Effect of bloodmeal hydrolysate on bloodmeal-based thermoplastic mechanical properties

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Abstract

Novotein thermoplastic (NTP) is a bloodmeal based plastic developed by the University of Waikato by mixing bloodmeal with water, additives and tri-ethylene glycol (TEG - a plasticiser and petroleum based product) so it can be extruded and injection moulded. In this research, bloodmeal was hydrolysed with alcalase, trypsin and pepsin. The hydrolysates were used in NTP as a substitute for TEG at four different concentrations. Tensile strength, modulus and glass transition temperature decreased with increasing hydrolysate, and also decreased with each subsequent hydrolysis step (Alcalase, then trypsin, then pepsin), consistent with a "plasticising" effect and the shorter chain lengths of the hydrolysate. While these trends were promising, the resulting material required a higher specific mechanical energy to extrude at higher hydrolysate contents and was very brittle. The effect of salt content in the hydrolysate and order of operations in preparing the material for extruding on specific mechanical energy would need to be explored further. Future work could look at fractionating the hydrolysate to recover the lower molecular weight peptides, or to increase extent of hydrolysis without increasing salt content in the hydrolysate.

Keywords: Thermoplastic, bloodmeal, hydrolysate, plasticiser, mechanical properties.

Introduction

Renewable and compostable bioplastics have been successfully developed from different protein sources such as wheat gluten, corn, sun flower, keratin, casein, soy, gelatine and whey (Verbeek and van den Berg 2009). A recent promising bioplastic is Novotein Thermoplastic (NTP) produced from bloodmeal. Bloodmeal is a highly aggregated, cross-linked and insoluble product from the meat processing industry with a glass transition temperature around 220^{o}C, too high for it to be extruded as a plastic without degrading the protein. Bloodmeal can be extruded successfully under relatively low temperatures when mixed with urea, sodium dodecyl sulphate, sodium sulphite and tri-ethylene glycol (TEG) to reduce hydrogen-bonding, hydrophobic interaction and cross-linking and increase protein chain mobility (Verbeek and van den Berg 2010). It is compostable and will lose half its mass within 12 weeks in a compost (Verbeek et al 2011).

NTP is in the process of being commercialized by the University of Waikato spin-off company Aduro Biopolymers. Potential applications include renderable plastic components for the meat processing industry and biodegradable pot plant containers.

TEG and water are used to plasticise NTP. Plasticisers are typically low molecular weight molecules that increase the space between polymer chains allowing for greater chain mobility. This lowers the glass transition temperature, making the material softer, more flexible and more durable. For

protein based plastics, plasticisers have to be compatible with the material, so typically have one or more -OH groups, e.g. TEG, glycerol, ethylene glycol, that allows hydrogen bonding and hydrophobic interaction with the protein (Bier et al 2014).

In NTP, 20 parts of TEG per 100 of bloodmeal is used as plasticiser, which in the final injection moulded product is about 13% by weight. NTP costs about $1.16 per kg to manufacture, of which $0.82 is bloodmeal and $0.17 per kg is the cost of TEG (TEG retails for $900 per 200L drum) (Sichone 2014). If the cost of plasticising the material could be reduced, this would reduce the cost of NTP. In addition, TEG is a petrochemical, so replacing the TEG with a plasticiser from biological material would also reduce the environmental impact in terms of non-renewable resource consumption.

Proteins can be readily hydrolysed into low molecular weight peptides for improving digestion, and food functional properties such as solubility, emulsification, and whip-ability (e.g. whey hydrolysates). Some common proteases include alcalase, trypsin and pepsin. Pepsin is a gastric acid protease present in the stomach, with an optimal pH between 1 and 3 and temperature around 38^{o}C. Pepsin preferentially hydrolyses peptide bonds formed by tyrosine, phenylalanine, alanine, leucine, cysteine, cysteine and glutamic acid, but has been found to cleave almost all peptide bonds except proline and isoleucine. Examples of use include minced chicken head and haemoglobin hydrolysis (Surowka and Fik 2006; Piot et al 2007). Alcalase (also called subtilisin) is a serine protease produced from *bacillus subtilis*, is specific for the amide group of serine, asparagine, alanine, and tryptophan and has an optimum pH of 8 and temperature of 60^{o}C (Polgar 1987). It is used for protein stain removal in laundry powders, cosmetics, and in contact lens cleaner. Trypsin hydrolyses the peptide bonds of lysine or arginine, and has an optimum ph of 8-9 and temperature of 38^{o}C (Rawlings and Barrett 1994).

While hydrolysates of collagen and soy have been manufactured into plastics (Pei et al 2013, Langmaier 2008) and hydrolysates of bloodmeal into fire-fighting foams (Bressler 2010), it appears only soy hydrolysate has been attempted as a plasticiser in soy isolate plastics (Vlad et al 2006) and ovoalbumin hydrolysates as plasticisers in suspensions of alumina powders (Schilling 2002).

The aim of this project was to explore the effect of using protein hydrolysates made from bloodmeal as an alternative plasticiser to TEG.

Materials and Methods

Materials

Bloodmeal was collected from the Wallace Corporation bovine rendering facility (Waitoa, New Zealand). Sodium dodecyl sulfate was purchased from Merck, sodium sulphite from BDH Labs and TEG from Orica Chemnet. Alcalase was purchased from Novozymes, and trypsin and pepsin from Sigma. All other chemicals were purchased from Ajax Finechem and were analytical grade.

Hydrolysate production

1.13 kg of bloodmeal (Wallace Corporation) was added to 30 litres of distilled water and the pH adjusted to 8. The solution was heated to 60^{o}C using a submerged heating coil and agitated using an overhead stirrer. 250 ml of alcalase was added and the reaction allowed to proceed for 8 hours while keeping the pH constant. 10 litres of solution (Alcalase hydrolysate) was removed and centrifuged to remove un-hydrolysed bloodmeal. Temperature was dropped to 38^{o}C and 10.8 g of trypsin was added to the remaining 20 L of solution, pH maintained at 8, temperature and the reaction allowed to proceed for another 8 hours. 10 litres again was removed for centrifuging (Trypsin hydrolysate). With the remaining 10 litres, the pH was adjusted to 1.5, temperature

lowered to 37.5°C, 15 g pepsin was added and the reaction allowed to proceed for another 8 hours before being neutralised and centrifuged (Pepsin hydrolysate).

Molecular weight distribution

Molecular weight distribution of proteins in each hydrolysate were analysed using gel filtration chromatography. A Superdex 200 10:300 column (GE Healthcare) was connected to an AKTA FPLC (GE Healthcare) and 50 μL of pre-filtered (using a 0.45 μm Minisart syringe filter) sample loaded and passed through the column at 0.5 ml/min. The running buffer was 0.02M phosphate buffer at pH 7. Protein concentration and conductivity in the effluent was recorded using an in-line UV spectrophotometer at 280 nm and an in-line conductivity meter. Average molecular weight for each hydrolysate was calculated converting volume to molecular weight using calibration data, multiplying molecular weight by the UV absorbence to obtain a weighted molecular weight that was summed, which was then divided by the sum of the UV absorbence.

NTP production

After centrifugation, each hydrolysate batch was concentrated to 50% solids by evaporating water on heating plates at 50°C. Each type of hydrolysate was added to a standard NTP formulation at four different concentrations (Table 1), resulting in 12 batches. Urea, sodium sulphite, sodium dodecyl sulphate and hydrolysate were added to water in a beaker according to the recipe in Table 1, and heated to 50-60°C on a hot plate for 15 minutes while being mixed. This was added to bloodmeal and the mixture mixed again for 5 minutes using both a blender and manual mixing. The actual mass of water used was adjusted to account for the water content in the hdyrolysate. The resulting mixtures were double bagged in zip-lock bags and refrigerated until used for extrusion.

Table 1. Recipe used for each batch of NTP.

	Amount	
Ingredient	Mass (g)	pph(BM)
Bloodmeal	300	100
Urea	30	10
Sodium sulphite	9	3
Sodium dodecyl sulphate	9	3
Water	120	40
Hydrolysate addition (dry weight)	20	6.7
	40	13.3
	60	20.0
	80	26.7

Extrusion of blended formulations

A twin screw Thermoprism TSE-16-TC extruder was used to extrude each NTP formulation. The temperature profile from the feeder to the die was 70, 100, 100, 100 and 120°C for each zone respectively (Figure 1). Extruder screw speed was 150 rpm and feed rate from the hopper to the extruder was 60 Hz. Mass flow rate from the extruder, torque and pressure were measured each minute for each batch. Specific mechanical energy (SME in kJ/kg) was calculated by:

$$SME = \frac{P_r}{M}\frac{\tau_a}{\tau_r}\frac{RPM_a}{RPM_r}$$

Where P_r is the rated power of the extruder (1.25 kW), M is the mass flowrate of extrudate (kg/s), τ_a and τ_r are the actual and rated torque of the extruder (Nm), and RPM_a and RPM_r are the actual and rated revolutions per minute of the extruder screws. τ_r was 12 Nm, RPM_a was 150 and RPM_r was 500.

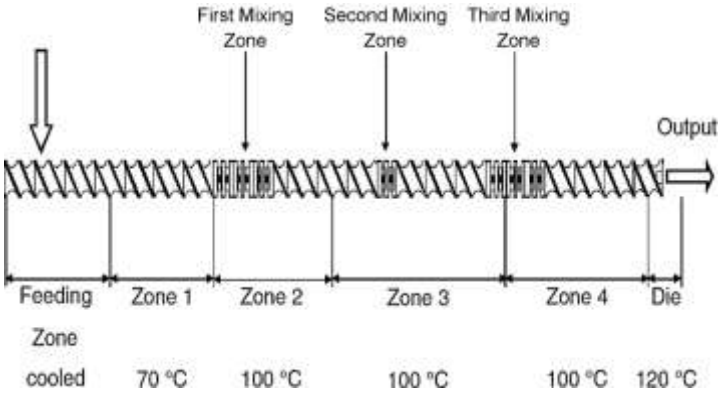


Figure 1. Extruder screw configuration.

Extruded material from each formulation was then pelletised using a tri blade granulator (Castin Machinery) and a 4 mm mesh screen and were double bagged in zip lock bags and refrigerated before injection moulding.

Each pelletised formulation was injection moulded into standard dog bone specimens (ASTM D638-03) and impact bars (12.8 x 6.0 x 3.4 mm) using a BOY-35A injection moulder. The temperatures for the zones from the feed to the nozzle were 100, 115, 120, 120, and 120^{o}C. The mould temperature was set at 65^{o}C. Injection moulder screw speed was 200 rpm with an injection pressure of 150 bar, and a residence time of 40 seconds in the mould. Specimens were conditioned for seven days at 50% relative humidity and 27^{o}C before tensile and impact testing.

Tensile testing

Tensile specimens were analysed according to the ASTM D638-86 method for tensile strength, modulus and elongation at break. An Instron 33R-4204 tensile tester was used, fitted with a 5 kN load cell, operating with a cross head speed of 5 mm per minute. A 50 mm extensometer attached to the middle of the specimen to measure strain. Five measurements were carried out for each formulation and average values and standard deviations obtained. Broken specimens were then oven dried to obtain moisture content.

Impact testing

Impact specimens were notched and impact tested on impact tester. Specimen width and thickness near the notch, and notch size were measured for each specimen before testing. The specimen was placed on the impact tester with notch facing away from the hammer which was placed at a set distance from specimen. Impact acceleration was set at 2.90 m per second. Energy required to break the specimen in kJ/m^2 was noted and recalculated with the sample dimensions.

Dynamic mechanical analysis

A Perkin Elmer Dynamic Mechanical Analyser was used to obtain glass transition temperatures of injection moulded samples. Impact bars were mounted in the DMA in a single cantilever bending system, enclosed by ceramic heater. Samples were heated to 180^{o}C from room temperature at a

rate of 2°C/minute. Data was collected for 1Hz, 10Hz and 30Hz at a dynamic displacement of 0.03mm. The storage modulus (*E*), loss modulus (*E''*) and loss factor (*tan δ*) were recorded by machine program interface. A peak in *tan δ* represented a transition temperature.

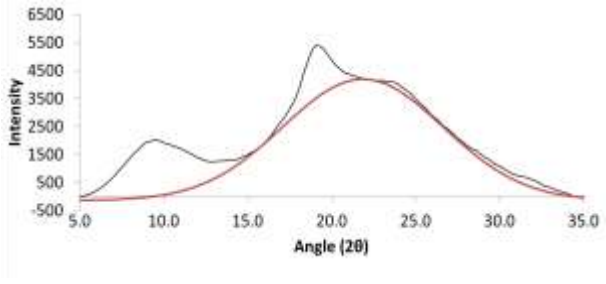
Percentage crystallinity

X-ray diffraction (XRD) (Panalytical Empyrean) was used to determine the percentage crystallinity of the tensile specimens using parameters listed in Table 2.

Table 2. Parameters for XRD.

Parameters	Value
Scan type	Single scan
Wavelength (Å)	1.541
X ray tube	Empyrean Cu LFF HR
Anode material	Cu
Voltage	45
Current	40
Soller slits (rad)	0.04
Mask (mm)	6.6
Filter	Large beta filter-Nickel
Scan mode	Continuous
Scan range	4-60

XRD graphs obtained (Figure 2) were baseline corrected between angles of 5 and 35 2Θ, a Gaussian curve fitted under the amorphous halo region between 10 and 35 2-theta, crystallinity obtained by subtracting the curve area from total peak area, and percentage crystallinity by dividing crystalline area by total peak area. Duplicates were done for each specimen.



a

Figure 2. A typical XRD graph for NTP that has been baseline corrected and a Gaussian curve fitted under the halo.

Results and Discussion

Bloodmeal hydrolysate

Conversion of bloodmeal into hydrolysate was about 70% by mass. Bloodmeal hydrolysate molecular weights ranged between 80 kDa down to very small sized peptides, with characteristic peaks around 15-18 ml (45 kDa to 12.5 kDa), major peak at 20 ml (4.3 kDa) for alcalase hydrolysate and 20.8 ml (2.8 kDa) for trypsin and pepsin hydrolysates, 24-25 ml (0.5 to 0.4 kDa) for alcalase and trypsin and 26.7 ml (0.170 kDa) for pepsin (Figure 3). Alcalase hydrolysate had an average molecular weight of 8.9 kDa, trypsin hydrolysate 5.5 kDa and pepsin hydrolysate went back up to 8.8 kDa. This could be due to the pepsin added; the peak at 15 ml on the pepsin hydrolysate chromatogram corresponds to 35 kDa which is close to the molecular weight for pepsin (34.5 kDa). The resulting

molecular weights are lower than haemoglobin (64 kDa) and bovine serum albumin (66 kDa), which makes up the majority of protein in bovine blood.

The Superdex 200 column used has a column volume of 24 ml, therefore there may have been some hydrophobic interaction between the column and some of the peptides retarding their passage through the column, resulting in them exiting between 25 and 40 ml.

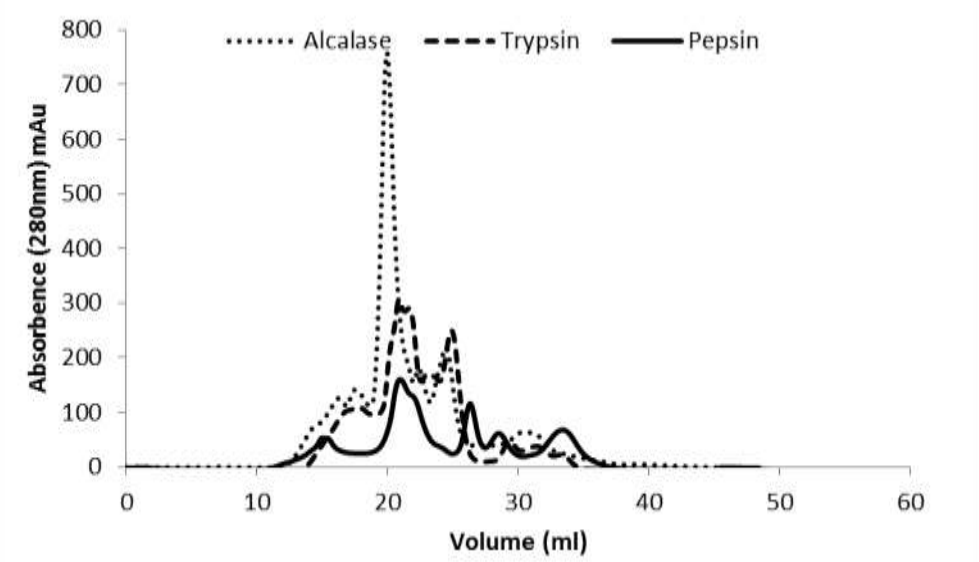
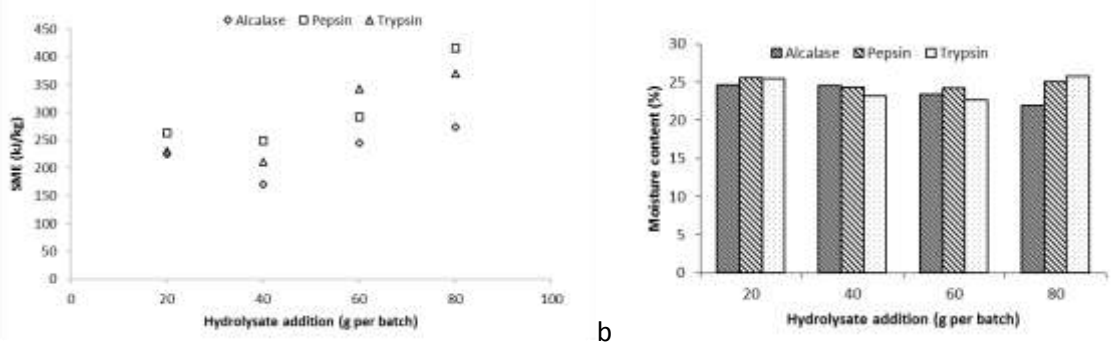


Figure 3. Gel filtration chromatogram for alcalase, trypsin and pepsin hydrolysates.

Processability of NTP with hydrolysate

NTP with hydrolysate added generally was able to be extruded and injection moulded, with the exception of NTP with 20 g alcalase hydrolysate. On this sample, the injection moulder blocked and only one partial tensile specimen was able to be produced. With increasing hydrolysate, foaming became an issue while preparing the mixtures for extrusion, and the material was increasingly harder to extrude with the extrudate coming out in small lengths. This was supported by the specific mechanical energy (SME) results (Figure 4a) which increased with increasing hydrolysate. Pepsin and trypsin hydrolysates gave higher SME than alcalase at the higher hydrolysate levels.



a b

Figure 4. a) Specific mechanical energy required to extrude the NTP with hydrolysates, and b) moisture content (% by weight) of the extrudate.

The higher SME could be caused by the slight reduction in water content in the extrudate (Figure 4b) due to the higher protein content in the pre-extruded mixture compared to water and other

additives (although water content for NTP extrudate with pepsin and trypsin hydrolysate at 80 g per batch was similar to the 20 g batch extrudate). Water also acts as a plasticiser and would explain the increase in SME. In addition, bloodmeal was added after the hydrolysate and other reagents had been mixed, so there could have been preferential interaction of the urea, sodium sulphite and sodium dodecyl sulphate with the proteins in the hydrolysate, the reducing their interaction with bloodmeal, also causing an increase in SME. Another possibility is the increased salt content in the hydrolysate from maintaining the pH during the hydrolysis reaction increasing the protein hydrophobic interaction in the material. Future experiments would need to explore whether or not the salt content in the hydrolysate contributed significantly to extrudate SME, either by desalting the hydrolysate or by reducing the amount of pH adjustment carried out which would reduce hydrolysis yield. Other experiments could also explore keeping total protein to water and other reagent ratios constant and also explore the effect of order of reagent mixing in NTP/hydrolysate production.

NTP mechanical properties

Increasing hydrolysate concentration in NTP lowered tensile strength and secant modulus (Figure 5), with the alcalase hydrolysate giving the higher tensile strength and modulus, followed by trypsin and pepsin (The result for alcalase at 20 g per batch should be ignored as the batch blocked the injection moulder and only one specimen could be produced for testing). This is consistent with the overall reduction in average protein molecular weight in the NTP/hydrolysate causing a reduction in mechanical properties. Conventional NTP in comparison has a tensile strength of around 9 MPa, a modulus of 620 MPa, toughness of 3.2 MPa and a strain at break of 0.7 (Shamsuddin 2013). With increasing hydrolysate we hoped to see an increase in strain at break, toughness and impact strength due to greater chain mobility, but toughness and impact strength marginally decreased with increasing hydrolysate while strain at break was 0.01 indicating an extremely brittle material.

Glass transition temperatures (T_g) (Figure 6a) obtained from the peak in *tan δ* from DMA results remained constant at around 78.5^{o}C for NTP with alcalase hydrolysate while they decreased with increasing hydrolysate addition for trypsin and pepsin, with pepsin giving the greatest decrease in T_g. from 81.5 to 77^{o}C. This showed that the pepsin and typsin hydrolysates did have a plasticising effect, with the pepsin hydrolysate having the greatest effect. In comparison, conventional NTP was around 61^{o}C (Shamsuddin 2013) to 66^{o}C (Bier 2014). A greater plasticising effect might be seen if the smaller peptides from hydrolysis could be separated from the higher molecular weight peptides, for example, by passing the hydrolysate through a low molecular weight cut-off ultrafiltration membrane, and concentrating and using the permeate in the NTP. Unfortunately, T_g of the hydrolysates were unable to be obtained as the powders after oven drying were very hygroscopic and rapidly absorbed moisture from the air, converting back into a viscous syrup.

Percentage crystallinity in NTP/hydrolysate samples (Figure 6b) were generally consistent around 20-23% while conventional NTP was around 19-20% for powdered samples (Shamsuddin 2013) to 26% (Bier 2013). Crystallinity in protein plastics can be attributed to α-helices and β-sheets (Bier et al 2013). An increase in crystallinity for NTP/hydrolysate might be expected as in previous work, Fourier Transform Infra Red analysis using Synchrotron light showed that NTP with TEG as a plasticiser had lower β-sheets than NTP without TEG (Bier et al 2013). Alternatively, the presence of low molecular weight peptides could reduce overall crystallinity by increasing the amorphous regions in the plastic. XRDs of the hydrolysates could not be obtained for the same reason as T_g could not be obtained.

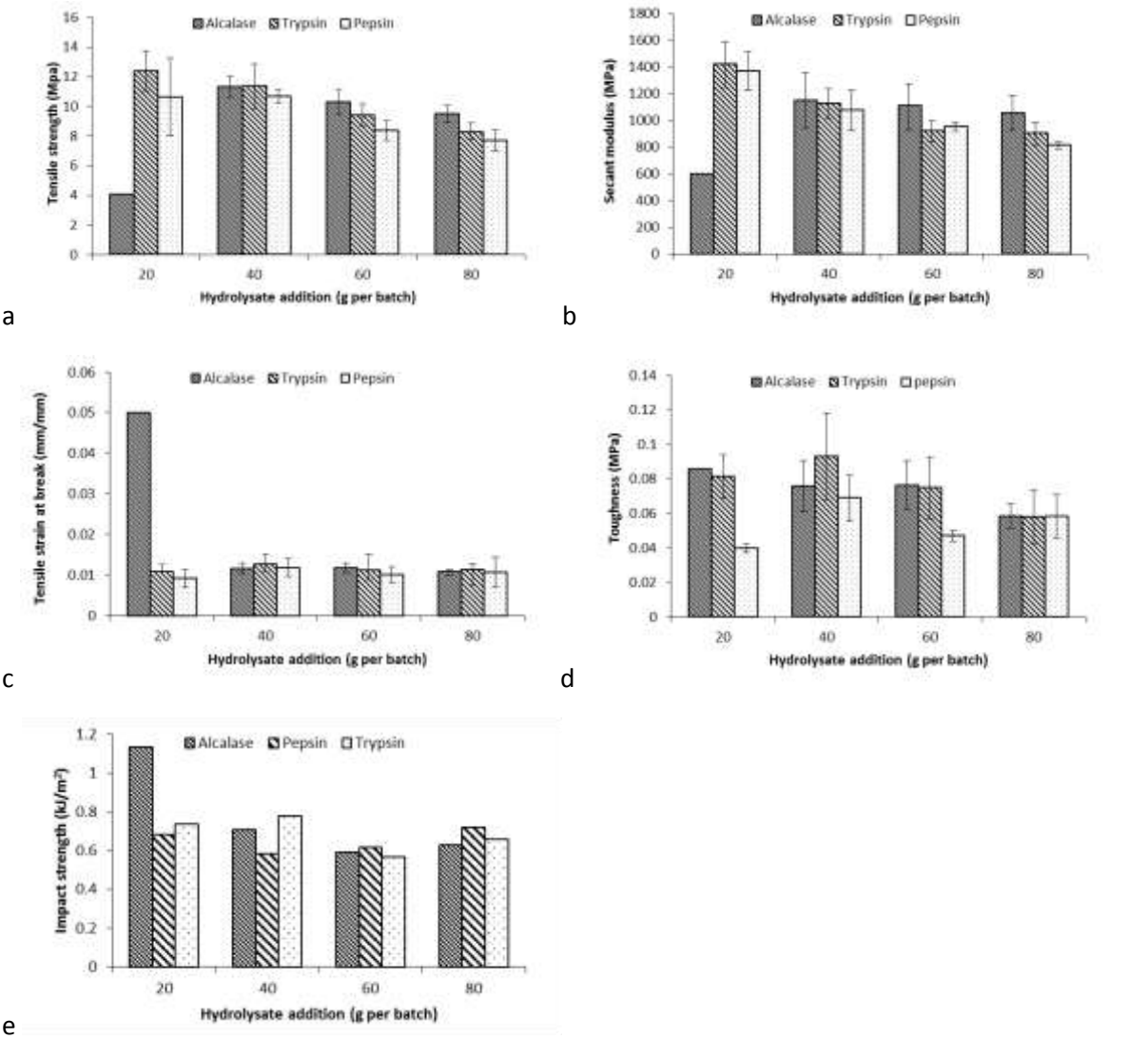


Figure 5. Mechanical properties of NTP/hydrolysate: a) tensile strength, b) secant modulus, c) strain at break, d) toughness (or energy to break) and e) impact strength.

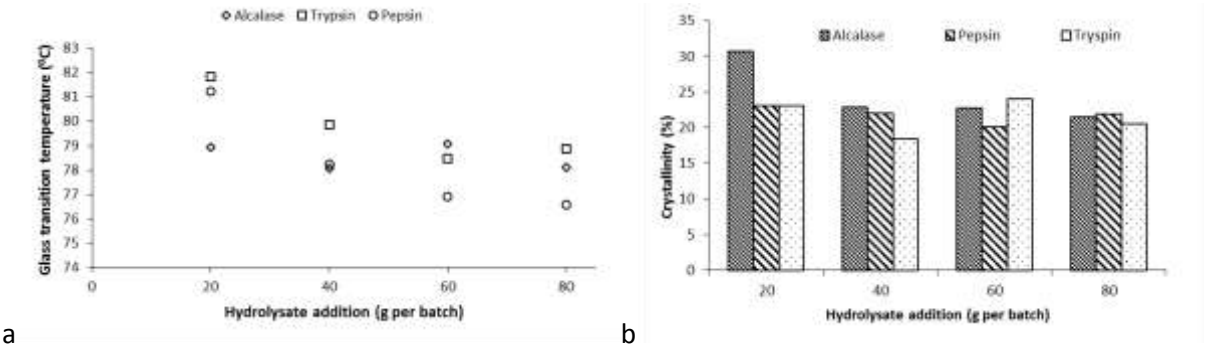


Figure 6. a) Glass transition temperature obtained from the peak in *tan δ* from DMA. b) NTP/hydrolysate crystallinity from XRD.

Conclusions

Thermoplastics could be successfully produced with NTP using bloodmeal hydrolysates as a substitute for TEG. Tensile strength, modulus and glass transition temperature decreased with increasing hydrolysate, and also decreased with each subsequent hydrolysis step (Alcalase, then trypsin, then pepsin), consistent with a "plasticising" effect and the shorter chain lengths of the hydrolysate. While these trends were promising, the resulting material required a higher specific mechanical energy to extrude at higher hydrolysate contents and was very brittle. The effect of salt content in the hydrolysate and order of operations in preparing the material for extruding on specific mechanical energy would need to be explored further. Future work could look at fractionating the hydrolysate to recover the lower molecular weight peptides, or to increase extent of hydrolysis without increasing salt content in the hydrolysate.

Biography

Mark Lay (Presenter).

Mark is a senior lecturer in Engineering at the University of Waikato, New Zealand and a member of the protein plastics/biocomposites research group led by Dr Johan Verbeek. Mark has a PhD in bioprocessing and his research interests are in protein plastics and protein recovery from process wastes.

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