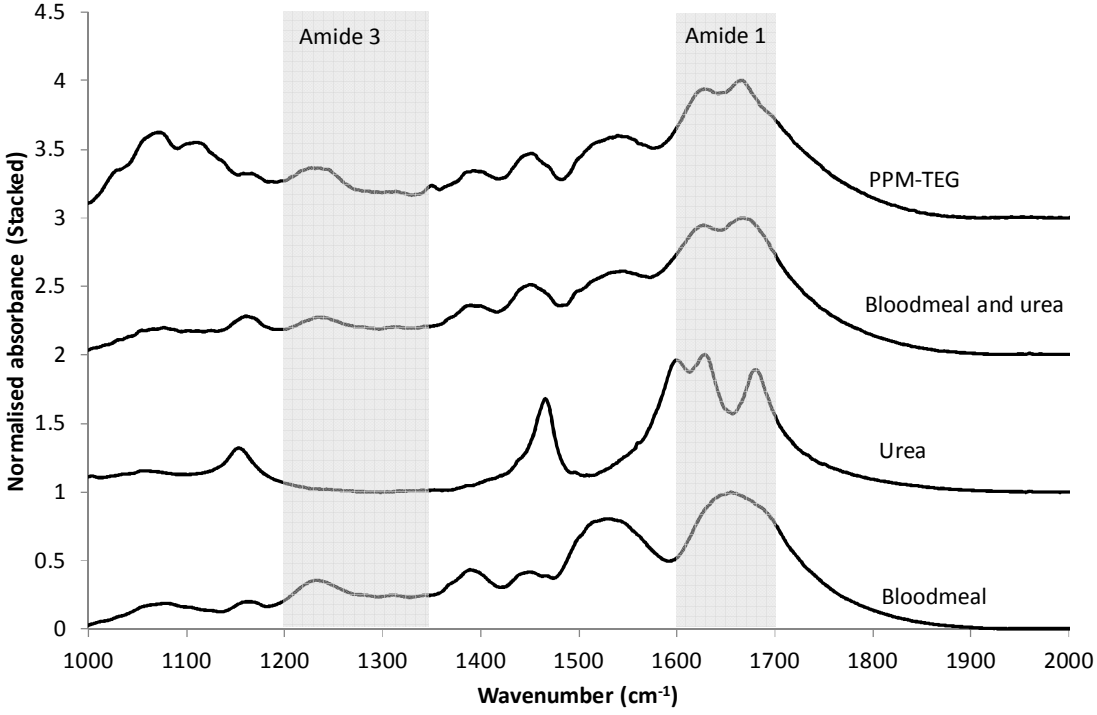

Fig. 2: Representative spectra collected by KBr disc method. Characteristic amide 1 and amide 3 absorbance bands are highlighted.

Although the amide 1 region is the most commonly used absorbance band for estimating secondary structure in proteins, there are several other bands also characteristic of peptide linkages. The amide 3 region between 1200 and 1350cm^{-1} is caused by in phase N–H bending and C–N stretching and, although typically 5-10 times weaker than the amide 1 region, is also sensitive to structural changes (Cai and Singh, 1999). The amide 3 region is sometimes used in conjunction with the amide 1 to give a more reliable determination than the amide 1 alone (Tu, 1986). Alternatively, due to advantages of greater peak separation and less interference from water, the amide 3 region may be used for samples in which structural determination from the amide 1 is not practical (Seabourn et al., 2008).

As can be seen from Fig. 2, urea does not have an absorbance peak within the amide 3 region and hence will not interfere with structural characterisation in the same way it obscures the amide 1 region. Fig. 3 shows a enlarged view of this region. The ratio of the height of the peak between 1295 and 1330 cm^{-1} has been compared to the height of the peak between 1220 and 1245 cm^{-1} in Tab. 1. These peaks have been assigned to α-helices and β-sheets respectively (Cai and Singh, 1999). It was found that PPM-TEG did show a decrease in α-helical content relative to β-sheets. From Tab. 1, it would appear that urea still had a small effect of the interpretation of secondary structure change, the change between bloodmeal and PPM-TEG was much greater and therefore suggest an actual change in structure after modification.

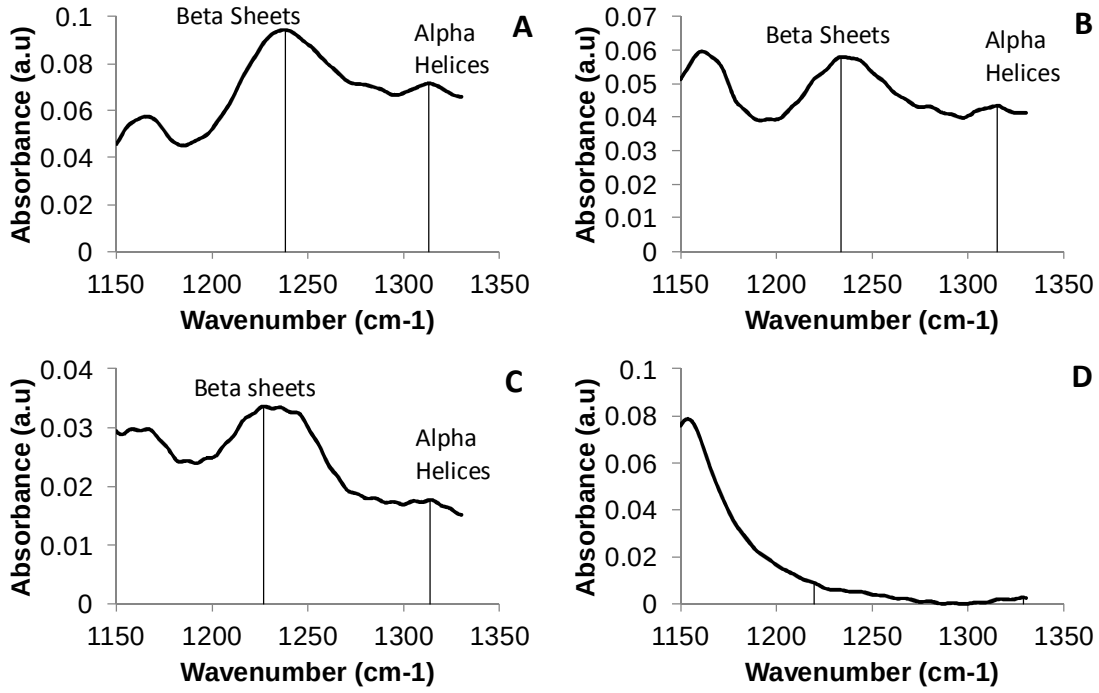

Fig. 3: Amide 3 region from KBr discs of A) bloodmeal, B) bloodmeal dry mixed with 10 pph_{BM} urea, C) PPM-TEG, D) urea alone.

Tab. 1: Ratio of amide 3 peak intensities characteristic of alpha helice and beta sheets in KBr discs.

Material	ratio (α/β)	stdev (n=3)
Bloodmeal	0.76	0.002
Bloodmeal dry mixed with 10 urea	0.67	0.08
PPM-TEG	0.53	0.005

Wide angle X-ray scattering (WAXS)

Inter-chain distances in glassy polymers can be estimated from the position of peaks in the inter-chain correlations region (between 1 and 2.5 $Å^{-1}$) of the WAXS scattering vector (s) where $s = 4\pi \sin\theta/\lambda$ (Elicegui et al., 1998). In proteins, two peaks are typically seen. Firstly, a peak at s ≈ 0.6–0.7 $Å^{-1}$ (d ≈ 10 Å), attributed to both inter helix packing in predominantly helical proteins and to inter-sheet separation in proteins that are predominantly beta sheets (Elshemey et al., 2010). Secondly, a peak s ≈ 1.5 $Å^{-1}$ (d ≈

4–5 Å), attributed to hydrogen bonding along the backbone of α-helical proteins and to hydrogen bonding between strands in beta sheets (Elshemey et al., 2010).

Fig. 4 shows the effect of processing additives on these peaks. The peak at s = 0.6 $Å^{-1}$ decreases in size, indicating disruption of longer range packing of secondary structures. Likewise the sharp peak at s = 1.3 $Å^{-1}$ (d spacing of 4.8) also reduces in size with the shoulder seen in bloodmeal at 1.5–1.7 $Å^{-1}$ (d spacing of 3.7–4.2) emerging as larger after treatment with the processing additives. This shows that some structural rearrangement is taking place, leading to a less ordered structure overall and affecting the hydrogen bonding environment of at least some of the protein backbone. As intra-helix spacing is wider than inter-chain packing in β-sheets, it was thought that the sharp peak at s = 1.3 $Å^{-1}$ would correspond to helical structures and the shoulder to β-sheets, further supporting the reduction in helical content (relative to sheets) suggested by FT-IR spectroscopy.

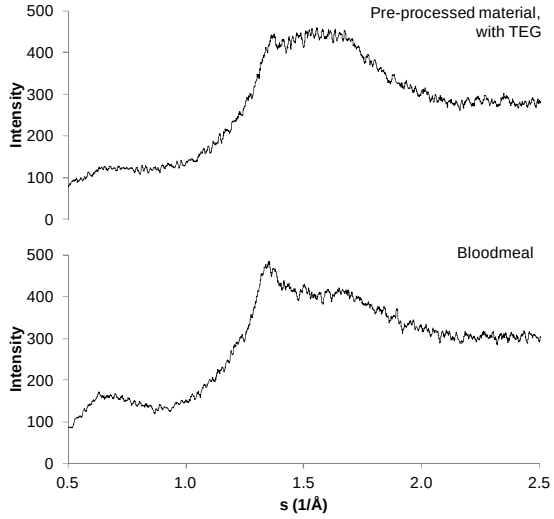

Fig. 4: WAXS scattering vector plots for bloodmeal and pre-processed material with TEG.

Synchrotron light based FT-IR microspectroscopy.

Structural mapping based on the ratio of amide 3 α-helix to β-sheet peak intensity is shown in Fig. 5 for bloodmeal and Fig. 6 for PPM-TEG. Mapping ratios in this way has the advantage of eliminating variations in spectral intensity arising from variation in thickness and enables relative changes in the ratio of secondary structures to be visualised across a sample (Yu et al., 2004).

It is apparent that there was variation of the amide 3 α-helix to β-sheet peak intensities across both bloodmeal and the pre-extruded thermoplastic particles prior to processing. Domains in bloodmeal appeared in the order of approximately 10–20 μm, whilst much larger domains were present in PPM-TEG. This would imply a gradual rearrangement of chains leading to a disruption in secondary structure. It is this rearrangement that leads to consolidation of individual bloodmeal particles.

In both materials, although there were regions where the α-helix peak intensity approached that of the β-sheet peak (reaching a maximum ratio of 0.9 in bloodmeal and 0.78 in PPM-TEG), for the most part it was considerably smaller, consistent with

bloodmeal being a thermally denatured aggregated protein with high beta sheet content. Averaging the peak intensity ratios from all spatially collected spectra supported earlier observations using globar-source FT-IR that processing additives reduced α-helical content relative to β-sheets (Tab. 2).

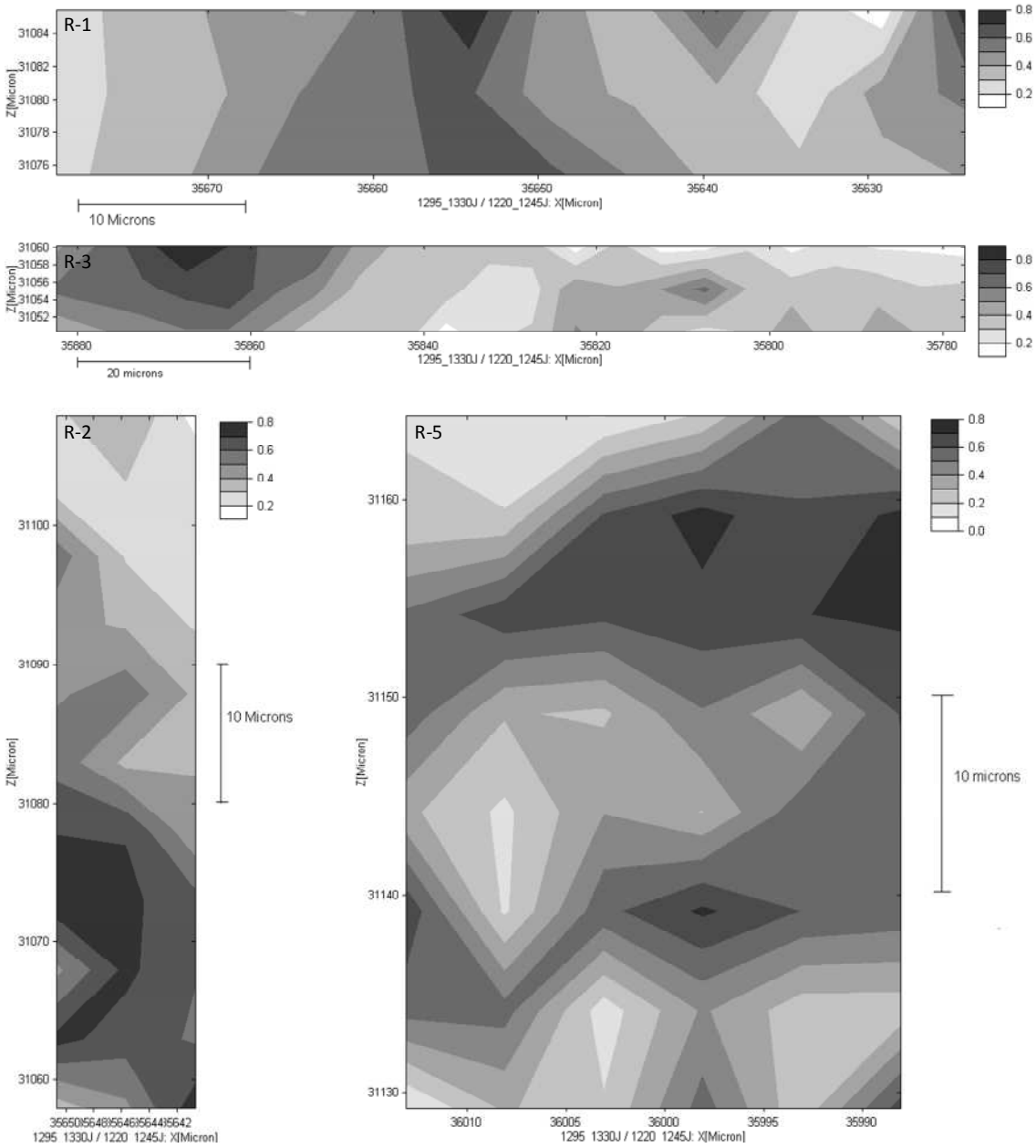

Fig. 5: Representative chemical maps of amide 3 α-helix peak height over β sheet peak height for bloodmeal. Contours are drawn at a distance of 0.1, but each map has been shaded independently. Rectangle numbers correspond to those in Fig. 1.

Tab. 2: Summary statistics for chemical mapping. The difference between the means was found to be significant at a >99% confidence interval.

		Bloodmeal	**PPM-TEG**
number of mapped points		291	564
α/β peak ratio:	average	0.54	0.25

standard deviation	0.22	0.19
minimum value	0.09	0.02
maximum value	0.90	0.78
median value	0.56	0.20

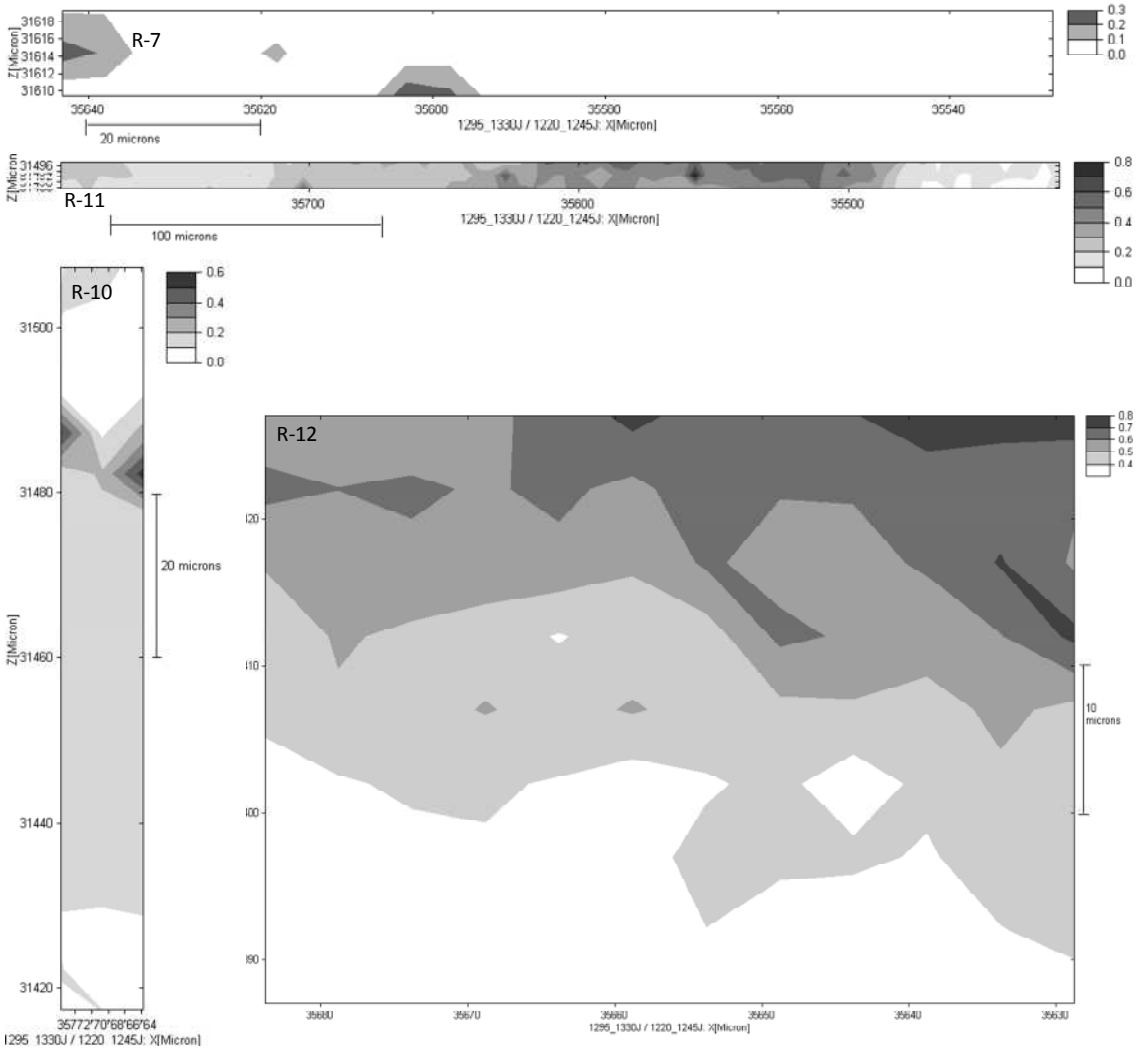

Fig. 6: Representative chemical maps of amide 3 α-helix peak height over β sheet peak height for PPM-TEG. Contours are drawn at a distance of 0.1, but each map has been shaded independently. Rectangle numbers correspond to those in Fig. 1.

CONCLUSIONS

Initial globar-source FT-IR experiments found the additives required for processing changed the shape of the amide I region. This change in shape suggested an increase of beta sheets and anti-parallel beta sheets at the expense of alpha helices, although it could also be attributed to interference from one of the additives, urea, which absorbs in this area. Examination of the amide 3 confirmed that the ratio of α-helices to β-sheets was decreasing.

WAXS revealed that the processing additives were indeed causing structural rearrangements prior to extrusion. In particular packing of secondary structures was

reduced and changes to the relative intensity of peaks at scattering vectors of 1.3–1.7 $Å^{-1}$ (d spacing 3.7–4.8 Å) suggested change to the backbone hydrogen bonding environment, thought to agree with a reduction in α-helices.

FT-IR micro spectroscopy at the Australian Synchrotron enabled collection of spatially resolved spectra for bloodmeal and PPM-TEG particles. Chemical maps of the amide 3 α-helix peak to β-sheet peak ratio showed spatial distribution of secondary structure in both materials. Bloodmeal showed domain separation on the approximate order of 10 μm, whilst PPM-TEG appeared to have larger phases and overall reduced α-helical content, relative to beta sheets.

Bloodmeal contains tightly packed heterogeneous secondary structures, consistent with its history as thermally aggregated protein. Consolidation of bloodmeal particles into a thermoplastic melt requires chain rearrangements to a more homogenous structure. Addition of urea, sodium sulphite, sodium dodecyl sulphate, tri-ethylene glycol and water disrupted this secondary structure packing, leading to a more homogenous distribution.

ACKNOWLEDGEMENTS

This research was undertaken on the infrared microspectroscopy beamline at the Australian Synchrotron, Victoria, Australia. Proposal number AS113/IRMFI/4267

Travel funding support was received from the New Zealand Synchrotron Group Ltd.

REFERENCES

CAI, S. W. & SINGH, B. R. 1999. Identification of beta-turn and random coil amide III infrared bands for secondary structure estimation of proteins. *Biophysical Chemistry,* 80**,** 7-20.

DE GIACOMO, O., CESARO, A. & QUARONI, L. 2008. Synchrotron based FTIR spectromicroscopy of biopolymer blends undergoing phase separation. *Food Biophysics,* 3**,** 77-86.

ELICEGUI, A., DEL VAL, J. J., MILLAN, J. L. & MIJANGOS, C. 1998. alpha and beta relaxation processes in internally plasticized poly (vinylchloride). *Journal of Non-Crystalline Solids,* 235**,** 623-627.

ELSHEMEY, W. M., ELFIKY, A. A. & GAWAD, W. A. 2010. Correlation to Protein Conformation of Wide-Angle X-ray Scatter Parameters. *Protein Journal,* 29**,** 545-550.

JACKSON, M. & MANTSCH, H. H. 1995. The Use and Misuse of FTIR Spectroscopy in the Determination of Protein Structure. *Critical Reviews in Biochemistry and Molecular Biology,* 30**,** 95-120.

OLIVIERO, M., MAIO, E. D. & IANNACE, S. 2010. Effect of molecular structure on film blowing ability of thermoplastic zein. *Journal of Applied Polymer Science.,* 115**,** 277-287.

SEABOURN, B. W., CHUNG, O. K., SEIB, P. A. & MATHEWSON, P. R. 2008. Determination of secondary structural changes in gluten proteins during mixing using Fourier transform horizontal attenuated total reflectance spectroscopy. *Journal of Agricultural and Food Chemistry,* 56**,** 4236-4243.

TU, A. T. 1986. Peptide backbone conformation and microenvironment of protein side chains. *In:* CLARK, R. J. H. & HESTER, R. E. (eds.) *Spectroscopy of biological systems.* Chichester: Wiley.

VERBEEK, C. J. R. & BIER, J. M. 2011. Synthesis and Characterisation of Thermoplastic Agro-polymers. *In:* SHARMA, S. K. & MUDHOO, A. (eds.) *A Handbook of Biopolymers: Synthesis, Degradation and Applications.* RSC Publishing, UK.

VERBEEK, C. J. R. & VAN DEN BERG, L. E. 2010a. Development of Proteinous Bioplastics Using Bloodmeal. *Journal of Polymers and the Environment.***,** 1-10.

VERBEEK, C. J. R. & VAN DEN BERG, L. E. 2010b. Extrusion Processing and Properties of Protein-Based Thermoplastics. *Macromolecular Materials and Engineering.*, 295**,** 10-21.

VERBEEK, C. J. R., VILJOEN, C., PICKERING, K. L. & VAN DEN BERG, L. E. 2007. *NZ551531: Plastics material*. New Zealand patent application. 13 AUG 2009

YU, P. Q. 2006. Synchrotron IR microspectroscopy for protein structure analysis: Potential and questions. *Spectroscopy-an International Journal,* 20**,** 229-251.

YU, P. Q., MCKINNON, J. J., CHRISTENSEN, C. R. & CHRISTENSEN, D. A. 2004. Using synchrotron-based FTIR microspectroscopy to reveal chemical features of feather protein secondary structure: Comparison with other feed protein sources. *Journal of Agricultural and Food Chemistry.*, 52**,** 7353-7361.