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**BLOODMEAL HYDROLYSATE IN NOVATEIN
THERMOPLASTIC PROTEIN**

A thesis

submitted in fulfilment

of the requirements for the degree

of

Masters of Engineering (Hons.)

in

Materials and Process Engineering

at

The University of Waikato

by

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THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2014

Dedicated to my ideals Albert Einstein, Richard Feynman and Bhagat Singh and specially
to my motherland INDIA

Abstract

Novatein 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. The aim of this research was to produce a bloodmeal hydrolysate that could be used in NTP as a substitute for TEG, and also used to treat sodium bentonite clay to improve its properties as a filler in NTP.

Bloodmeal was hydrolysed with pepsin and alcalase to determine the reaction rate, degree of hydrolysis and optimum conditions. Bloodmeal could be readily hydrolysed giving up to 80% hydrolysis yield with 25-40% degree of hydrolysis, with average peptide molecular weight ranging between 2-12 kDa for alcalase, and 20-25% degree of hydrolysis and average molecular weights of 20-80 kDa for pepsin. Large scale hydrolysis with alcalase, trypsin and then pepsin gave 80% hydrolysis yield, and peptide average molecular weights at 8.9 kDa for alcalase, to 5.5 for trypsin.

Adsorption of hydrolysate on to sodium bentonite gave 127 mg/g clay adsorption for alcalase, but was low for trypsin, and no adsorption occurred for pepsin hydrolysate.

Specific mechanical energy required to extrude NTP increased with increasing hydrolysate content but only slightly increased with clay content, but in both cases increased with extent of hydrolysis, i.e. trypsin hydrolysate gave greater SME than alcalase, and pepsin hydrolysate gave greater SME than trypsin, which could be due to the increasing salt content in the hydrolysate.

Tensile strength, secant modulus, crystallinity, thermostability and glass transition temperature decreased with increasing hydrolysate content in NTP, likely due to the shorter average protein chain length, indicative of some plasticisation. Glass transition temperature did not change for NTP with alcalase hydrolysate. Toughness, strain at break, and impact strength were low indicating a very brittle material.

Highly variable results were obtained for the NTP with treated and untreated clay as a filler, but generally gave lower mechanical properties than conventional NTP. Alcalase treated clay was particularly detrimental on NTP composite strain at break and toughness. Thermostability of the composites increased within the 450-600°C region with increasing clay concentration for all treated clays, but showed a much more rapid decrease in mass loss.

Acknowledgements

First of all, I would pay homage to my motherland India for sending me down here to Aotearoa for my studies.

Secondly, I sincerely feel gratitude towards my chief supervisor, Dr Mark Lay and special thanks to my secondary supervisor Dr Johan Verbeek for paying me surprise visits and helping me with my stuff and giving suggestions on how to proceed (mainly beating the sh*t out of me!). Both of them provided me with intellectual prowess and kept trust in me for completing my work in proper time with ease (an exaggeration as they have well understood my way of working!). A token of appreciation again goes to Dr Lay, in whom I found a dear friend, fatherly figure and a person who introduced me to New Zealand and all the adventurous kind of stuff we could do here. His dedication and helpfulness is inexpressible, except bowing my head in front of him (as per Indian tradition!). A lot of thanks to Dr Jim Bier, who was a source of information on anything related to NTP, providing me with deep insights for the material and helping me out with some technical problems and helping me get a grasp on the instruments.

I am also thankful to my juniors, especially Rohit Giridhar, Matt Cooke and Aaron Giles who helped and spent some sleepless nights in the lab alongside me for performing hydrolysis experiments. Special thanks also goes to Shrestha Singh for providing a helping hand in the last phases of the experiments.

A special thanks goes to all lab assistants, Indar Singh, Lisa Li, Helen Turner for help on the SEM, Yuanji Zhang for helping out on the tensile tester and impact tester, and a special thanks to Liz, for having discussions with me and sharing thoughts (I remember your coffee!!). Thanks to Ivan Bell, science store keeper, and big thanks to Cheryl Ward for helping out with Endnote and various publications.

In the end, special thanks to my flatmates Geoffery Wood and Catherine Milson (for teaching how important money is and their special English ways!). Thanks everyone in office especially Chanelle, Kavwa, Matt Smith, Geome, Herman, Jussi, Marscilla, Arun, Tim, De Wet and Talia for putting up with me and my silly talks. Thanks to Rashid and Aaron Low as well.

Not forgetting, my family, parents Mrs and Mr Ahuja and brother Munny (Paroo) for their support round the clock. Special thanks to my girlfriend Isha; Harman and Bhawna for listening to my obscure ramblings.

And in the end special thanks to McDonalds who provided this cash stripped guy with constant inflow of money.

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Chapter 1 Introduction

1.1 Introduction

Plastic has dramatically changed human lifestyle, providing a material which is used from packaging to fabricating new tools, household appliances and even as replacements for broken limbs and hip joints.(Greenemeier 2007)

Plastic comes from Greek word ‘plastikos’ meaning able to be moulded in different shapes (Shah, Hasan et al. 2008). They are man-made materials manufactured from long polymeric chains of molecules. In the 1970’s, plastic materials started to replace natural materials in almost every area (Shah, Hasan et al. 2008) due to their exceptional properties and performance over metals and wood (Shimao 2001). The plastic we use today comes from inorganic and organic raw materials manufactured by petrochemical industries. This consumes 270 million tons of oil and gas worldwide (Slater 2000) to produce 140 million tons of plastic (Shimao 2001, Ren 2003), with a per capita consumption of 80-100 kg a year in developed countries (Ren 2003), which is increasing at 12% per annum (Shah, Hasan et al. 2008).

These synthetic polymers are highly stable, have a large volume to weight ratio, and are resistant to microbial degradation. They do not degrade easily, polluting water, cause “White Pollution”, i.e. white plastic material accumulating on land, and increase landfill depletion (Shimao 2001, Ren 2003). Accumulation of plastic residues in soil can decrease crop yields. Plastic bags floating on the water are also a threat to fish, birds and animals and can cause navigation issues as well (Ren 2003).

Plastic biodegradation has been a much studied subject in last three decades (Luengo, García et al. 2003). Bio-based plastics are considered to be an alternative for petroleum based plastics, reducing the pressure on landfills and help preserve non-renewable resources (Shimao 2001, Ren 2003, Olsen 2013).

Bio-based plastics are “commercial or industrial goods, (other than feed or food), composed in whole or in significant part of biological products, forestry material or renewable domestic agricultural materials including plant, animal or marine materials” as defined by Department of Agriculture (US). The American Society for Testing and Materials (ASTM) defines a bioplastic as “an organic material in which carbon is derived from a renewable resource via a biological process” (Olsen 2013). Bio-based materials are inclusive of all animal and plant mass derived from CO₂ via photosynthesis as per the definition of renewable resources (Ren 2003).

With the increase in the fossil fuel prices, pollution and drive to decrease dumping plastics in landfills (Iles and Martin 2013), and new legislature for developing environmentally and economically viable manufacturing (Soroudi and Jakubowicz 2013) for reuse and recycling (Iles and Martin 2013), the bioplastics and biopolymers industry is growing rapidly. The production of European bioplastics is expected to exponentially increase from 700,000 tonnes in 2010 to 1.7M tonnes in 2015 (Soroudi and Jakubowicz 2013).

Bio-based plastic production started in the 1970's, with investment in research into starch based biopolymers (Mohanty, Misra et al. 2002). These biopolymers are called agro-based biopolymers, because the raw material comes from plants and animals (Gaspar, Benkő et al. 2005). Biodegradable plastics can also be produced from transgenic plants and microorganisms (Scheller and Conrad 2005). Some examples of bio-degradable plastic are polyhydroxyalkanoates (PHAs), polylactic acid (PLA), thermoplastic starch (TPS), bio-urethanes (BURs), cellulose and lignin, corn zein and soy protein (Cuq, Gontard et al. 1998, Shima 2001). These are called "first generation" bio-products, derived directly from edible biomass especially corn and soybeans. This raises crop demand, raising the price of animal feed and food.

"Second generation" bio-products are produced from waste materials, for example cellulose from plant waste to produce biofuels, which could later be used to produce plastics which are bio degradable. Proteins available as a by-product or wastes from the agricultural, horticulture (wheat, soy, sunflower) and animals (gelatins, keratin and whey) are being used to produce plastics (Cuq, Gontard et al. 1998, Wang, Auty et al. 2010).(Le Tien, Letendre et al. 2000).

The principal driving force of the New Zealand economy is the red meat industry with exports worth \$8 billion a year (MIA 2013). The meat processing industry produces by-products such as connective tissue and skin, bone, tallow, and blood. Edible by-products comprise almost 10 to 30 percent of the live weight of cattle and sheep (Ockerman and Hansen 1999). Two to five percent of a cattle's live weight is blood (Ockerman and Hansen 1999). Blood has the potential for producing food ingredients, emulsifying agents and colorants (Parés, Saguer et al. 2011), but because of aesthetic values of society and the taste and dark color of blood, blood is commonly dried and

processed as a low value but nitrogen rich bloodmeal for fertilizer (Ciavatta, Govi et al. 1997).

At the University of Waikato, bloodmeal has been successfully converted into a thermoplastic (Verbeek and van den Berg 2011), called Novatein Thermoplastic (NTP) with comparable mechanical properties to low density polyethylene. This involves mixing bloodmeal with water, urea, sodium sulphite, sodium dodecylsulphate and triethylene glycol (TEG) to reduce protein-protein interactions so it can be extruded and injection moulded at temperatures that do not degrade the protein chains. NTP is in the process of being commercialized by the University spin-off company Aduro Biopolymers. Potential applications include render able plastic components for the meat processing industry and biodegradable pot plant containers. It is compostable and will lose half its mass within 12 weeks in a compost (Verbeek, Hicks et al. 2012). Current and past research is on improving NTP properties for broader commercial applications, creating NTP blends with other polymers, making films and foams, making composites by including nano-clays and fiber reinforcement. Research has also examined making a decoloured NTP (Low, Verbeek et al. 2014), and assessing NTP environmental impact by life cycle assessment (Verbeek and van den Berg 2011, Verbeek, Hicks et al. 2011, Bier, Verbeek et al. 2012, Verbeek and Koppel 2012, Bier, Verbeek et al. 2013).

1.2 Problem Statement

Plasticization of NTP with TEG and water is necessary as, while unplasticized NTP can be extruded and injection moulded, the resulting product is very brittle. This is due to poor protein chain mobility due to weak bonding, disulphite bonding and hydrophilic and hydrophobic interactions between proteins (Sothornvit and Krochta 2001). To reduce these interactions water and tri ethylene glycol (TEG) are added to bloodmeal based bioplastics (Verbeek and Koppel 2012), reducing protein-protein interactions and increasing space between protein chains resulting in more flexibility (Lawton 2004, Vieira, da Silva et al. 2011, Wihodo and Moraru 2013). TEG as a plasticizer is costly and is a petroleum product, so the aim of this thesis was to produce NTP using peptides from enzyme hydrolyzed bloodmeal as an alternative to TEG. This would help reduce cost and potentially reduce the environmental impact (Bier, Verbeek et al. 2012). Bloodmeal was chosen as a feedstock for hydrolysis as it can be sold internationally

because the blood proteins have undergone thermal denaturation. Any potential pathogens should have been destroyed during bloodmeal manufacture.

Previous research has also shown NTP mechanical properties can be improved by treating sodium bentonite clay with protein (gelatin peptides) from meat rendering plant stick water and including it as a filler in NTP, while treating clay with food grade gelatin was either detrimental or not as effective (Shamsuddin 2013). Therefore the second aim of this thesis was to treat sodium bentonite clay with peptides from bloodmeal hydrolysate and incorporate the clay as a filler in NTP.

1.3 Research Objectives and Thesis Structure

The research objectives of this study are:

- Assess the effectiveness of pepsin, alcalase and acid and optimal conditions in producing peptides from bloodmeal. This will be quantified by measuring reaction rates, degree of hydrolysis, hydrolysis yields and peptide size.
- Produce large quantities of hydrolysate of varying size distribution by sequentially treating bloodmeal with alcalase, trypsin and pepsin and recovering some of the hydrolysate at each step.
- Absorb some of the hydrolysate from each step to treat sodium bentonite clay for use as a filler in NTP.
- Assess the effect of hydrolysate as a substitute for TEG in NTP, and the effect of the hydrolysate treated bentonite as a filler in NTP. This would be determined by producing tensile specimens and impact bars containing different concentrations of each type of hydrolysate and hydrolysate treated bentonite, and measuring tensile strength, modulus, impact strength, toughness, strain at break, glass transition temperature, thermal stability, and crystallinity. Effect on plastic processability and morphology will also be examined.

This thesis will follow a conventional layout. Coverage of relevant literature to this research will be provided in Chapter Two, methods used in the research in Chapter Three, results and discussion presented in Chapter Four, and conclusions and recommendations will be given in Chapter Five.

Chapter 2 Literature Review

2.1 Introduction

The aim of this thesis was to produce NTP using peptides from enzyme hydrolyzed bloodmeal as an alternative to TEG and to treat sodium bentonite clay with peptides from bloodmeal hydrolysate and incorporate the clay as a filler in NTP. Therefore this literature review will briefly cover blood and its conversion into bloodmeal, amino acids and proteins, and conversion of proteins into plastics, the role and use of plasticizers in protein plastics, and the role and use of fillers and modified fillers in plastics. It will also cover enzymes and enzyme hydrolysis and provide some applications of enzyme hydrolysates in materials. Finally it will briefly cover some of the previous research on NTP.

2.2 Blood

Two to eight percent of a live weight of an animal is blood. Blood is the main carrier of nutrients and the engine of various functions in the body. It transfers nutrients and distributes heat throughout the body and carries waste products to the kidneys. It transports oxygen from the lungs to every organ of the body and returns CO₂ to the lungs for exhalation (Hyun and Shin 1998, Mandal, Rao et al. 1999, Ockerman and Hansen 1999). Blood is also responsible for carrying hormonal and chemical signals from one part of the body. Blood consists of many types of cells ranging from those for circulating oxygen to producing antibodies. These can be classified into three types (Stryer 2007, Cox 2008, Grisham 2010) (Table 1).

- Erythrocytes: (red cells), filled with hemoglobin and specialized for carrying O₂ and CO₂.
- Leukocytes: (white cells), helps in the fighting of the infections especially lymphocytes.
- Platelets: helps in clotting.

18-19% of blood is protein and the remainder is water (Mandal, Rao et al. 1999). Approximately 45% by volume is red blood cells and the remainder plasma. The red blood cell fraction contain 34-38% solids, the majority of which is protein, while plasma contains 7-8% protein.

Table 1: Blood and its components (Cox 2008)

Type of cell	Main functions	Typical concentration in blood per litre
Red blood cells (erythrocytes)	Transport O ₂ and CO ₂	5x10 ¹²
White blood cells (leucocytes)		
Neutrophils (polymorphonuclear leucocytes)	Phagocytose and destroy invading bacteria	5x10 ⁹
Eosinophils	Destroy larger parasites and modulate allergic inflammatory responses	2x10 ⁸
Basophils	Release histamine (and in some species serotonin) in certain immune reactions	4x10 ⁷
Monocytes	Become tissue macrophages, which phagocytose and digest invading microorganisms and foreign bodies as well as damaged senescent cells	4x10 ⁸
Lymphocytes		
B cells	Make antibodies	2x10 ⁹
T cells	Kill virus infected cells and regulate activities of other leucocytes	1x10 ⁹
Natural killer cells	Kill virus infected cells and regulate activities of tumour cells	1x10 ⁸
Platelets (cell fragments arising from megakaryocytes in bone marrow)	Initiate blood clotting	3x10 ¹¹

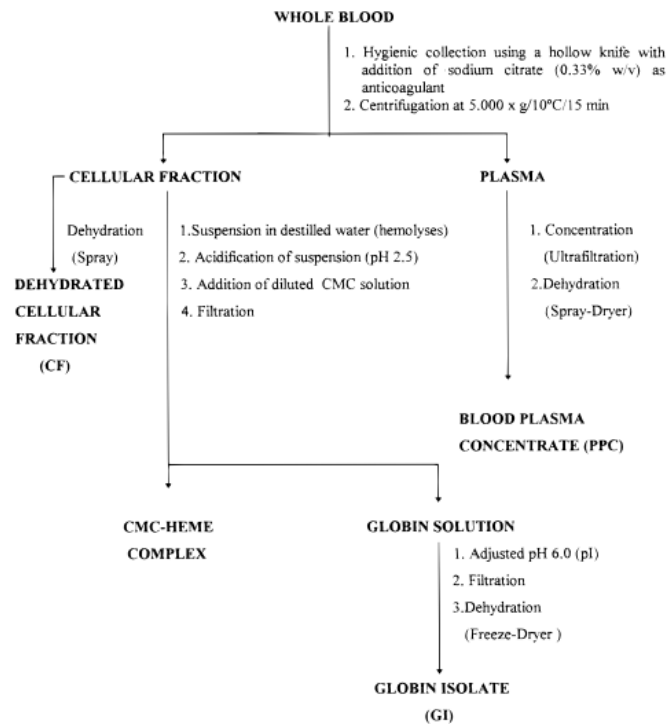


Figure 1 Blood component fractionation (Duarte, Carvalho Simões et al. 1998)

Table 2: Quantity of raw and dried blood in various species (Fernando 1992)

Species	Raw blood (kg/animal)	Dried blood (kg/animal)
Lamb	1.17	0.193
Sheep	1.76	0.266
Ox	23	3.5
Bull	16.52	2.51
Cow	18.41	2.8
Pig	2.84	0.432

2.3 Bloodmeal

A large amount of blood is produced in abattoirs when slaughtering animals. This is very rich source of nutrients and amino acids such as lysine (Waibel, Cuperlovic et al. 1977), and can be used as feed for animals such as weaning pigs (Hansen, Nelssen et al. 1993, DeRouchey, Tokach et al. 2002). Blood collected in abattoirs is dried to produce bloodmeal. Bloodmeal is a dark brown and odorous powder with a moisture content of 5-10% and protein concentration of 75-85% (Ockerman and Hansen 1999, Low 2012). It contains high amounts of hydrophobic amino acids such as valine, methionine, isoleucine and leucine. Bloodmeal has a low solubility in water due to disulphite and non-disulphide crosslinks formed during coagulation and drying (Finley, Wheeler et al. 1982, van den Berg 2009, Low 2012).

Bloodmeal can be produced using the following methods:

Direct drying: This method is used for smaller plants, where the number of animals killed per day is small, and gives almost 100 percent recovery of collected blood. Normally blood is diluted to 85 percent water content and dried in large batch cookers at a constant temperature 100 to 140°C for up to 10-15 hours. Bones are also introduced in the cooker to scour the surface and decrease drying time (Kramer, Waibel et al. 1978, Fernando 1984).

Batch coagulation followed drying: Blood is coagulated in a tank using injected steam. The coagulum is drained of water. Product losses can occur from incomplete coagulation and drainage. In addition attaining a optimum coagulation temperature of 90°C is not easy and the steam does not distribute uniformly through the tank resulting in low product quality and yield (Fernando 1984).

Continuous coagulation and mechanical dewatering before drying: This method is the most common method of drying blood. Blood is preheated to 60°C in a stainless steel holding tank with slow agitation for almost an hour. It is then passed through a tube with orifices for steam injection for rapid heating up to 90°C, the optimum temperature for coagulation. The coagulum is separated using a decanter centrifuge and sent to a dryer (Kramer, Waibel et al. 1978).

Spray drying: Blood is first dried to 40-50 % solids by evaporators with a lower heating temperature of 49°C and then spray dried with hot air at 316°C (L 1954, Kramer, Waibel et al. 1978, Teixeira, Castro et al. 1995).

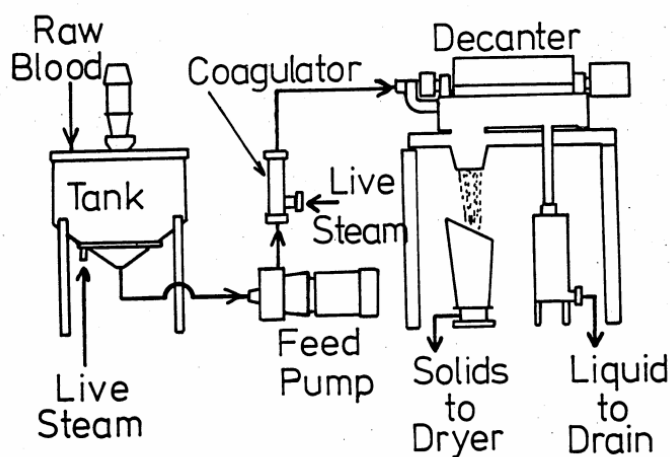


Figure 2 Diagram depicting continuous coagulation and mechanical dewatering process (Fernando 1984)

The different methods for drying blood results in slightly different amino acid profiles (Table 3).

2.4 Amino acids, proteins and peptide bonds

“The word Protein that I propose to you..... I would wish to derive from proteios, because it appears to be primitive or principal substance of animal nutrition that’s plants prepare for the herbivores, and which the latter furnish to carnivores.” –J.J. Berzelius, letter to G.J. Mulder, 1838 (Cox 2008).

Table 3 Amino acids present in differently produced bloodmeal (Kramer, Waibel et al. 1978, Low 2012)

Amino Acids	Dried Bloodmeal	Coagulated bloodmeal	Spray dried bloodmeal
Lysine	7.55	9.52	10.37
Histidine	3.76	44.23	6.38
Arginine	3.89	4.12	2.07
Aspartic acid	9.58	11.04	11.03
Theonine	3.36	5	5.11
serine	3.36	4.78	5.47
Glutamic acid	8.7	10.11	8.09
Proline	4.02	4.16	3.24
Glycine	4.91	4.48	4.51
Alanine	7.17	8.74	9.47
Cysteine	0.57	1.02	-
Valine	7.78	9.76	8.5
Methionine	0.62	0.95	0.36
Isoleucine	1.01	0.92	-
leucine	11.25	13.82	13.92
Tyrosine	2.22	3.1	2.39
Phenylalanine	6.06	7.9	8.19
Tryptophan	-	-	-

All the mechanisms taking place in biological organisms are mediated by proteins (Cox, 2008). All proteins are constructed from combinations of 20 different amino acids, linked covalently together by a condensation reaction to form a linear sequence (Figure 3).

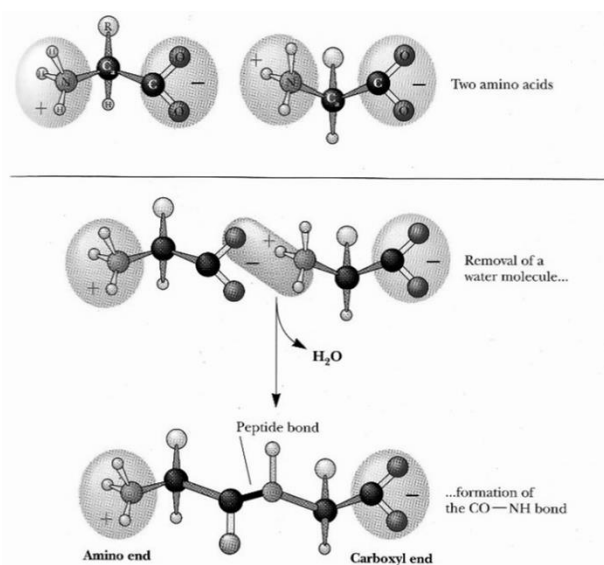


Figure 3 Formation of a peptide bond (Grisham, 2010)

2.4.1 Amino Acids

The general structure of an amino acid is shown in Figure 4. An amino acid consists of an alpha (α) carbon (C_α), covalently linked to the amino group (NH_3^+) and carboxyl group (COO^-). The central carbon atom (C_α) is also attached to a hydrogen atom and a variable side chain, called the R group, which differs for each amino acid, giving amino acid its identity. At neutral pH the amino acid is present as a neutral molecule called a Zwitterion, containing one positive and negative charge. All the amino acids are chiral molecules.

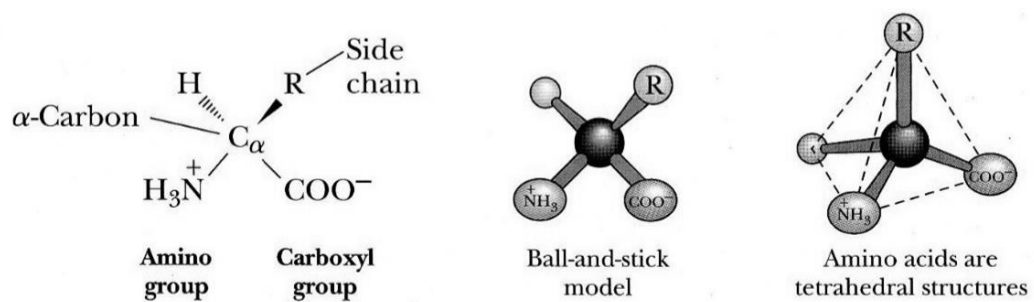


Figure 4 Various models of amino acids (Grisham, 2010)

The ability of the amino acids to polymerize to form peptides and proteins is due to the presence of the amino and carboxyl group. These groups react in a head to tail fashion (Stryer 2007, Cox 2008), with the removal of a water molecule and formation of a covalent amide link which is called a peptide bond.

The peptide bond has partial double bond character (Cox 2008), which restricts free rotation around peptide bond and leaves backbone of peptide with two degrees of freedom of movement per amino acid group.

There are commonly 20 amino acids present in the proteins, with their molecular mass shown in Table 4.

Table 4 List of amino acids and their molecular weight (Hernandez-Izquierdo and Krochta 2008)

Name	Abbreviations	Molecular Weight (Da)
Neutral		
Alanine	Ala	89
Asparagine	Asn	132
Cysteine	Cis	121
Glutamine	Gln	146
Glycine	Gly	75
Isoleucine	Ile	131
Leucine	Leu	131
Methionine	Met	149
Phenylalanine	Phe	165
Proline	Pro	115
Serine	Ser	105
Threonine	Thr	119
Tryptophan	Try	204
Tyrosine	Tyr	181
Valine	Val	117
Basic		
Arginine	Arg	174
Histidine	His	155
Lysine	Lys	146
Acidic		
Aspartic acid	Asp	133
Glutamic acid	Glu	147

All the amino acids have free carboxyl and amino groups, except proline. The amino acids are classified into different categories, but the best way to categorize them is based on the polarity of the side chains.

Nonpolar amino acids, are important in “folding” the protein chains. In this category are alanine, valine, leucine, methonine, tryptophan, phenylalanine, isoleucine and proline. These amino acids cluster together within proteins and stabilize the protein structure by hydrophobic interaction.

Polar, uncharged amino acids, form hydrogen bonds and are more soluble in water. This class of amino acids includes glycine, serine, asparagine, glutamine, threonine, cysteine and tyrosine.

Polar, acidic amino acids, aspartic acid, glutamic acid have a net negative charge at neutral pH.

Polar, basic amino acids, histidine, arginine and lysine have a net positive charge at neutral pH.

There are a few other classifications of amino acids based on interaction with water:

Hydrophobic amino acids: Alanine, glycine, isoleucine, leucine, phenylalanine, proline and valine.

Hydrophilic amino acids: Arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, histidine, serine and threonine.

Amphipathic: Lysine, methionine, tryptophan and tyrosine.

Another classification is based on the **R groups**:

Nonpolar, aliphatic R groups: Alanine, glycine, isoleucine, leucine, methionine and proline.

Aromatic R Groups: Phenylalanine, tryptophan and tyrosine.

Amphipathic amino acids tend to form micelles, which contain molecules in the form of a sphere causing it to form very stable structure. The nonpolar regions of the molecules are stabilized with hydrophobic interactions. These hydrophobic interactions are very important for stabilizing the molecule and helps determines the structure of the biological molecules. Another kind of weak interaction are Van der Waals interactions also called the London forces. These forces come into interaction when the electron cloud of molecules come close enough so they start repelling.(Stryer 2007, Cox 2008, Grisham 2010). The different structures of amino acids are shown in Figure 5 and Figure 6.

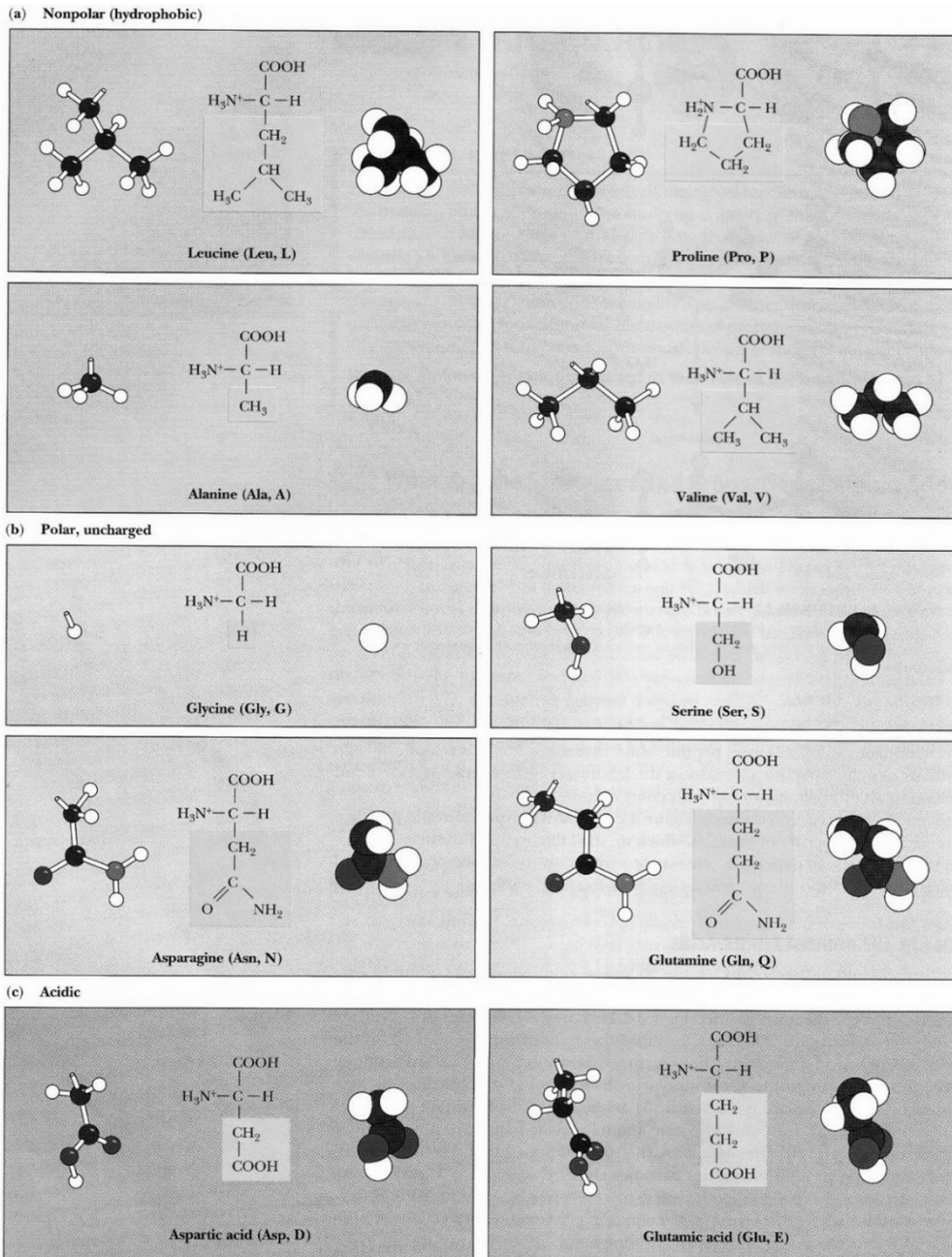


Figure 5 Twenty amino acids, which form proteins and polypeptides (continued on next page) (Grisham, 2010)

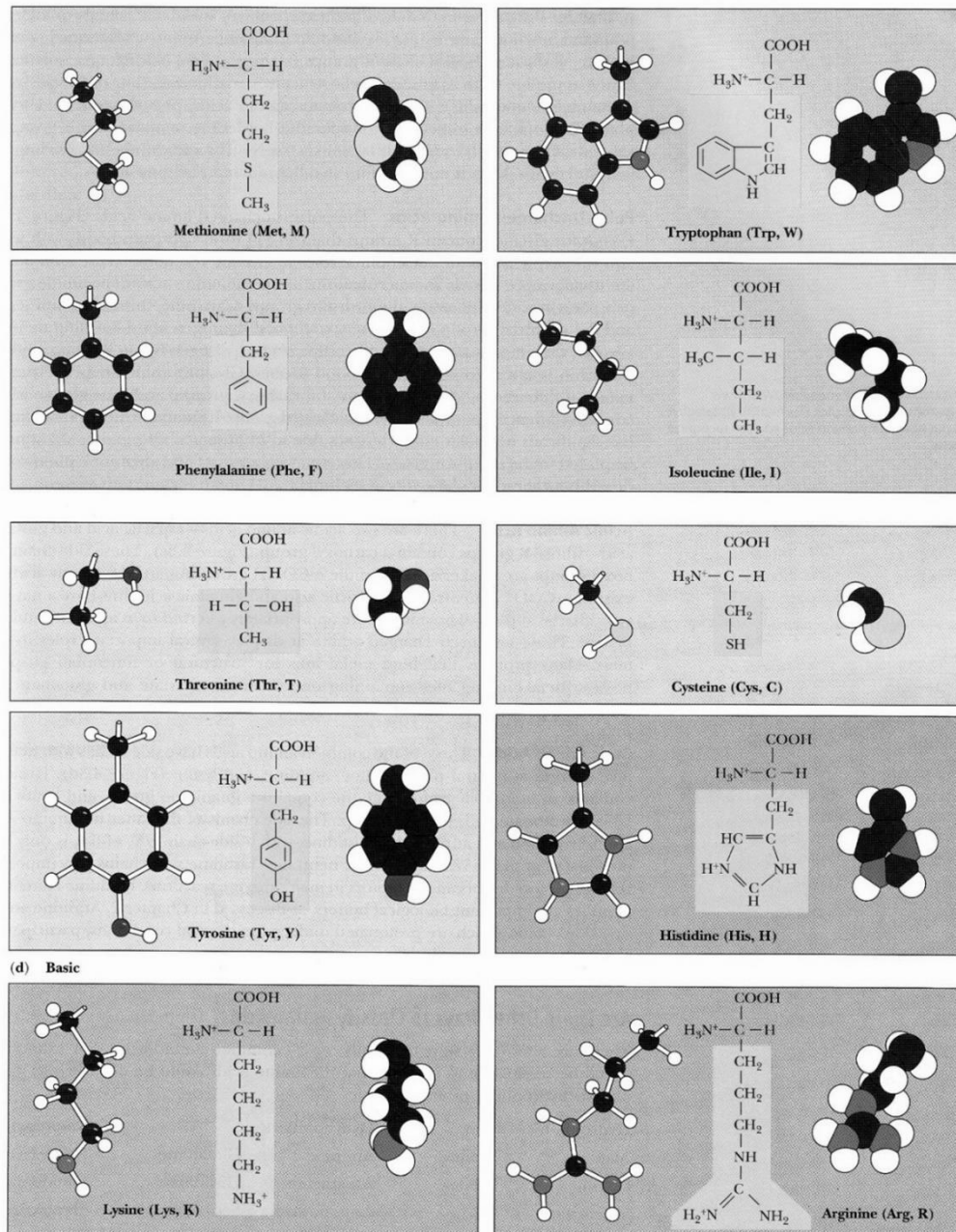


Figure 6 Continued from Figure 5

There are a few more amino acids, commonly derivatives, which perform important functions. 4-hydroxyproline and 5-hydroxylysine are found in collagen, the fibrous protein in connective tissue. 6-N-Methyllysine is in myosin, a contractible protein in muscle. γ -carboxyglutamate is found in prothrombin, a blood clotting protein. Desmosine is found in elastin in connective tissue (Figure 7).

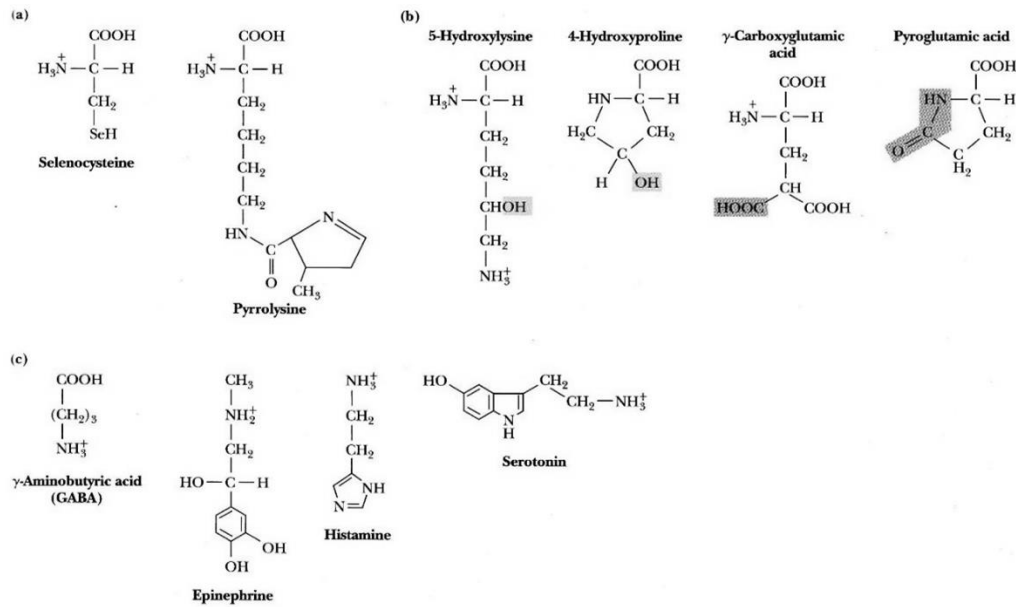


Figure 7 Uncommon amino acids (a) Selenocysteine, pyrrolysine (b) 5-Hydroxylysine, 4-Hydroxyproline γ -Carboxyglutamic acid, Pyroglutamic acid (c) γ -Aminobutyric acid (GABA), Epinephrine, Histamine, Serotonin(Grisham, 2010)

Almost 300 additional amino acids are found in cells, each having different functions, but all of them are not constituents of proteins (Cox 2008).

Amino acids behave as weak acid and bases, when an amino acid is dissolved in a solution at neutral pH, it remains as a dipolar ion or Zwitterion (Figure 8), acting as acid or base. These kind of substances are called amphoteric or ampholytes.

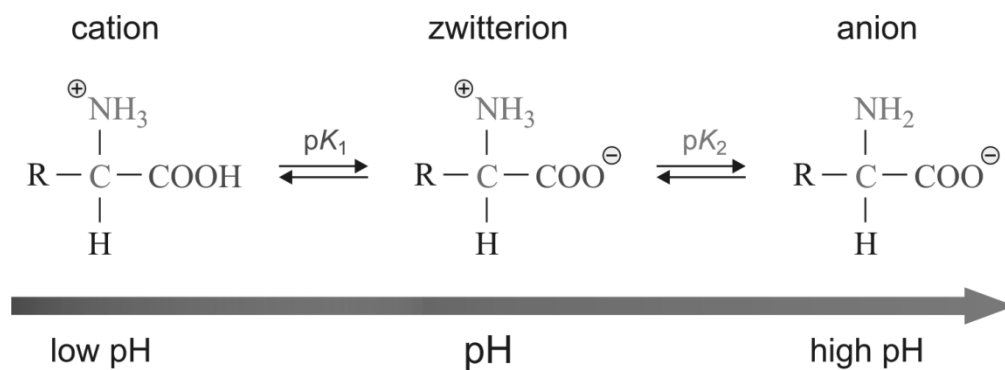


Figure 8 Amino acid shuffling between a zwitterion (Generalic 2014)

Amino acids are also chiral molecules, and have two possible configurations for α carbon atom, which are non-superimposable mirror images or enantiomers. These molecules have a special property called optical property, to rotate the plane of polarization of plane polarized light. Clockwise rotation of incident light is referred as dextrorotary and counter clockwise rotation is called levorotary. All the amino acids

are L-configuration with some dextrorotary and levorotatory at a given pH (Cox 2008, Grisham 2010).

Amino acids with aromatic rings absorb light at 280 nm. Measurement of light absorption using a spectrophotometer is commonly used to measure protein concentration in solution. The relation is defined by Lambert-Beer law,

$$\log (I_0/I) = \epsilon cl$$

where I_0 is the intensity of the incident radiation, I is the intensity of the light transmitted and ratio (I_0/I) is called transmittance, ϵ is the molar extinction coefficient, c is the concentration of the absorbing species and l is the length of the light absorbing sample.

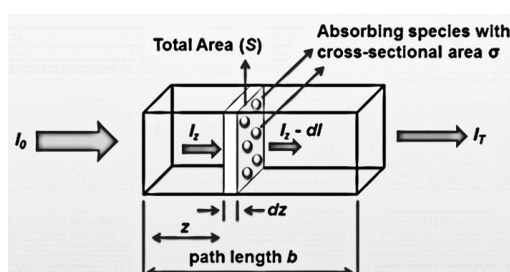


Figure 9 Depiction of Beer Lambert law (PharmaXChange)

2.4.2 Proteins

Proteins are long polypeptides of 100 to thousands of amino acid residues linked in series with peptide bonds. They consist of primary, secondary, tertiary and sometimes quaternary structures (Figure 10).

The primary structure of a protein is the amino acids joined together by peptide bonds. Regions of this may coil or fold to form secondary structures called α -helixes and β -sheets, which are stabilized by hydrogen bonds. Hydrophobic and ionic interactions fold the primary and secondary structures into the individual protein final shape, the tertiary structure, which may be globular, and will act to reduce contact between hydrophobic regions and polar regions of the protein or the surrounding environment (Figure 10 and Figure 11). Covalent cross links such as disulphide bonds between cysteine amino acids that are close to each other may form and add strength to the tertiary structure. This protein may then join with other proteins of the same or different type to form a quaternary structure (Figure 10) (Stryer 2007, Cox 2008, Grisham 2010).

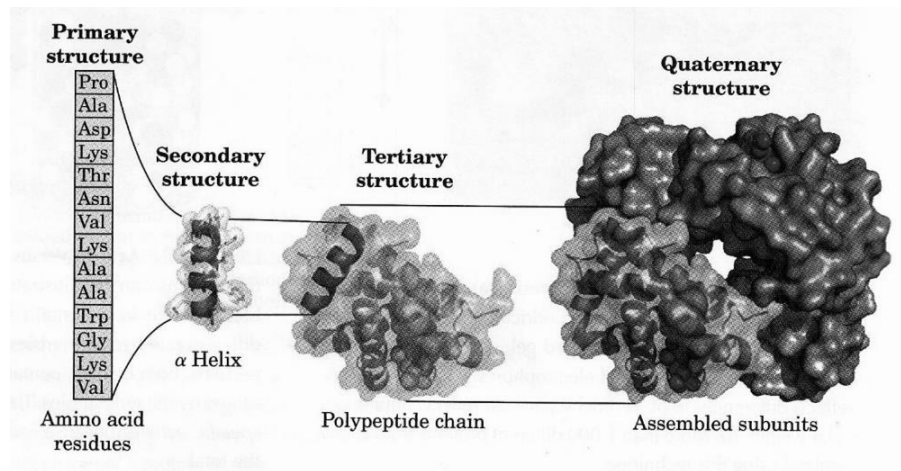


Figure 10 Representation of primary, secondary, tertiary and quaternary structure of proteins

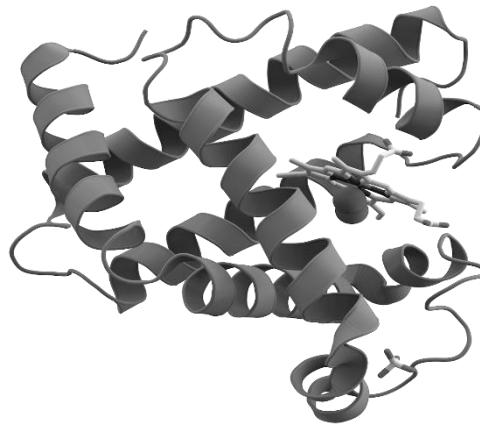


Figure 11 A depiction of a protein structure in conformational form (Cox 2008)

The end result is a protein conformation which is energetically stable (Stryer 2007, Cox 2008, Grisham 2010, Verbeek and van den Berg 2010) for the environment it exists in. These proteins can be classified into:

- Fibrous proteins: contain simple, regular structures and serve in structural roles in and outside the cells.
- Globular proteins: spherical in nature, consisting of hydrophilic and hydrophobic regions inside the molecule, and are readily soluble in water.
- Membrane proteins: consist of hydrophobic residues on the outside and are soluble in detergents rather than in water.

These proteins are very sensitive to temperature, pH and ionic changes, and the presence of organic solvents or surfactants that may reduce the polarity of the environment. An increase in temperature may overcome the interactions that hold a protein in its native shape, cause it to unfold or denature exposing hydrophobic regions, which may then interact with hydrophobic regions of other nearby proteins to produce a new stable structure. This will result in a loss of the original function (denaturation) and may give rise to aggregated and insoluble proteins (e.g. in bloodmeal production) (Adler-Nissen 1976). Heating can also give rise to more β -sheet structures in the protein or aggregate (Somero 1995).

pH changes can result in individual polar, acidic or basic amino acid side groups changing their charge from uncharged to a positive or negative charge or vice versa. This may also cause a change in protein tertiary structure resulting in unfolding and protein aggregation, particularly when the pH is taken past the protein isoelectric point or point of overall neutral charge. Increase in ionic strength by adding salt will increase hydrophobic interactions between proteins and may cause salting out, while adding non-polar organic solvents will reduce ionic strength and may also result in the protein unfolding (Cox 2008).

2.5 Proteins as Plastics

Plastics consist of long polymeric molecules that are entangled together. The polymers consist of repeating units manufactured from petroleum products that are joined together by condensation, poly-addition and cross linking reactions (Young and Lovell 2011).

Plastics can be classified into thermoplastics and thermosets. Thermoplastics do not undergo chemical change when heated and can be melted and reformed into new shapes repeatedly. Examples include polyethylene, polypropylene, polystyrene, and polyvinyl chloride (Elias 1993, Young and Lovell 2011).

Thermosets form crosslinks when heated that holds the plastic in its final shape. They cannot be remolded. Examples include rubber, that is formed from vulcanized polyisoprenes, and polyurethanes.

Plastics have amorphous (areas of disorder) and crystalline (areas of ordered structure) regions. They have a glass transition temperature (T_g) where the amorphous region

changes from a glassy state that is brittle to a molten or rubber like state, where the polymer chains in the amorphous region can move relative to each other when a force is applied. They may also have a melting temperature (T_m) where even the crystalline regions melt. Side groups on polymers such as benzene will interfere with polymer chain movement raising the T_g of the plastic. As a thermoplastic is heated, its viscosity reduces, and it more readily flows when force is applied (Nicholson 2012). Examples of material T_g are shown in Table 5.

Table 5. Glass transition temperatures of some plastics (Caprio, Fino et al. 2001, Wilkes, Summers et al. 2005, Ibeh 2011, Nicholson 2012)

Material	T_g (°C)
Low-density polyethylene (LDPE)	-125
Tire rubber	-70
Polyvinylidene fluoride (PVDF)	-35
Polypropylene (atactic)	-20
Polyvinyl fluoride (PVF)	-20
Polypropylene (isotactic)	0
Poly-3-hydroxybutyrate (PHB)	15
Poly(vinyl acetate) (PVAc)	30
Polychlorotrifluoroethylene (PCTFE)	45
Polyethylene terephthalate (PET)	70
Poly(vinyl chloride) (PVC)	80
Poly(vinyl alcohol) (PVA)	85
Polystyrene	95
Poly(methyl methacrylate) (atactic)	105
Acrylonitrile butadiene styrene (ABS)	105
Polytetrafluoroethylene (PTFE)	115

Proteins behave in similar way to thermoplastics. When they are heated beyond their ability to maintain their overall shape, their tertiary structure destabilizes and “melts”. For most proteins this melting temperature depends on the number of stabilizing interactions within the protein, and is close to the body temperature from which the protein came. For example human haemoglobin has partial unfolding temperatures of 37.2°C compared to 34°C for a duckbilled platypus and 42°C for a spotted nutcracker (Stadler, Garvey et al. 2012). Generally heating beyond this will result in a denatured protein, leading to an insoluble precipitate or aggregate of proteins. With appropriate additives to control inter and intra-protein interactions, proteins can be formed into thermoplastics. For example, bloodmeal has a glass transition temperature of 220°C. Additives can include water to hydrate and plasticize the protein, urea to reduce

hydrogen bonding between and within proteins, sodium sulphite or other reducing agents to break disulphide bonds, surfactants to reduce hydrophobic interaction (Verbeek and van den Berg 2011), and plasticizers (e.g. glycerol, sorbitol and triethylene glycol (TEG)) (Pommet, Redl et al. 2003, Verbeek and van den Berg 2011, Vieira, da Silva et al. 2011), which results in the glass transition temperature of the material dropping to 75°C.

Proteins that have been successfully converted into plastics include soy, whey, wheat, egg white, and bloodmeal (Scheller and Conrad 2005, Zhao, Torley et al. 2008, van den Berg 2009, Verbeek and van den Berg 2011, Verbeek, Lay et al. 2013). Two methods to process proteinaceous materials are dry processing and wet processing (Cuq, Gontard et al. 1998, Hernandez-Izquierdo and Krochta 2008). Wet processing involving dispersing the protein in a solvent and then removing the solvent to form the product, for example in thin films (Cuq, Gontard et al. 1998). Dry processing involves the addition of additives and plasticizers to the material and mechanical shaping using injection moulding, extrusion and or compression moulding into the final shape (Cuq, Gontard et al. 1998). Mechanical processes such as extrusion, injection moulding and addition of surfactants and plasticizers results in denaturation of proteins (if they are in their native state) (Verbeek and van den Berg 2010).

2.5.1 Compression moulding

Compression moulding involves placing preheated material into an open heated mould, which is then closed with a plug that forces the material into its final shape. This is commonly used for thermosets or fibre reinforced thermoplastics. It is a low cost method and intricate or large parts are able to be formed (Gällstedt, Hedenqvist et al. 2011).

2.5.2 Extrusion and Injection Moulding

An extruder consists of one or two rotating screws in a heated barrel, with a hopper and feeder at one end, and a die at the other. Powder or pellets are fed through feeder into the barrel, heated and pushed by the screw to the end out through the die. The temperature profile, screw speed and feed rate can be manipulated to get the desired extrudate. The shearing and mixing caused by the screw reduces the heat required to make the material less viscous and flow. In addition, kneading blocks on the screw

improve mixing of materials, allowing plastics made from combinations of materials to be extruded (Gällstedt, Hedenqvist et al. 2011, Verbeek and van den Berg 2011).

The injection moulder operates on a similar principle, but instead of going through a die, a “shot” of the material is forced into a temperature controlled mould where it cools and hardens in the final shape, after which the mould opens and the part ejected from the mould (Gällstedt, Hedenqvist et al. 2011).

2.5.3 Plasticizers

A plasticizer is a low molecular weight compound used as an additive in polymers to improve flexibility and processing of polymers by lowering the glass transition temperature of the mixture (Altenhofen da Silva, Adeodato Vieira et al. 2011, Vieira, da Silva et al. 2011). They typically have a cyclic or linear chain consisting of carbon atoms. They have a high boiling point making them ideal for injection moulding and extrusion at high temperatures. Plasticizers work by fitting in between polymer chains increasing the space between chains, and reducing the interactions between chains, allowing the chains to move more easily, lowering the temperature at which the polymer can be processed (Vieira, da Silva et al. 2011).

Plasticizers need to be compatible with or soluble in the material, so for protein based plastics, they typically have one or more -OH groups, e.g. TEG, glycerol, ethylene glycol, that allows hydrogen bonding and hydrophobic interaction with the protein (Bier, Verbeek et al. 2014). Where the plasticizer is not compatible, it may be mixed with another plasticizer which is compatible with the material, making it a secondary plasticizer (Table 6).

Table 6 Plasticizer classification (Vieira, da Silva et al. 2011)

Plasticizer Classification	
Primary	Are soluble in the material at high concentrations
Secondary	Have low solubility in the material, but are soluble in the primary plasticizer
Water soluble	e.g. glycerol, glycols
Water insoluble	e.g. fatty acids

Examples of plasticizers are listed in and plasticizers for protein based films in Table 8.

Table 7 Plasticizers and their molecular weight (Prescott and Dunn 1949, Wang, Zhuge et al. 2001, Teo, Suzuki et al. 2006, Verbeek and van den Berg 2010).

Plasticizer	Molecular mass (g/mol)	Origin
Water	18	Water
Sorbitol	182	Sugar alcohol (biological)
Lactic acid	90	Lactic acid bacteria (biological)
Glycerol (GLY)	92	Triglycerides (biological) or propylene
Ethylene glycol (EG)	62	Ethylene
Diethylene glycol (DG)	106	Ethylene
Propylene glycol (PG)	76	Propylene or glycerol (biological)
TEG	150	Ethylene
		Acetylene and formaldehyde, propylene, maleic anhydride, butadiene (petroleum or biological), allyl acetate, succinic acid (petroleum or biological) or 4-hydroxybutyrate (biological)
1, 4-butanediol	90	
Dibutyl tartrate	262	
Dibutyl phthalate	278	n-butanol (from propylene) and phthalic anhydride
Octanic or caprylic acid	144	1-octanol (from ethylene) or mammalian milk (biological)
Palmitic acid	256	Palm tree oil (biological)

Table 8 Plasticizers used in protein based films (Vieira, da Silva et al. 2011)

Protein based film	Plasticiser
Zein	Oleic and linoleic acids
Caseinate-pullulan	Water and sorbitol
Whey protein	GLY and sorbitol
b-Lactoglobulin	Sorbitol, EG, TEG, DEG, Polyethylene glycol (PEG), glycerol, sucrose, propylene glycol
Sunflower protein	Saturated fatty acids
Peanut protein	Glycerin, sorbitol, PEG, PG
Wheat gluten	Glycerin
Feather keratin	GLY
Fish mince from Atlantic sardines (<i>Sardina pilchardus</i>)	Sorbitol, GLY, sucrose
Fish skin protein	Fatty acids and sucrose esters
Water-soluble fish proteins	GLY, EG, PEG, sucrose and sorbitol
Fish muscle proteins	GLY, PG, DEG and EG
Fish myofibrillar protein	Glycerin and water
Gelatin	GLY, sorbitol, mannitol, sucrose, oleic acid, citric acid, tartaric acid, malic acid, EG, DEG, TEG, PEG, diethanolamine
Pigskin gelatin	GLY, sorbitol
Bovine gelatin	Fatty acids, sorbitol, GLY

Hydrolysis products from enzymatic treatment of proteins could also be potentially used as plasticizers. However it appears that only soy hydrolysate has been attempted as a plasticizer in soy isolate plastics (Vlad, Jane et al. 2006) (Section 2.6.2.5) and ovoalbumin hydrolysates as plasticizers in suspensions of alumina powders (Schilling, Tomasik et al. 2002). Enzymes, hydrolysis and applications of hydrolysates are discussed in Section 2.6.

2.5.4 Fillers

Another method to manipulate plastic properties is to add fillers such as talc, mica, clays (e.g. kaolin, bentonite, montmorillonite), carbon nanotubes, cellulose nanowhiskers, and synthetic and natural fibres (Zhao, Torley et al. 2008, Verbeek and Christopher 2012, Shamsuddin 2013). These can be used as bulk extenders to reduce the overall cost of the plastic, or as functional fillers to improve mechanical properties such as impact strength, tensile strength, and modulus (Croce, Persi et al. 2001). Filler addition can also change toughness, reduce gas and liquid permeability, increase thermal stability, and change electrical conductivity (depending on filler and matrix). The fillers have a much larger tensile strength than the polymer matrix. The matrix binds to the filler improve overall tensile strength of the composite. The fillers increase modulus by obstructing polymer chain movement. They also increase impact strength by stopping crack propagation in the matrix, because the fractures will stop at the filler surface. The fillers are also more impermeable to liquids and gas diffusion, forcing liquid or gas to diffuse around the filler, reducing overall permeability of the composite (Verbeek and Christopher 2012)

Nano-composites are plastics containing nano-sized organic or inorganic fillers. Clays for example are made up of platelets with high aspect ratios and high surface areas that can exfoliate in the plastic. Exfoliation is thought to proceed by the polymer intercalating between the platelets, forcing the platelets apart resulting in exfoliation (Figure 12). Nano-composites have improved strength and stiffness, but decrease in toughness (Shamsuddin 2013).

Nano-fillers are used to produce nano composites using three techniques (Shamsuddin 2013):

1. Solution blending produces nano-composites by mixing the polymer and filler in a solvent and then removing the nano-composite from the solvent.

2. Polymerization, where clay is mixed with monomers, the monomers polymerise and exfoliated nano-composites are produced.
3. Melt processing, where high temperature and shear pressure is used to exfoliate clay into the mixture.

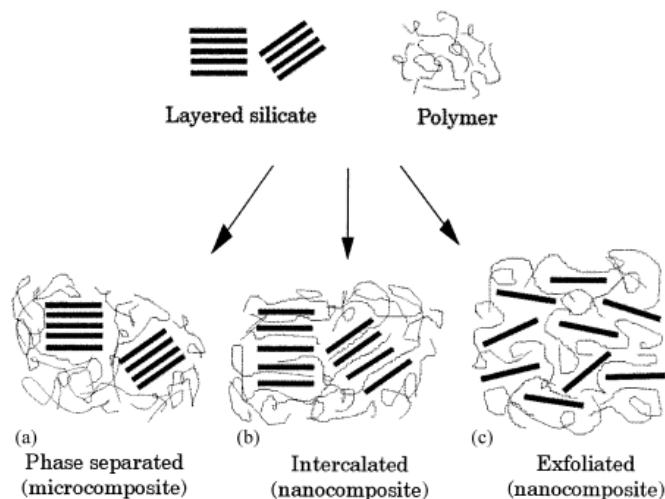


Figure 12 Schematic diagrams of different type of composites arising from the mode of nanoclay-polymer interaction (Shamsuddin 2013)

Exfoliation is dependent on the compatibility of the filler material with the polymer. If the filler is incompatible with the polymer, the polymer will not adhere to the surface, and the filler and polymer will phase separate. For example, clay surfaces are negatively charged while the polymer may be non-polar or hydrophobic. In the case of protein plastics, proteins contain a mixture of hydrophobic and hydrophilic regions. Polymer/filler compatibility can be increased by adding compatibilisers such as maleic anhydride which interact both with the clay surface and polymer. In the case of protein plastics, the clay surface can be modified by adsorbing octodecylamine to the surface (Verbeek and Klunker 2013), or suspending the clay in solution and adsorbing soluble protein on to the surface (e.g. gelatin or stickwater) (Shamsuddin 2013).

2.6 Enzymes

To sustain life, two important functions are required, reproduction and catalysis of a chemical reaction within stipulated time frame to sustain life. Enzymes are proteins which help catalyse and accelerate a reaction. Enzyme and catalyst promoted reactions reduce the free energy (ΔG) required for a reaction to proceed (Figure 13). Enzymes have a high specificity for their substrates and normally work under mild pH and temperature (Stryer 2007, Cox 2008, Grisham 2010).

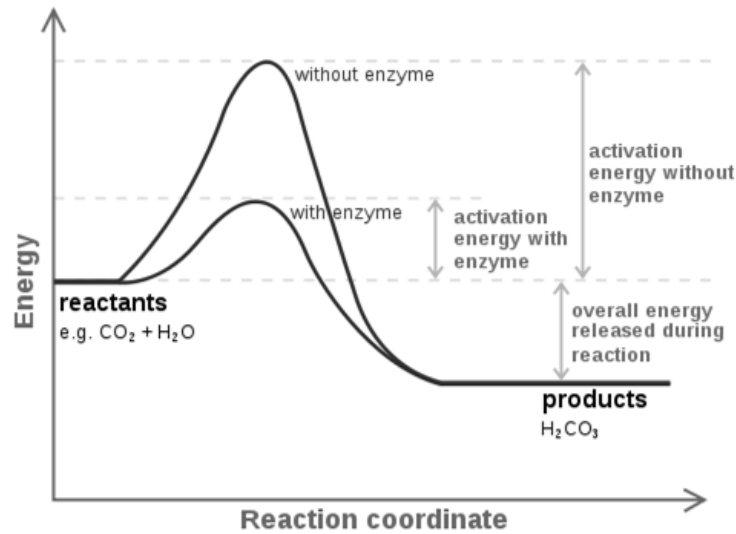


Figure 13 Transition state and activation energy with and without enzyme (Activation energy is also said as free energy in some cases as detailed in paragraph above).

Biological catalysis was recognized in late 1700's and early 1800's, but a major breakthrough came when Louis Pasteur's conclusion that fermentation of sugar to alcohol was not possible without yeast. This idea was known as Vitalism, but in 19th century Eduard Buchner concluded that sugar can be fermented without yeast if the molecules responsible for fermentation are present. The molecules detected by Buchner were named enzymes by Friedrich W. Kuhne. Haldane made a suggestion that weak interactions between substrate and enzyme catalyses the reaction at an accelerated rate.

Almost all enzymes are proteins with an exception of RNA molecules, their weight ranging from 12000 to more than a million Daltons. Enzymes often contain an additional chemical component called a cofactor or an organometallic molecule called a coenzyme which aid in the attachment of the enzyme to substrate.

Enzymes have been classified with various terminologies, but international community designates them with the type of reaction catalyzed by enzymes (Table 9).

An example of simple enzymatic reaction could be written as



Table 9 International Classification of Enzymes (Cox 2008)

Class no.	Class Name	Type of reaction catalysed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reaction (transfer of functional to water)
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of bonds
5	Isomerases	Transfers of groups within molecules to yield isomeric forms
6	Ligases	Formation of C-C, C-S, C-O and C-N bonds by condensation reaction coupled with cleavage of ATP or similar cofactor

According to recent theory enzymes work on the principle of a stick model, where the substrate (stick) is bent by the enzyme rendering it more susceptible to reaction. The substrate enters the active site of the enzyme, which is specific for the substrate, and reaches a transition state between substrate and product. The enzyme active site can be subdivided into two categories: enzyme complementary to substrate and enzyme complementary to the transition state. An enzyme complementary to substrate (stick) delays the bending of the stick, stabilizing the substrate, but increases interactions that increase free energy and increases rate of reaction. Whereas enzymes complementary to the transition state destabilize the substrate (stick) resulting in catalysis of reaction (Cox 2008).

2.6.1 Enzyme hydrolysis

Native protein tends to be resistant to hydrolysis by enzymes, but denatured or partially denatured proteins are more prone to cleavage (Adler-Nissen 1976). The Linderstrom-Lang model of attack by enzymes on globular and denatured protein subdivides hydrolysis into the one by one type and zipper attack. In this model, the protein oscillates between the native and denatured state, the rate of oscillation may be fast or slow (Figure 14). When it is in the denatured state it is more susceptible to hydrolysis. The cleavage of one or more peptide bonds may result in the protein irreversibly unfolding exposing more peptide bonds to enzymatic attack, resulting in extensive degradation into peptides. If the initial rate of oscillation is slow, it will appear that “one by one” each protein is broken down into peptides with little or no intermediates. If the initial rate of oscillation is fast, it will appear that all of the protein has denatured

and unfolded at the start of hydrolysis (i.e. unzipped), and subsequent cleavage of the protein into peptides will be slow with the appearance of a large number of intermediates (Adler-Nissen 1976, Panyam and Kilara 1996).

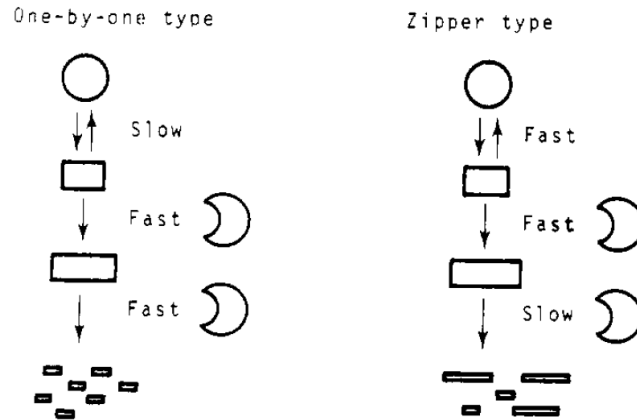


Figure 14 Linderstrom-Lang hydrolysis model (globular proteins are shown by circles, rectangles represent denatured proteins and small rectangles shows polypeptide chains of varied length) (Adler-Nissen 1976)

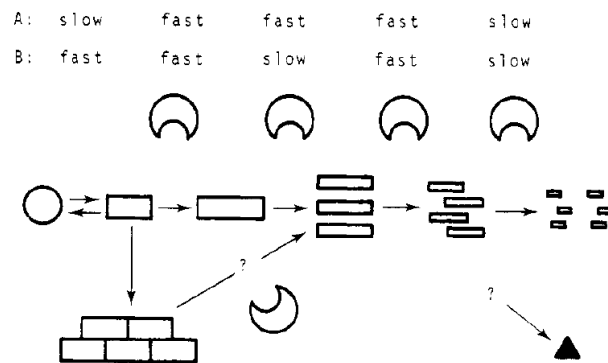


Figure 15 Hydrolysis of denatured proteins (a) one by one method (b) Zipper method. The triangle represents the insoluble residue (Adler-Nissen 1976)

The extent and rate of hydrolysis can be measured by the pH stat method, where hydrolysis is measured by the amount of base or acid added to keep the pH constant (Panyam and Kilara 1996, Spellman, McEvoy et al. 2003). The amount of acid or base added can be converted into degree of hydrolysis by:

$$DH\% = \frac{(B \times M) \times MP}{H} \times 100$$

where B is amount of base added (ml); M molarity of the base; MP is mass of protein (g), H been amount of peptide bonds present in the molecule and α the degree of dissociation of the α -amino group calculated by

$$\alpha = (10^{\text{pH}-\text{pK}\alpha}) / (1 + 10^{\text{pH}-\text{pK}\alpha})$$

Alternatively degree of hydrolysis can be calculated by (Ruan, Chi et al. 2010):

$$\text{DH} = (h/H_{\text{tot}}) \times 100$$

Where h is the equivalent of peptide bonds broken, calculated from addition of acid or base, assuming 1 mol of bond broken is equal to 1 mol of H⁺ or OH⁻ ions added.

Other approaches include measuring the amount of liberated amino groups using the photometric ninhydrin method, or by measuring the increase in solubility of proteins after trichloroacetic acid precipitation. While the different approaches are not comparable to each other, base consumption methods are easy to perform (Panyam and Kilara 1996).

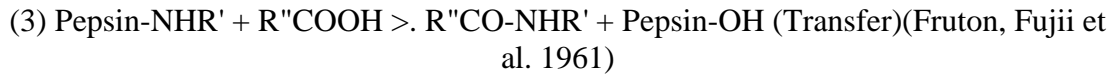
2.6.2 Applications of Enzyme Hydrolysis

Enzymes have been used to modify the structure and functional properties of proteins to improve solubility, digestibility, and foaming, or produce hydrolysates from various feed stocks including blood, soy protein, egg white, fish proteins, chicken heads and other animal parts, casein and whey (Piot, Guillochon et al. 1988, Surowka and Fik 1994)(Chobert, Bertrand-Harb et al. 1988, Achouri, Zhang et al. 1998, Kim, Ki et al. 2007). Protein hydrolysates have been used in animal feeds for centuries (Clemente 2000), and their antioxidant properties and use in human nutrition have been studied (Clemente 2000, Peña-Ramos and Xiong 2001). Enzymes used for producing hydrolysates include pepsin, alcalase, and trypsin (Kim, Ki et al. 2007, Ruan, Chi et al. 2010).

2.6.2.1 Pepsin

Pepsin is a gastric acid protease present in the stomach. It was the first enzyme discovered and named in 1823 by T. Schwann (Northrop 1920, Antonov, Ginodman et al. 1978, Fruton 2006). Pepsin is an endopeptidase with broad specificity, with optimum pH 3.5 (Lin, Fusek et al. 1992).

Pepsin preferentially hydrolyses peptide bonds formed by tyrosine, phenylalanine, alanine, leucine, cysteine, cysteine and glutamic acid (Hirs 1967), but has been found to cleave almost all peptide bonds except proline and isoleucine. A mechanism of pepsin action consists of:



There are differences in literature (Schlamowitz and Peterson 1959) as to pepsin's optimal pH, with pH cited ranging from 1.5 to 3. Certain books suggest the highest rate of pepsin reaction is at pH 3 (Cox 2008).but according to Ruan et al who used pepsin to hydrolyse egg white, the optimum pH which gave the best degree of hydrolysis was pH 1.5 (Ruan, Chi et al. 2010). Peptide hydrolysis rate decreases with increasing peptide length (Pohl and Dunn 1988).

Examples of pepsin use include preparing hydrolysates from egg white (Ruan, Chi et al. 2010), whey protein (Kananen, Savolainen et al. 2000), milk protein and soybean whey proteins (Terada, Kato et al. 1975, Peñas, Préstamo et al. 2004), and chicken heads (Surowka and Fik 1994)

2.6.2.2 *Alcalase*

Alcalase (also called subtilisin) is produced from *bacillus subtilis* (Hirs 1967). It belongs to a class of serine proteases that use the catalytic triad of Asp32, His64 and Ser221 in its active site to hydrolyse proteins. The enzyme is specific for the amide group of serine, asparagine, alanine, and tryptophan (Hirs 1967). The rate limiting step is acylation for amide bond hydrolysis and deacylation of the ester bond. Alcalase optimal hydrolysis reaction pH is 8 to 8.5 (Adamson and Reynolds 1996, Kristinsson and Rasco 2000) and temperatures ranging from 60 to 90°C.

Examples of alcalase use include producing hydrolysates from food proteins, soy protein, whey protein, sunflower proteins and porcine haemoglobin (Adler-Nissen 1977, Villanueva, Vioque et al. 1999, Sousa Jr, Lopes et al. 2004, Chang, Wu et al. 2007).

2.6.2.3 *Trypsin*

Trypsin is considered as a prototype of the serine endo-peptidases of family S1, which hydrolyses peptide bonds between the COOH group of lysine or arginine and the adjacent NH₂ group of the next amino acid. Cleavage is slow when lysine or arginine is adjacent to an acidic amino acid such as aspartic acid or glutamic acid, and stops if proline is an adjacent amino acid on either the N or C terminal side (Hirs 1967). The

optimum pH for trypsin hydrolysis to occur is between 7.0 to 9.0 and at a temperature of 39°C (Jost and Monti 1977, Pintado, Pintado et al. 1999, Galvão, Souza Silva et al. 2001, Yin, Tang et al. 2008).

Trypsin has been used to hydrolyse whey protein (Kananen, Savolainen et al. 2000), oak leaves and cannabis (Feeny 1969, Yin, Tang et al. 2008).

2.6.2.4 Examples of protein hydrolysates in materials

Ovoalbumin hydrolysates have been investigated as a plasticiser in micrometric and nanometric ceramics (Schilling, Tomasik et al. 2002). Egg white hydrolysate was used as a low cost binder in ceramics (Dhara and Bhargava 2001)(Lyckfeldt, Brandt et al. 2000, Sigmund, Bell et al. 2000). Collagen and soy protein hydrolysates are used in producing biodegradable films and packing materials (Surowka and Fik 1994, Swain, Biswal et al. 2004, Langmaier, Mokrejs et al. 2008, Bressler 2010, Song, Tang et al. 2011) and hydrolysates of bloodmeal for fire-fighting foams (Bressler 2010).

2.6.2.5 Soy protein hydrolysates as a plasticizer

In Vlad et al (2006) paper, soy protein isolate (SPI) were mixed with soy protein hydrolysates as plasticizers. The hydrolysates were produced by two types of alkali hydrolysis and added in concentrations up to 30 parts per 100 (pph) of SPI along with water at 80 pph. Compression moulded samples had tensile strengths ranging between 9-11 MPa with strain at break between 2-2.5%. Injection moulded samples tensile strength ranged between 5.5 to 10 MPa, with strain at break between 1.4 to 2.5% (Vlad, Jane et al. 2006). The tensile strengths did not decrease as would be expected with increasing hydrolysate, and strain at break decreased for the compression moulded samples, while only one of the injection moulded samples with 30 pph hydrolysate increased in strain at break to 2.5%.

2.7 Previous work done on NTP

Bloodmeal was first converted into a thermoplastic in 2008 (van den Berg 2009, Verbeek and van den Berg 2011). Bloodmeal has a glass transition temperature of 220°C, which is too high for it to be melted and extruded as a plastic without degrading the protein. It is also crystalline in nature with about 30-40% crystallinity. To reduce the glass transition temperature, a combination of urea (which interferes with H-bonding within and between proteins and also acts as a plasticiser), sodium sulphate

(with breaks disulphide bonds), sodium dodecyl sulphate (a surfactant that reduces hydrophobic interactions), and tri-ethylene glycol and water (both form H-bonds with proteins and act as plasticisers) is mixed with bloodmeal. This reduces the glass transition temperature of the mixture to 61°C enabling it to be extruded and injection moulded as a thermoplastic. NTP has similar mechanical properties to low density polyethylene and is currently being commercialized as NTP by Aduro Biopolymers, a University of Waikato spin-off company.

Currently, TEG in NTP makes up 13% by weight of the final product. TEG retails at NZ\$4.5 per litre and contributes NZ\$0.17 to the material cost per kg of NTP, about 10% of the total material cost. TEG is a petroleum product which increases the environmental impact of the material. A cradle to gate life cycle assessment carried out by Bier et al (2010) showed that omitting TEG would reduce non-renewable process energy required for NTP by 55% and reduce greenhouse gas emissions by 45% (Bier, Verbeek et al. 2012).

Various studies have examined decolouring bloodmeal and the effect of decolouring on protein properties, plastic processability and mechanical properties (Verbeek and van den Berg 2010, Verbeek and van den Berg 2011, Verbeek and van den Berg 2011, Verbeek, Hicks et al. 2011, Verbeek and Koppel 2012). Other studies have looked at the effect of the additives used in NTP and processing on protein structure, transition temperatures and mechanical properties. Other studies have examined blending NTP with other plastics and using fillers to improve NTP mechanical properties, while others have looked at the environmental impact, UV degradation and compostibility of NTP (Low, Verbeek et al. 2014, Marsilla and Verbeek 2013, Verbeek and Klunker 2013, Verbeek, Hicks et al. 2011, Bier, Verbeek et al. 2013, Bier, Verbeek et al. 2012).

2.7.1 Water adsorption, biodegradation and UV degradation

NTP will readily adsorb moisture and is biodegradable. Tensile specimens of NTP were mixed in compost at a green waste composting facility to examine biodegradability. Specimens plasticized with TEG lost half their weight over 12 weeks while unplasticized samples lost one third their mass (specimens shown in Figure 16a). The samples increased into water content from 10% up to 60% by weight within four weeks, and Fourier Transform Infra-Red analysis showed that significant hydrolysis of protein chains had occurred (Verbeek, Hicks et al. 2012). Accelerated weathering tests

by exposing the samples to UV testing showed that there was little chemical change, but that the samples became brittle due to moisture loss, with an increase in modulus, and decrease in tensile strength and strain at break (Verbeek, Hicks et al. 2011)

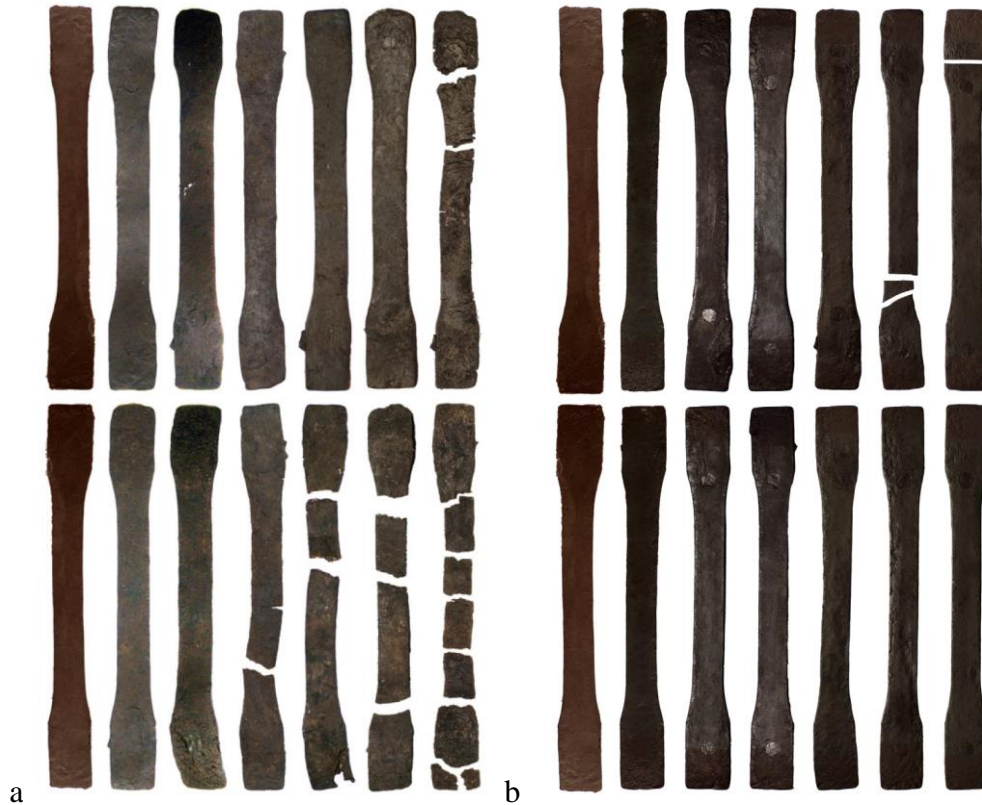


Figure 16 NTP samples with and without plasticizer (top and bottom rows respectively) over 12 weeks of a) composting and b) accelerated weathering by UV exposure.

NTP will also readily equilibrate with the water in the atmosphere. Experiments carried out on NTP with different plasticizer contents exposed to different relative humidities showed that NTP would gain moisture up to 23% at high relative humidities and lose moisture at low humidities (Verbeek and Koppel 2012). The rate and amount of water adsorbed was dependent on the amount of TEG added with high concentrations of TEG slowing water adsorption, but increasing the amount of water adsorbed (Verbeek and Koppel 2012, Lay, Verbeek et al. 2013). NTP mechanical properties are dependent on water content, with tensile strength and modulus decreasing with increasing water content (Verbeek and Koppel 2012).

2.7.2 NTP blends

To improve NTP mechanical properties and reduce water adsorption, NTP was blended with polybutylene succinate (PBS) (Marsilla and Verbeek 2013) and linear low density polyethylene (LLDPE) (Marsilla and Verbeek 2013). Poly (phenylisocyanate) co-formaldehyde (pMDI) and poly-2-ethyl-2oxazoline (PEOX) were added to compatibilise PBS with NTP at different amounts (Table 10). Polyethylene with grafted maleic anhydride was used to compatibilise LLDPE with NTP. Compatibilisation allows mixing of the two phases and produces a strong mechanical interface (Utracki 1990). When immersed in water for five days, the PBS blends reduced water adsorption from 90% for NTP to 7% for the blend (Table 11). The compatibilised blend also improved tensile strength compared to normal NTP (Figure 17) while glass transition temperature did not change (Figure 18).

Table 10 Formulations of NTP with PBS and compatibilizer pMDI and PEOX (Marsilla and Verbeek 2013).

Sample Name	NTP (wt%)	PBS (wt%)	pMDI (wt%)	PEOX (wt%)	PEOX (pph) dissolve in NTP formulation
PBS	0	100	0	0	0
NP _{0/0}	50	50	0	0	0
NP _{7/0}	50	43	7	0	0
NP _{7/3}	50	40	7	3	0
NP _{7/3*}	50	43	7	0	3
NTP	100	0	0	0	0

Table 11 Water absorption in various blends over five days (Marsilla and Verbeek 2013).

Blends	Water absorption (wt%) over 5 days
100 PBS	0.91
NP _{0/0}	18.78
NP _{7/0}	5.333
NP _{7/3}	8.48
NP _{7/3*}	7.57
100 NTP	90.42

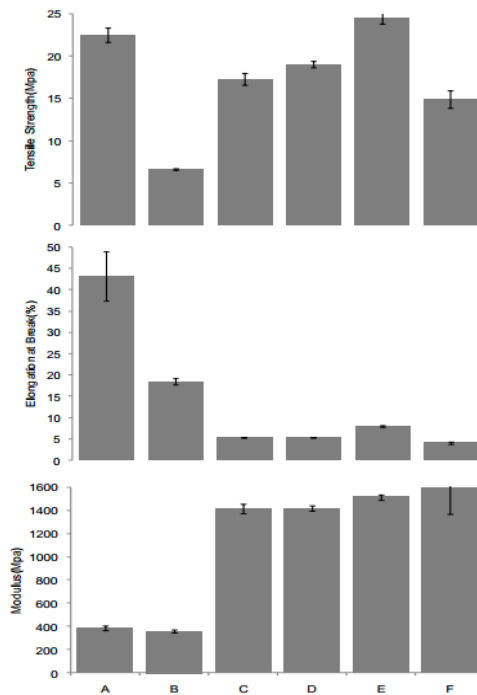


Figure 17 Tensile strength, percentage elongation and secant modulus of NTP/PBS blends [A:PBS; B:NP0/0; C:NP7/0; D:NP7/3; E:NP7/3*; F:NTP(Marsilla and Verbeek 2013)]

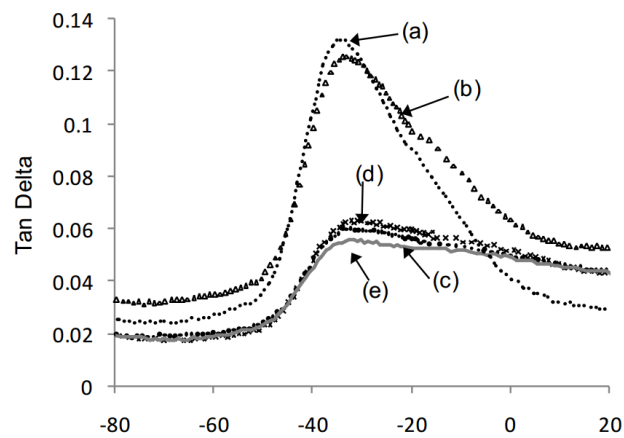


Figure 18 Glass transition temperatures of various blends [a: PBS; b: NP0/0; c: NP7/0; d: NP7/3; e: NP7/3*](Marsilla and Verbeek 2013)

NTP blended with LLDPE with the compatibiliser did not improve tensile strength until NTP content was 40% by weight or lower but did increase elongation at break (Figure 19)(Marsilla and Verbeek 2013).

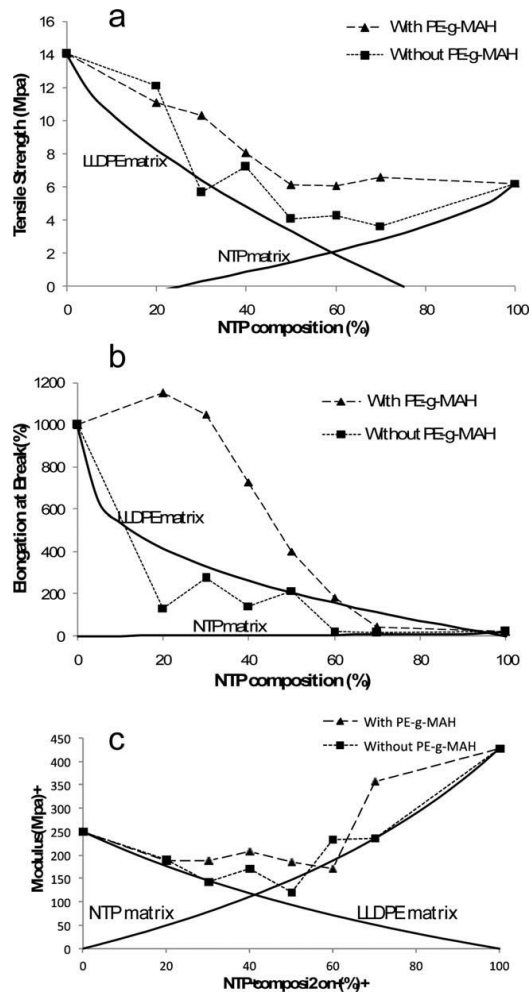


Figure 19 Mechanical properties of blends a. Tensile strength b. Elongation at break (%) c. Modulus

2.7.3 Decolouring bloodmeal and manufacturing decoloured NTP

Bloodmeal has been treated with peracetic acid (PAA), a strong oxidizing agent used in sanitisers, to produce a decolourised NTP (Figure 20) and reduce odour (Low, Verbeek et al. 2014). 3 wt % PAA solution was enough to remove the odour and decolour bloodmeal (Figure 21). With PAA treatment, molecular mass of the bloodmeal increased slightly (Figure 22) and XRD analysis showed that crystallinity decreased to 27-31% from 35%. Glass transition temperature was reduced 50⁰C from 225⁰C (**Error! Reference source not found.**), and solubility also increased in SDS and sodium sulphite and phosphate buffer solution (Figure 24).

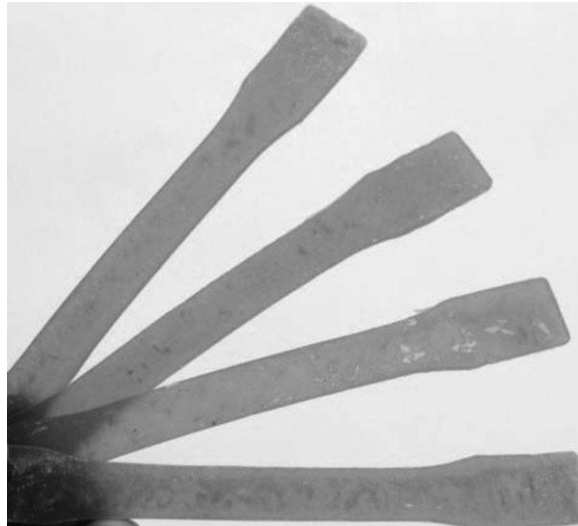


Figure 20 Sample of peracetic acid treated bloodmeal bioplastic (Low, Verbeek et al. 2014)

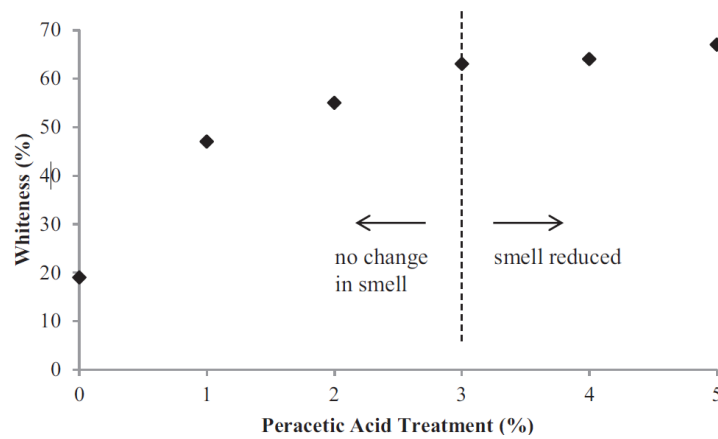


Figure 21 Percentage whitening of bloodmeal treated with 1-5% PAA (Low, Verbeek et al. 2014)

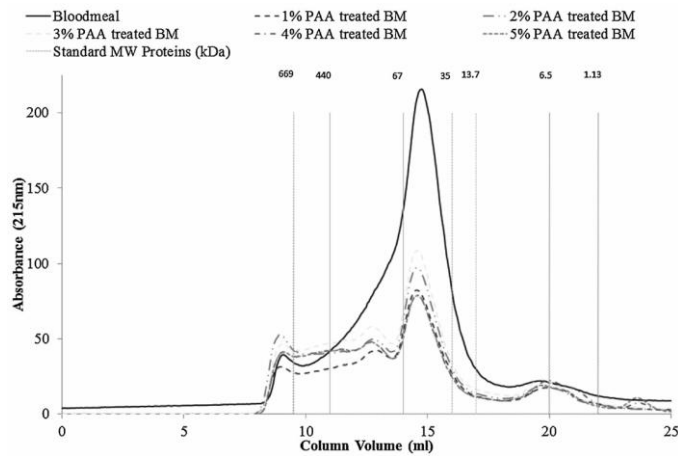


Figure 22 Elution profile of bloodmeal treatment with 1-5wt%PAA (Low, Verbeek et al. 2014)

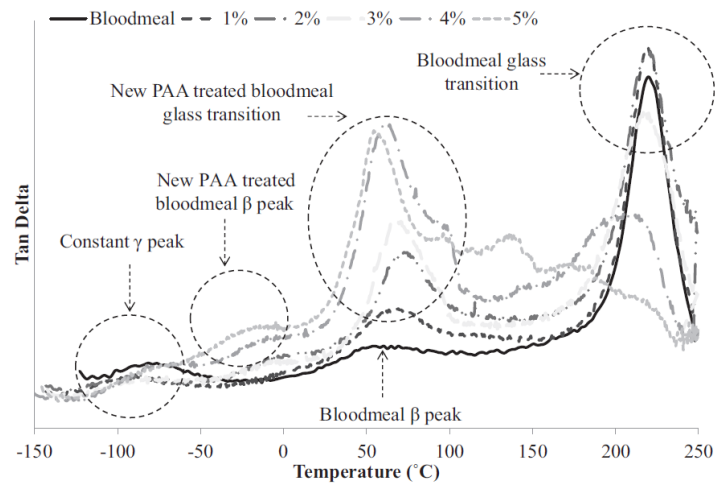


Figure 23 Comparison of peaks for $\tan \delta$ from DMA (Low, Verbeek et al. 2014)

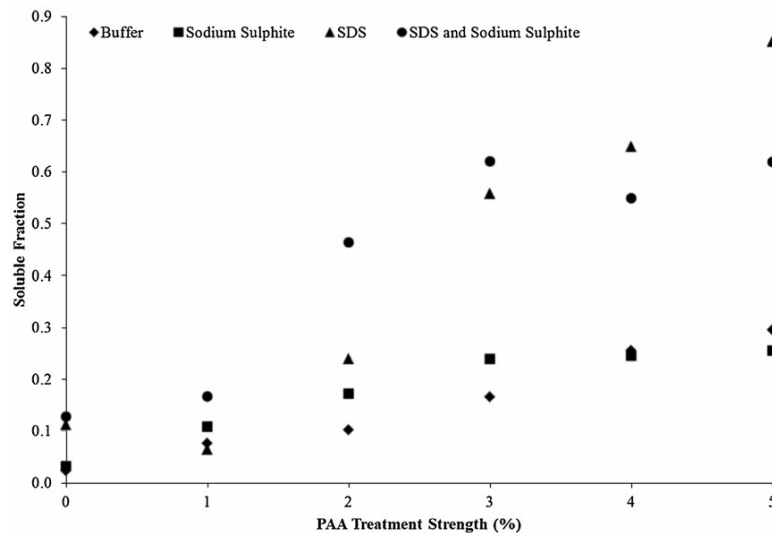


Figure 24 Solubility of treated bloodmeal in different solutes at 100°C (Low, Verbeek et al. 2014)

2.7.3.1 nano-composites using bentonite as a filler

In Verbeek and Klunker's (2013) paper, NTP was mixed with untreated and octadecylamine treated bentonite clay and extruded at different specific mechanical energy (SME). Octadecylamine treatment increased clay basal spacing from 13.5 to 31 Å (Figure 25), and gave an increase in tensile strength from 7.69 to 9.26 MPa with the addition of 2 pph clay, while Young's Modulus generally increased when clay was added (Table 12). Composites with 2 pph clay showed complete exfoliation while 7 pph showed two distinct XRD peaks between the position of unmodified clay and octadecylamine clay (Figure 26).

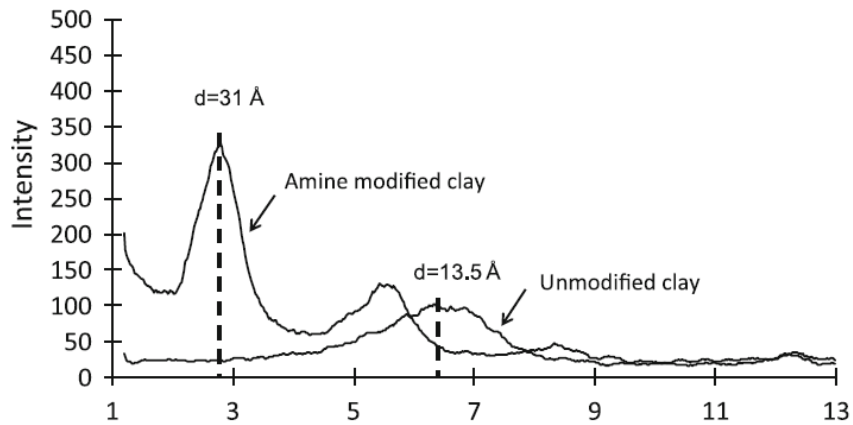


Figure 25 XRD pattern for modified and unmodified clay (Verbeek and Klunker 2013)

Table 12 Mechanical properties of modified and unmodified bentonite based composites at high and low SME_{ExR_T} (Verbeek and Klunker 2013)

Bentonite amount (pph _{BM})	SME × R _T	Young's Modulus (MPa)	Strength (MPa)	Elongation at yield (%)
Polymer only				
0	Low	458 (18.3)	8.19 (0.30)	5.55 (0.25)
0	High	418 (32.2)	7.69 (0.31)	6.85 (0.39)
OAmine modified bentonite				
2	Low	454 (50.5)	8.00 (0.20)	6.42 (1.20)
7	Low	808 (44.9)	7.23 (0.32)	1.29 (0.15)
2	High	556 (38.6)	9.26 (0.32)	5.55 (0.37)
7	High	670 (13.2)	6.41 (0.60)	1.36 (0.22)
Unmodified bentonite				
2	Low	532 (33.0)	8.82 (0.15)	5.45 (0.28)
7	Low	517 (74.4)	7.67 (0.95)	5.66 (1.64)
2	High	464 (29.5)	8.02 (0.23)	6.61 (0.42)
7	High	657 (52.4)	9.31 (0.24)	5.86 (1.12)

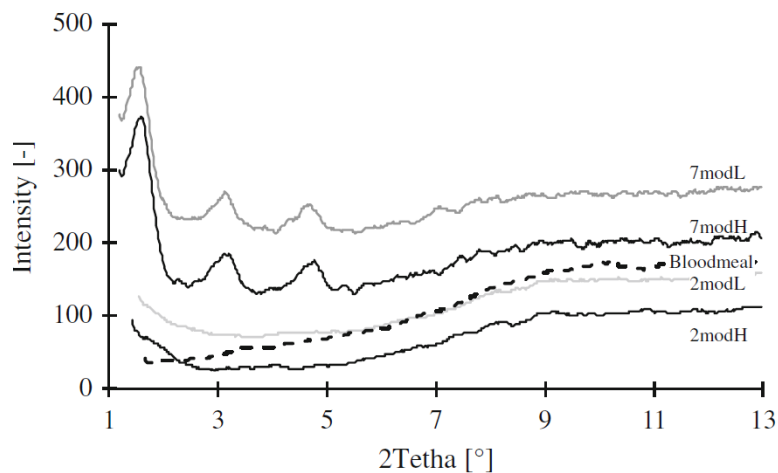


Figure 26 XRD patterns (basal spacing) of modified composites, (H-High SME_{ExR_T}, L-Low SME_{ExR_T}) (Verbeek and Klunker 2013)

NTP was also reinforced with sodium and calcium bentonite treated with stickwater, a meat rendering plant by-product (Shamsuddin 2013). Stickwater increased the compatibility of the clay with NTP, resulting in an improvement in tensile strength of the composite by 23% and modulus by 17%. while untreated clay addition reduced or had little effect on mechanical properties (Figure 27).

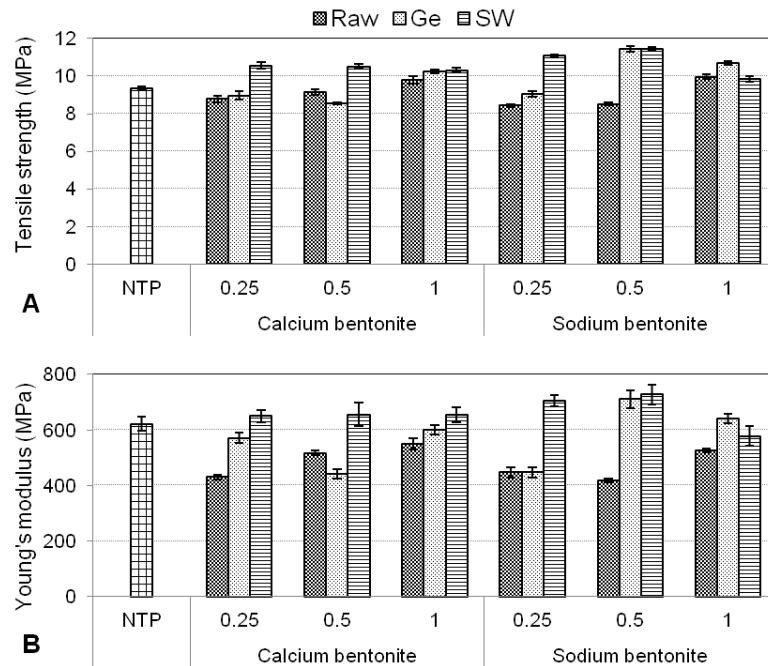


Figure 27 Graphs for A). Tensile strength B) Young's modulus with comparison of standard NTP and addition of different doses of clay (Shamsuddin 2013)

2.8 Conclusion

NTP was developed in 2008 and is in the process of being commercialized. It has been well researched with regard to mechanical and thermal properties, protein structural behavior, and incorporating bentonite clay that had been treated with gelatin, stickwater and octodecylamine. It has also been blended with LLDPE with and without compatibiliser. Other research is examining the production of NTP with decoloured bloodmeal to increase its commercial applications.

Currently, TEG is used in NTP as a plasticizer and makes up 13% by weight of the final product and about 10% of the total material cost. TEG is a petroleum product which increases the environmental impact of the material. One avenue which has not been explored is to use bloodmeal hydrolysate produced by enzymatic hydrolysis as an

alternative plasticizer in NTP. Another area which has also not been explored is surface modification of bentonite clay with bloodmeal hydrolysate to improve NTP mechanical properties. In terms of material applications, bloodmeal hydrolysate has only been used for producing foams used in fire retardants (Bressler 2010). Protein hydrolysates in general have not been used as plasticizers in plastics with the exception of soy protein isolate hydrolysate in soy protein plastics (Vlad, Jane et al. 2006) and ovalbumin hydrolysates as plasticizers in suspensions of alumina powder (Schilling, Tomasik et al. 2002).

The aim of this thesis is to produce bloodmeal hydrolysates and incorporate them into clay and NTP and explore the effect of using hydrolysates and hydrolysate modified clay on NTP mechanical properties.

Chapter 3 Methodology

3.1 Introduction

The research objectives of this research are:

- Assess the effectiveness of pepsin, alcalase and acid and optimal conditions in producing peptides from bloodmeal. This will be quantified by measuring reaction rates, degree of hydrolysis, hydrolysis yields and peptide size.
- Produce large quantities of hydrolysate of varying size distribution by sequentially treating bloodmeal with alcalase, trypsin and pepsin and recovering some of the hydrolysate at each step.
- Absorb some of the hydrolysate from each step to treat sodium bentonite clay for use as a filler in NTP.
- Assess the effect of hydrolysate as a substitute for TEG in NTP, and the effect of the hydrolysate treated bentonite as a filler in NTP. This would be determined by producing tensile specimens and impact bars containing different concentrations of each type of hydrolysate and hydrolysate treated bentonite, and measuring tensile strength, modulus, impact strength, toughness, strain at break, glass transition temperature, thermal stability, and crystallinity. Effect on plastic processability and morphology will also be examined.

Experimental methods were divided into five parts.

1. Enzymatic and acidic hydrolysis of bloodmeal to explore the effect of acid and enzyme type and concentration and bloodmeal concentration on hydrolysis rate and conversion.
2. Large scale production of bloodmeal hydrolysate.
3. Adsorption of bloodmeal hydrolysate onto bentonite clay.
4. Production of NTP plastics with hydrolysate as a replacement for TEG as a plasticizer.
5. Production of NTP plastics using hydrolysate treated clay as a filler.

NTP plastics were produced using extrusion and injection moulding, and plastic properties, such as tensile strength, modulus, toughness, glass transition temperature, thermostability, crystallinity and fracture surface morphology were examined using

tensile and impact testing, dynamic mechanical analysis, thermogravimetric analysis, x-ray diffraction, and scanning electron microscopy.

Equipment and materials used are shown in Table 13 and Table 14.

Table 13 List of instrument used

Equipment	Manufacturer and model
Glass beakers (250ml)	Duran
Centrifuges	Sigma Centrifuges Eppendorf Minispin Plus
Conditioning chamber	Lloyd Instruments
Dynamic mechanical analyser	Perkin-Elmer-DMA 8000
Oven	Contherm-Thermoter 2000
FE-SEM	Hitachi-S- 4700
Electronic balance	Sartorius -CP225D
Food processor, blender	Kenwood Multipro
Injection moulder	BOY-35A
Magnetic stirrer	Chiltern Scientific-MM31
pH meter	Eutech Intsruments- Cyberscan 100
Tensile tester	Instron-33R-4204
Notch maker	T-Verter
Impact tester	Rayran
Tri-blade granulator	Castin Machinery
Twin screw extruder	Thermo Prism-TSE-16-TC
UV spectrophotometer	Shimadzu UV-Pharma Spec
Water bath	Global Science
AKTA FPLC	Pharmacia Biotech
Filters	Sartorius Stedim Biotech
Syringes	BD
Water pump	
X ray diffraction	Panalytical Empyrean
3 ml graduated pasteur pipettes	Raylab
Micropipettes	Eppendorf Research

Table 14 List of materials used

Materials	Grade	Supplier
Bloodmeal (Bovine origin)	Industrial	Wallace Corporation Ltd
Alcalase	Analytical	Novozymes
Pepsin	Analytical	Sigma Life Sciences
Trypsin	Analytical	Sigma Life Sciences
Sodium bentonite	Industrial	Transform Minerals, New Zealand
Sodium phosphate (dibasic)	Analytical	Ajax Finechem
Sodium dihydrogen phosphate	Analytical	Ajax Finechem
Hydrochloric acid	Analytical	Merck
Sodium hydroxide	Analytical	Scharlau
Urea	Analytical	Ajax Finechem
Sodium dodecyl sulphate	Technical	Merck
Sodium sulphite	Technical	BDH Lab
Tri-ethylene glycol (TEG)	Technical	Orica Chemnet

3.2 Hydrolysis

3.2.1 Small scale hydrolysis

The aim of the small scale hydrolysis experiments was to determine which out of alcalase, pepsin and nitric acid gave the best hydrolysis of bloodmeal, and to explore the effect of enzyme/acid and bloodmeal concentration on hydrolysis kinetics.

Each set of small scale hydrolysis experiments were carried out in five 250 ml glass beakers, agitated at 120 rpm using a Boltac overhead stirrer unit (Figure 28). The beakers were covered using plastic petri dish lids. Each lid had a hole in the centre for the agitator shaft. The beakers were placed in a water bath to control temperature. The water bath consisted of a lined plastic tray filled with warm water circulated from a hot water bath using a peristaltic pump. The water overflowed from the tray through a drainage port back into the hot water bath. Temperature in the tray and beakers was monitored using a temperature probe.

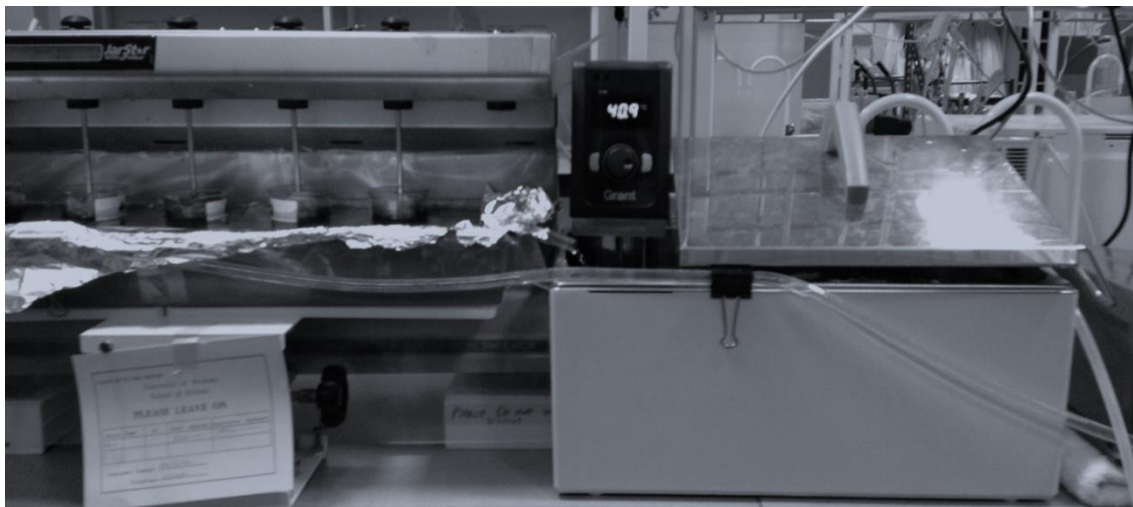


Figure 28 Apparatus setup for enzymatic and acidic hydrolysis

Bloodmeal masses used for each experiment were 5, 10, 15, and 20 g per 200 ml of distilled water. The solutions were allowed to reach the required temperature, before pH adjustment and enzyme or acid addition. Hydrolysis was carried out using pepsin, alcalase and nitric acid.

Pepsin hydrolysis for each bloodmeal concentration was carried out at pH 1.5 and 3, 37.5°C, and at 0 (pH 1.5 only), 60, 120, 180, 240 and 300 (pH 3 only) mg enzyme per 200 ml.

Alcalase hydrolysis was carried out at pH 8, 60°C, and at 0, 0.5, 1.0, 1.5, and 2.0 ml of enzyme per 200 ml.

Solution pH for enzyme hydrolysis was monitored and adjusted as needed by titrating with 1M NaOH for alcalase or 1M HCl for pepsin using an auto-pipette. Reaction rate was measured with time by noting the amount of NaOH or HCl added to keep the pH constant. pH was adjusted every 15 to 30 minutes for the first two hours, and once each hour thereafter. The total reaction time for each run for alcalase was 12 hours and 36 hours for pepsin. Alcalase hydrolysis was halted by cooling the solution to room temperature and pepsin hydrolysis halted by raising the pH to 8 by adding 1M NaOH.

For acid hydrolysis, nitric acid was added to make the solution up to 0, 0.1, 0.5, 1 and 2M nitric acid, and it was carried out at 37°C. Reaction rate was measured by collecting and centrifuging three 1 ml samples of solution every hour, diluting the samples by adding 100 µl of sample to 900 µl of distilled water, placing the sample in a quartz cuvette and measuring UV/Vis absorbance at 280 nm, 540 nm and 580 nm. The UV/Vis absorbances were converted to concentration by comparing the absorbances against a calibration curve constructed from solutions of known hydrolysate concentration (Figure 64). Total reaction for each run was seven hours. Acid hydrolysis was halted by raising the solution pH to 7 by adding 1M NaOH.

After each hydrolysis experiment, the solution was centrifuged (Section 3.3) to separate the unhydrolysed bloodmeal, weight of supernatant and pellet noted, and samples of each dried to determine solids content for hydrolysis yield calculations (3.4). Samples of the supernatant were also taken for protein molecular weight determination (3.5).

3.2.2 Large scale hydrolysis

The aim of the large scale hydrolysis experiments was to produce three sets of hydrolysate of sequentially smaller peptides by reacting bloodmeal with alcalase, then trypsin, and then pepsin. These hydrolysates after each run were later divided into two fractions, the first being adsorbed onto sodium bentonite, and the bentonite added as a filler in NTP, and the second concentrated and incorporated into NTP as a substitute for TEG.

For large scale hydrolysis, two 20 L buckets were each filled with 15 litres of distilled water, 1125 g of bloodmeal, the pH adjusted to 8, and the solution heated to 60°C using copper coils connected to a hot water bath and a peristaltic pump. The solutions were

agitated using overhead stirrers at 160 rpm. 125 ml of alcalase was added to each bucket, the reaction allowed to run for 8 hours, and the pH maintained at 8 by adding 1M NaOH. The buckets were covered with aluminium foil to reduce evaporation. 10 litres of solution was removed for centrifuging (Section 3.3). 10.8 g of trypsin was added to the remaining 20 L of solution, pH maintained at 8 and the reaction allowed to proceed for another 8 hours. 10 litres again was removed for centrifuging. With the final 10 litres, pH was adjusted to 1.5, temperature lowered to 37.5°C, 150 g pepsin was added and the reaction allowed to proceed for another 8 hours.

Each batch of hydrolysate after being centrifuged was divided into two fractions, one for concentrating (Section Preparation of clay and hydrolysate based NTP3.7) and incorporation into NTP (Section 3.7), and the other for adsorption onto bentonite clay (Section 3.6.3).

3.3 Centrifugation

The bloodmeal hydrolysate from each hydrolysis experiment was centrifuged in 400 ml centrifuge bottles at 4000 rpm for 20 minutes in a Sigma Laboratories Centrifuge. The weight of the bottles, hydrolysate added, pellet and supernatant were noted. Where needed, distilled water was added to the bottles prior to centrifugation so the weight of each bottle was the same. In each case, the mass of water added was noted. Samples of supernatant and pellet from each bottle were taken for dry weight and moisture content determination (Section 3.4). The supernatant was also analysed for molecular weight distribution (Section 3.5).



Figure 29 Samples in centrifugation bottles with labels

3.4 Dry weight calculations

Samples of pellet and hydrolysate were added to pre-weighed 50 ml plastic sample containers, and total weight noted. These were placed in a Contherm 2000 series oven for three days at 65°C, after which the dry mass was checked. All weights were measured using a Sartorius fine balance series P225D. Moisture content was calculated using the formula given below.

$$\text{moisture content} = \frac{\text{mass}(\text{wet}) - \text{mass}(\text{dry})}{\text{mass}(\text{wet})}$$

3.5 Molecular weight distribution

Molecular weight distribution of proteins in each hydrolysate sample 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 absorbance to obtain a weighted molecular weight that was summed, which was then divided by the sum of the UV absorbance.

3.6 Rate Calculation

Rate of reaction was calculated using underlined formula

$$\ln(C_s - C_t) / C_s * (-1) = kt$$

Where C_s and C_t are concentration at final time and time taken respectively, k is a constant and t is time, k provides the slope of the straight line giving the rate of reaction.

3.6.1 Preparation of phosphate buffer

Phosphate buffer stock solution (PBS) was prepared at 0.1 M concentration by adding 3.2 g of NaH_2PO_4 and 10.9 g of Na_2HPO_4 to one litre of distilled water. Solution pH

was adjusted to 7 using 1M HCl or 1M NaOH solution. The stock solution was diluted to 0.02M and pH adjusted as required

3.6.2 Hydrolysate concentration

The supernatants from the large scale hydrolysis experiments were placed into 5 L beakers and heated to 65°C on a hot plate while being mixed using a magnetic stirrer, and concentrated until about 50% solids (about a 10 fold reduction in volume assuming a starting concentration of 5% solids). The final solids content was then calculated using the method in Section 3.4. The concentrates were then refrigerated or frozen until needed for incorporation into NTP (Section 3.7).

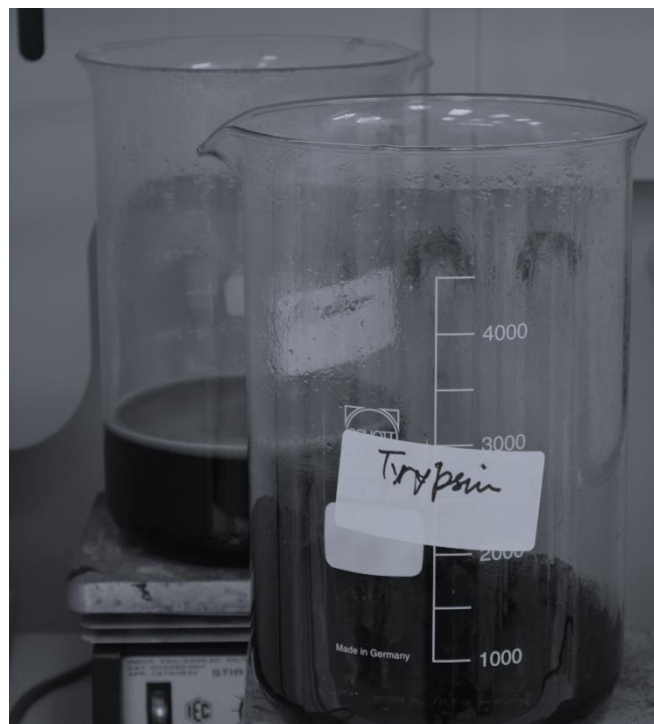


Figure 30 Depicting reduction to 50 percent solids

3.6.3 Intercalation of hydrolysate with sodium bentonite (NaBt)

The remaining fractions of hydrolysate from the large scale hydrolysis experiments were mixed for 24 hours with sodium bentonite at 3 g bentonite per 100 ml of hydrolysate at pH 7. Solutions were stirred using a magnetic stirrer. The mixture was centrifuged at 4000 rpm for 15 minutes, masses of pellets and supernatant noted, samples retained for dry weight calculations. The pellet was stored in the freezer before use as a filler in NTP.

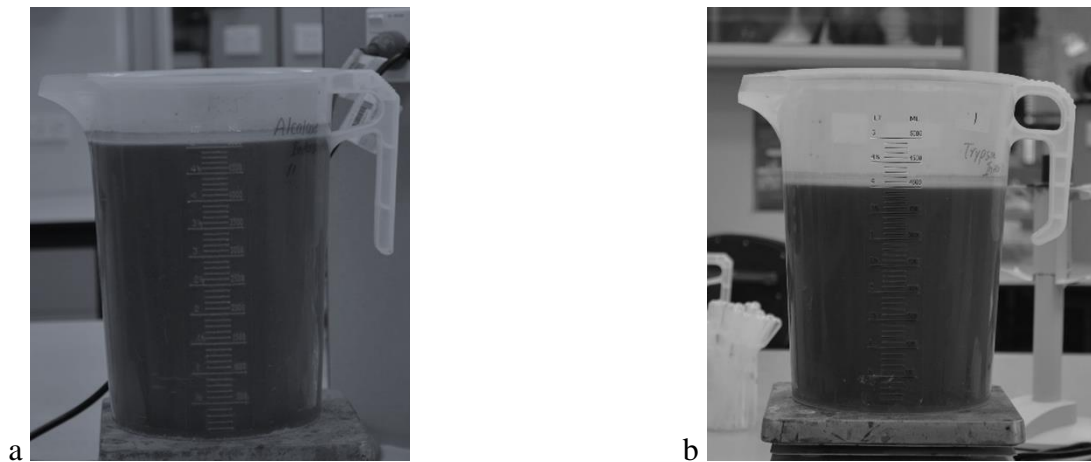


Figure 31 Depicting intercalation a) Alcalase intercalation b) Trypsin intercalation

3.7 Preparation of clay and hydrolysate based NTP

3.7.1 Formulation of NTP composites

Urea, sodium sulphite and sodium dodecyl sulphate were added to water in a beaker according to the recipe in Table 4, and heated to 50-60°C on a hot plate for 15 minutes while being mixed. Bloodmeal and clay was mixed together in a blender, the dissolved mixture added, mixed for 5 minutes, TEG added and the mixture mixed again for 5 minutes using a combination of the blender and manual mixing using a spatula. The actual mass of clay and water used was adjusted to account for the water content in the clay. Four types of clay were used: untreated, alcalase, typsin and pepsin hydrolysate modified clay, at three concentrations ranging from 1 to 3 g of clay per mixture. The resulting mixtures were double bagged in zip-lock bags and refrigerated until used for extrusion.

Table 15 Standard recipe for NTP composites with untreated and treated clay as a filler

Ingredient	Amount	
	Mass (g)	pph(BM)
Bloodmeal	300	100
Urea	30	10
Sodium sulphite (SS)	9	3
Sodium dodecyl sulphate (SDS)	9	3
Tri ethylene glycol (TEG)	60	20
Water	120	40
Clay addition (dry weight)	1	0.33
	2	0.67
	3	1.00

3.7.2 Formulation of NTP with hydrolysate as a plasticiser

NTP was prepared using the same method as in Section 3.7.1 according to the recipe in Table 5, but using alcalase, trypsin and pepsin hydrolysate at four different concentrations (20, 40, 60 and 80 g per batch) in the NTP as a substitute for TEG. The actual mass of water and hydrolysate added was adjusted to account for the water content in the hydrolysate. The resulting mixtures were double bagged in zip-lock bags and refrigerated until used for extrusion.

Table 16 Standard recipe for NTP and variability of ingredients with different specimens

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



Figure 32 Material in blender after being blended

3.7.3 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 33). Extruder screw speed was 150 rpm and feed rate from the hopper to the extruder was 60 Hz. The torque depended on the amount of material present in extruder tubing, but normally ranged from 50 percent to 80 percent of maximum (12 Nm per screw). Bridging in the hopper occurred for some of the samples and was remedied by using a push rod to break the bridge.

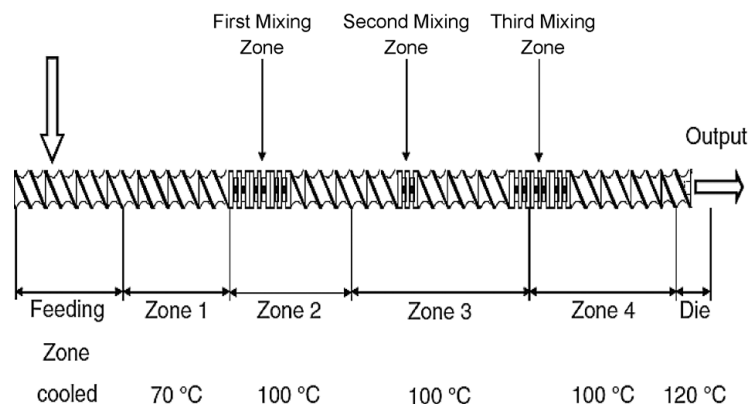


Figure 33 Extruder screw configuration

Extruder torque and pressure, hopper feed rate and mass flow of extrudate were noted each minute. Mass flow was measured by breaking the extrudate at the die and weighing the length extruded on an electronic balance. Extrudate quality and processability were also noted. Samples of extrudate from each formulation were oven dried for three days at 70°C to determine moisture content

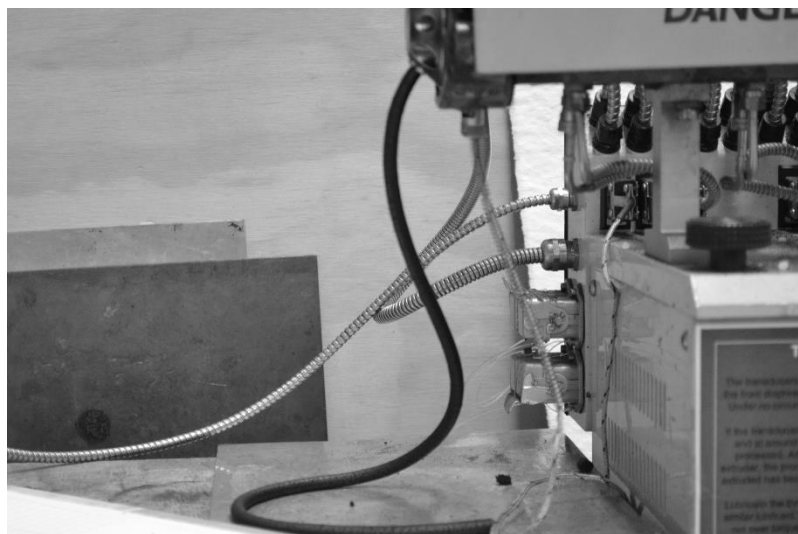


Figure 34 Material coming out of extruder

3.7.4 Granulation of extruded blends

Extruded material from each formulation was then passed through a tri blade granulator (Castin Machinery) and a 4 mm mesh screen to produce pellets which were double bagged in zip lock bags before being put in the freezer for injection moulding the next day.

3.7.5 Injection moulding of granulated blends

Each pelletised formulation was injection moulded into ASTM D638-03 standard dog bone specimens and impact bars using a BOY-35A injection moulder. The temperatures for the zones from the feed to the nozzle were 100, 115, 120, 120, and 120°C. The mould temperature was set at 60°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. The injection moulder was operated in a semi-automatic mode and material was removed from the mould manually as required. Injection moulded samples were labelled and the length, width, thickness and weight of each specimen were taken before and after conditioning for seven days at 50% relative humidity and 23°C. Shrinkage after conditioning was calculated by dividing the conditioned length, width and thickness by the original dimension value for the sample.



Figure 35 BOY 35A Injection Moulder



Figure 36 Specimen for injection moulded material

3.7.6 Composites Tensile and impact testing

3.7.6.1 Tensile testing

After conditioning, tensile specimens were analysed according to the ASTM D638-86 method for tensile strength, Young's 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.

3.7.6.2 Impact testing

After conditioning, 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 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.

3.7.7 X ray diffraction

X ray diffraction (XRD) was used to find out basal spacing (d) of clay samples treated with hydrolysed bloodmeal and percentage crystallinity of tensile specimens. A Panalytical Empyrean XRD was used for powdered samples with the parameters shown in Table 17 Parameters for XRD. Powdered clay samples were mounted on rotating sample holders while tensile specimens were mounted on a fixed sample holder. Scan time ranged between 15 to 20 minutes.

Bragg's law was used to find the basal spacing:

$$n\lambda = 2d\sin\theta$$

Where, where n is an integer, λ is wavelength of incident wave, d is the spacing between the planes in the atomic lattice, and θ is the angle between the incident ray and the scattering planes

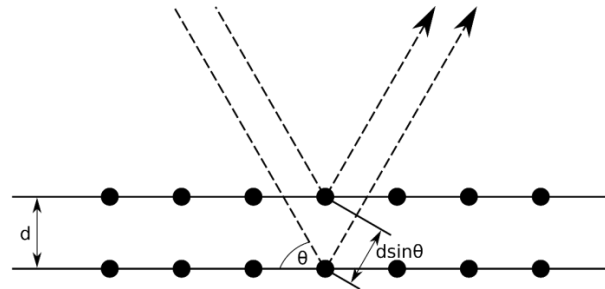


Figure 37 Calculation of d (basal spacing)

Table 17 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
Movement	Rotating at 1 rps for powders, 0 for solid samples
Filter	Large beta filter-Nickel
Scan mode	Continuous
Scan range	4-60

XRD graphs obtained (Figure 12) were baseline corrected between angles of 5 and 35 2-theta, 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 (Figure 39). Duplicates were done for each specimen.

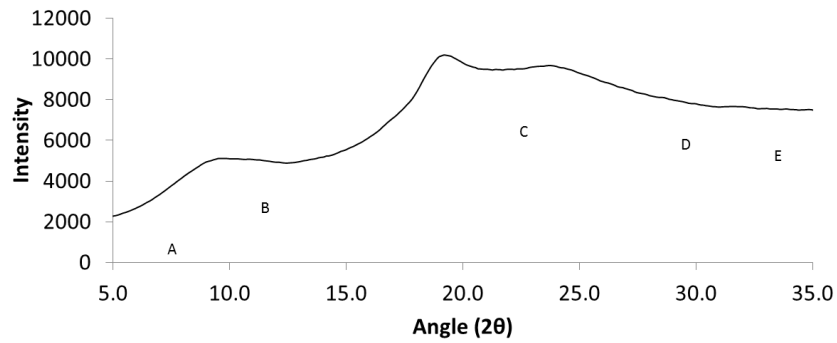


Figure 38 A typical XRD graph for NTP.

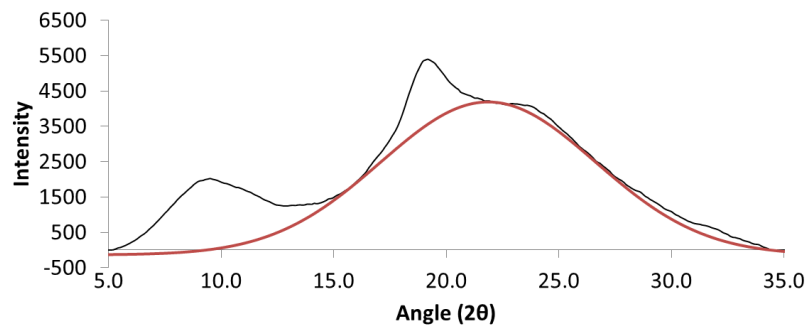


Figure 39 XRD graph that has been baseline corrected and a Gaussian curve fitted under the halo.

3.8 Surface morphology using Scanning electron microscopy

SEM (Scanning electron microscope) was used to examine surface morphology of fracture surfaces for both impact bars and tensile test specimens. Specimens were cut with a hacksaw blade and placed on double sided adhesive carbon tape on aluminium stubs. The samples were then sputter coated with platinum under vacuum and the stub sides coated with carbon to increase electron conductivity. SEM images were taken on the 50 μm , 100 μm and 500 μm scale with the SEM operating at 3kV (Figure 40). Extra images were taken if any unusual or notable surface features were found.

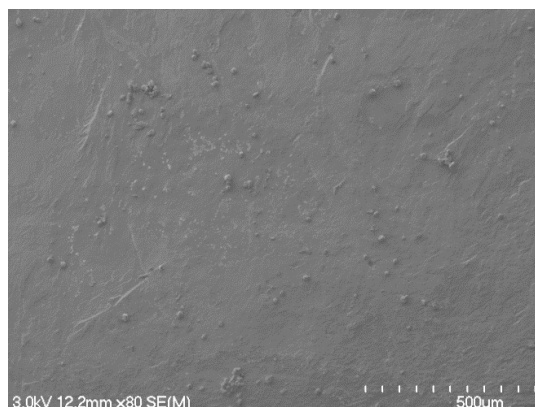


Figure 40 An example of SEM image at 500 μm

3.9 Thermogravimetric analysis (TGA)

TGA was carried out on samples by placing 5 mg-20 mg sample of specimen in an aluminium crucible, placing the sample in a DTA-TGA analyser along with an empty reference crucible. Temperature was raised at 10°C per minute from 20°C to 800°C with air flow of 150 ml per minute for complete combustion of samples.

3.10 Dynamic mechanical analysis

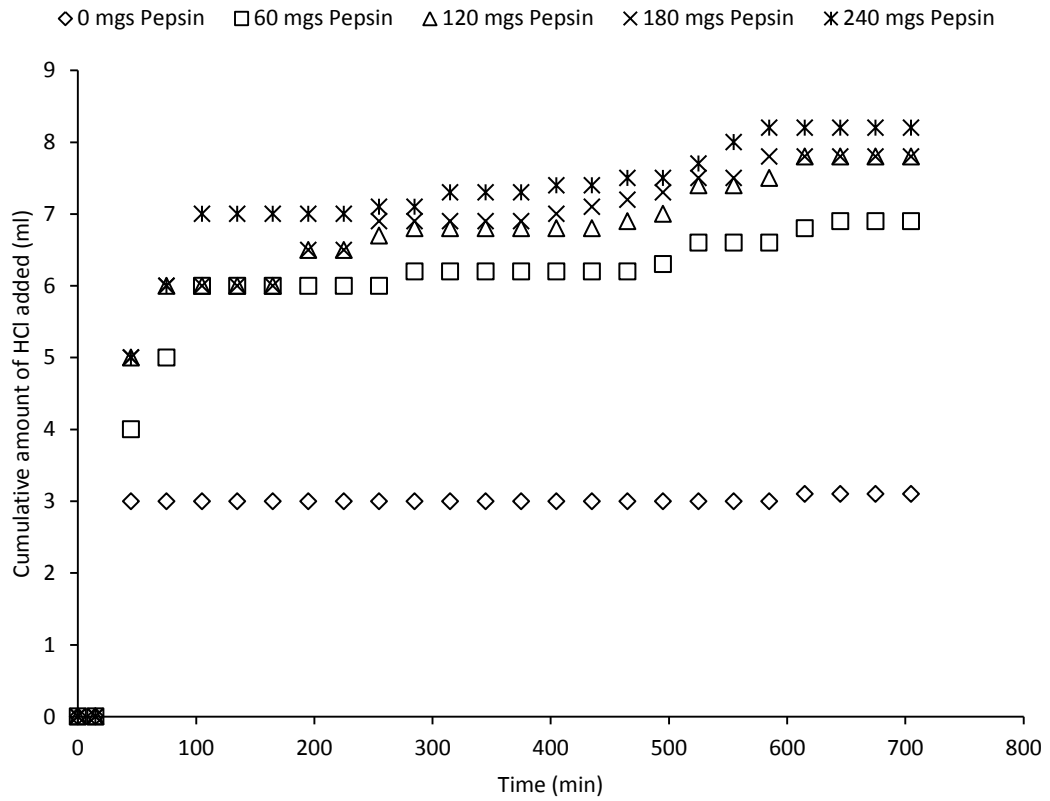
A Perkin Elmer Dynamic Mechanical Analyser was used to obtain glass transition temperatures of injection moulded samples. Impact bars of approximately 12.8 x 6.0 x 3.4 mm were mounted in the DMA in a single cantilever bending system, enclosed by ceramic heater heated incrementally to 150°C from room temperature at a rate of 2°C/minute. Data was collected for 1Hz, 10Hz and 30Hz for dynamic displacement of 0.03mm. The storage modulus (E), loss modulus (E'') and loss factor ($\tan \delta$) were recorded by machine program interface. A peak in $\tan \delta$ represented a transition temperature.

Chapter 4 Result and Discussion

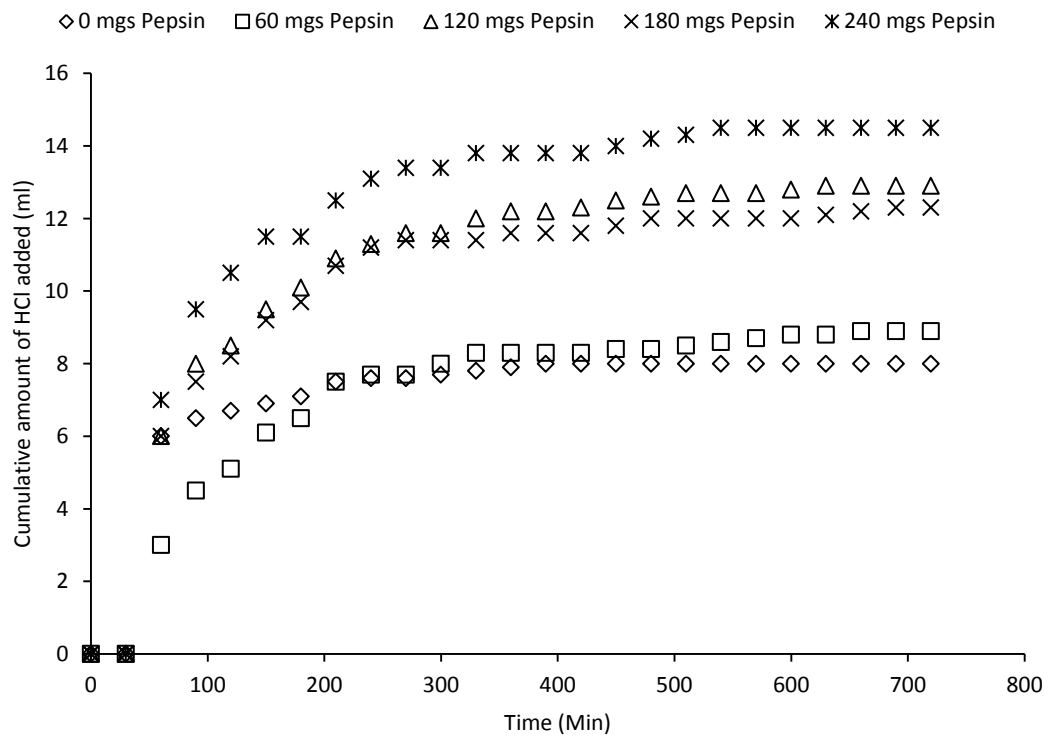
4.1 Hydrolysis experiments

4.1.1 Pepsin

For each pepsin hydrolysis experiment, the initial rate of reaction was fast for pH 1.5 and plateaued after 100-300 minutes (Figure 41 and Figure 42). The amount of acid required for maintaining pH increased with both increasing enzyme concentration and bloodmeal concentration, with the exception of 20 g, where the amount of acid required for constant pH was slightly less than that for 15 g of bloodmeal. Pepsin hydrolysis at pH 3 was much slower than pH 1.5, showed an initial fast hydrolysis which slowed after 100 minutes to a constant rate, and continued to proceed after 42 hours (Figure 43 and Figure 44), whereas the reaction for pepsin at pH 1.5 had completed within 10 hours.

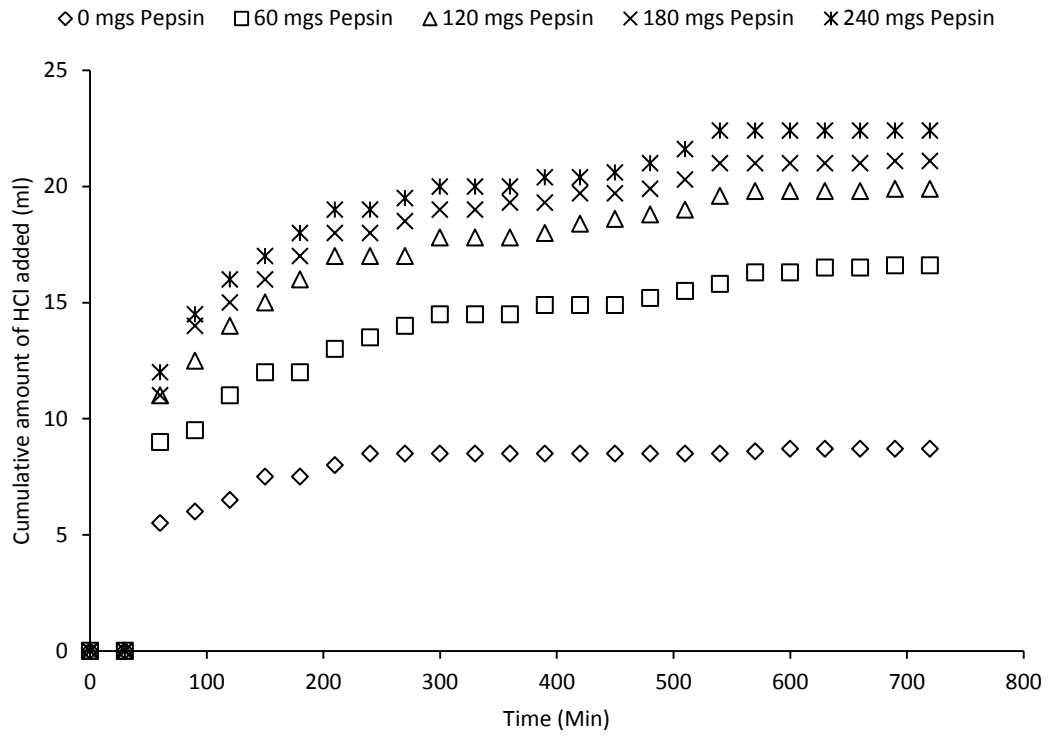


a

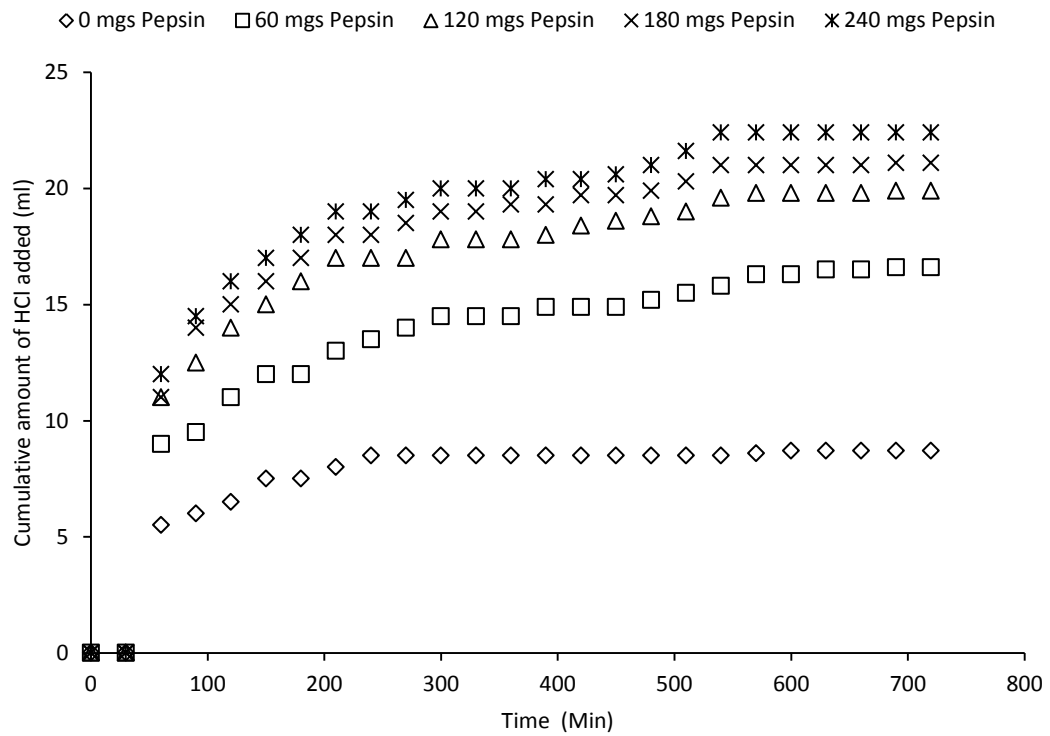


b

Figure 41 Bloodmeal hydrolysis with for a) 5 g bloodmeal and b) 10 g bloodmeal, at pH 1.5 and 37°C for different amounts of pepsin in 200 ml.

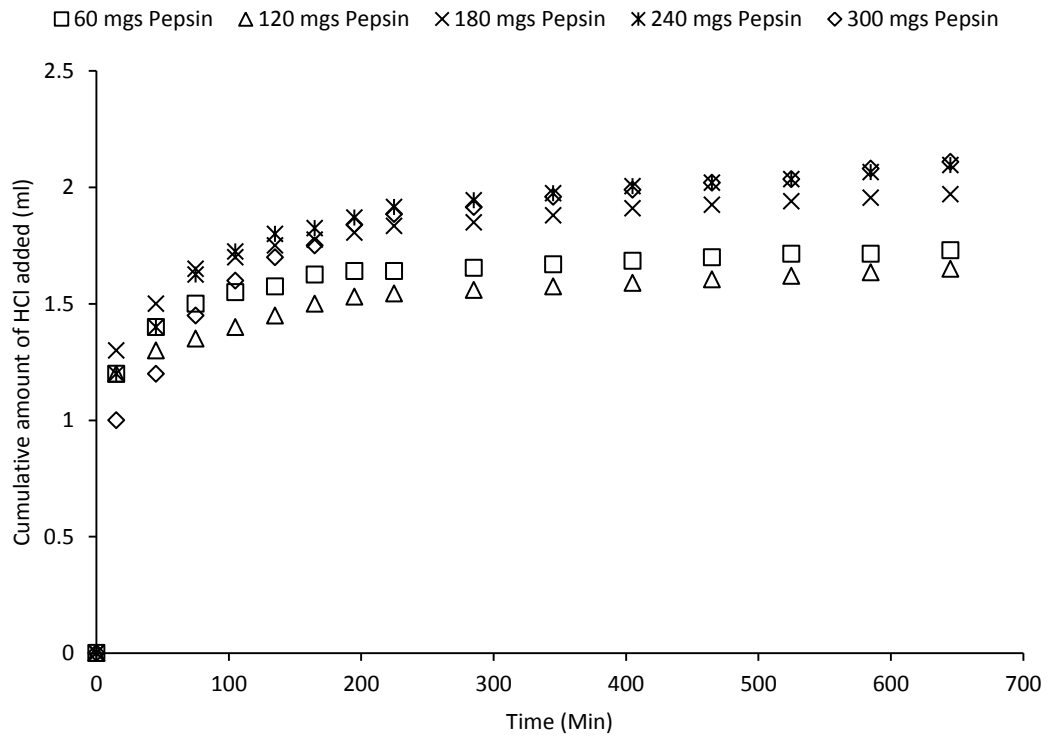


a

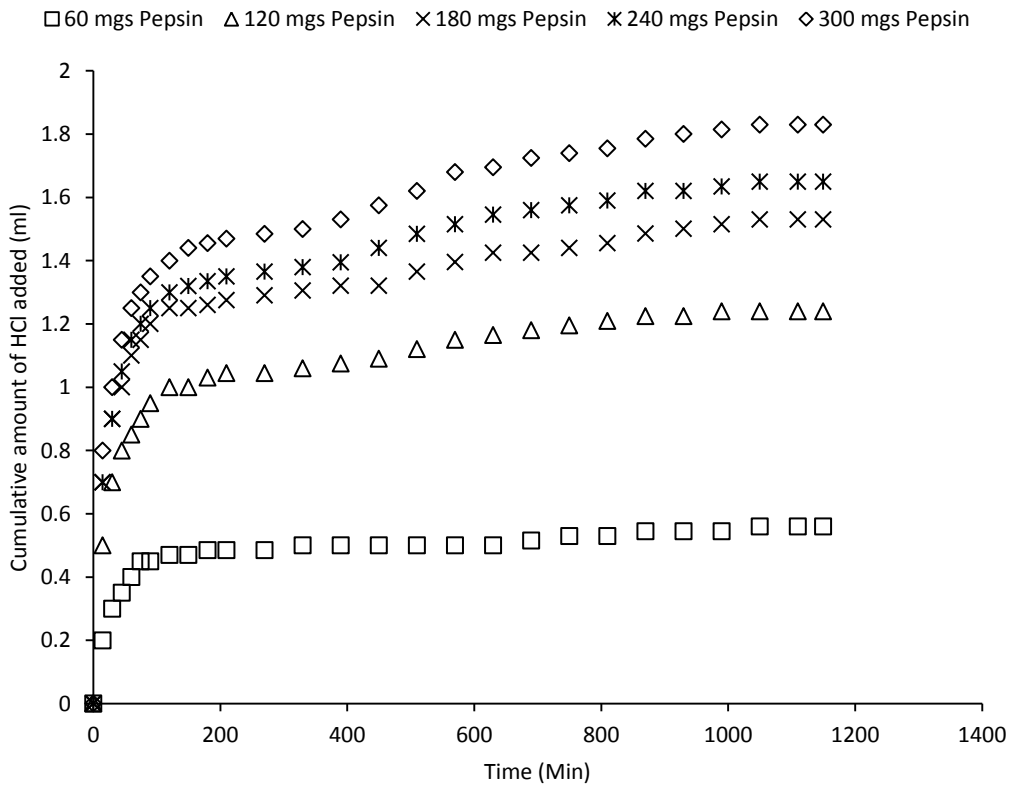


b

Figure 42 Bloodmeal hydrolysis with for a) 15 g bloodmeal and b) 20 g bloodmeal, at pH 1.5 and 37°C for different amounts of pepsin in 200 ml.

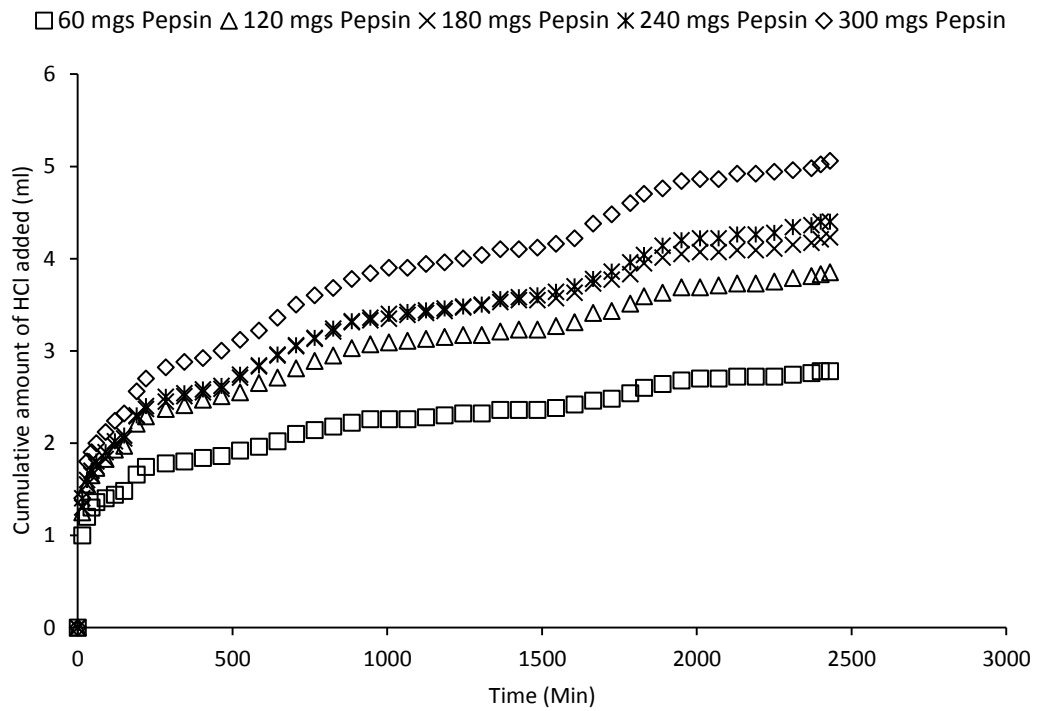


a

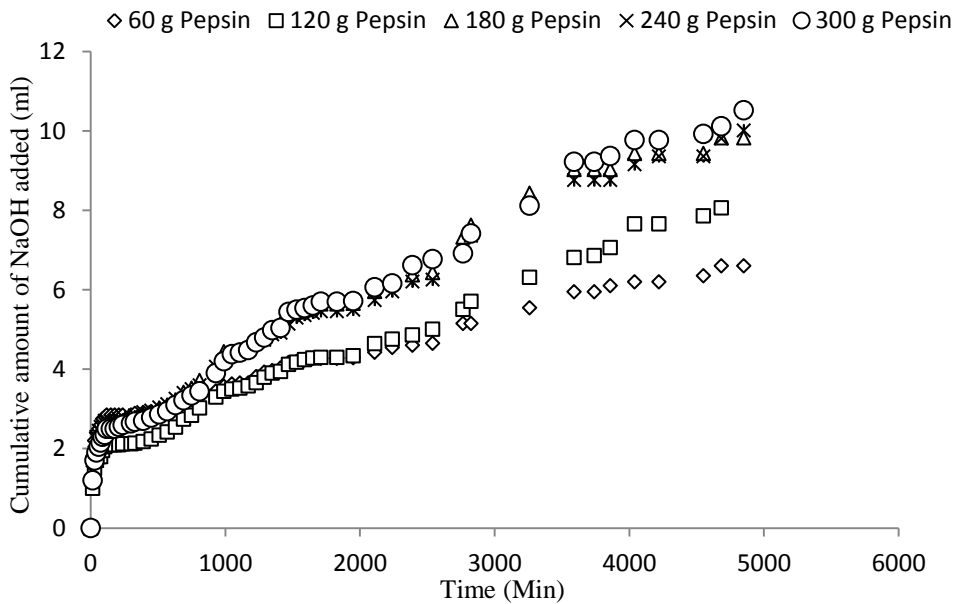


b

Figure 43 Bloodmeal hydrolysis with for a) 5 g bloodmeal and b) 10 g bloodmeal, at pH 3 and 37°C for different amounts of pepsin in 200 ml.



a



b.

Figure 44 Bloodmeal hydrolysis with for a) 15 g bloodmeal and b) 20 g bloodmeal, at pH 3 and 37°C for different amounts of pepsin in 200 ml.

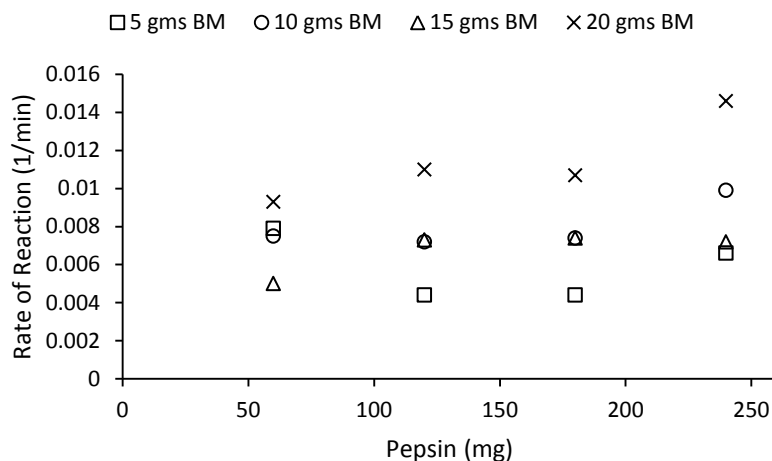


Figure 45 Pepsin rate of reaction at pH 1.5 vs pepsin concentration at different bloodmeal concentrations.

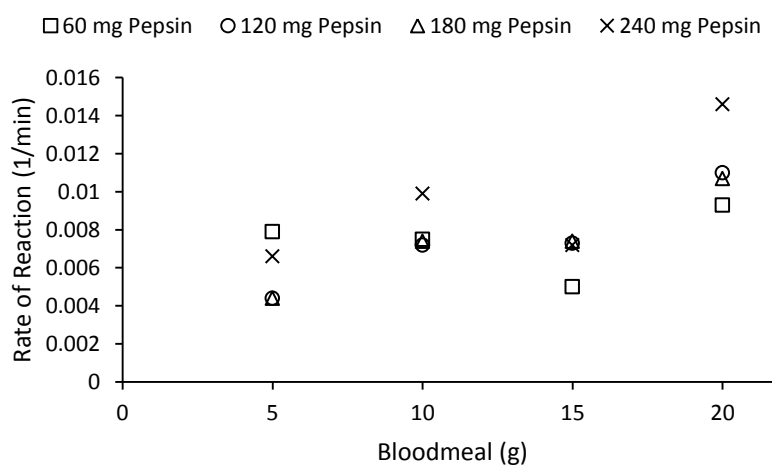


Figure 46 Pepsin rate of reaction at pH 1.5 vs bloodmeal concentration at different pepsin concentrations.

For pH 1.5, pepsin reaction rate increased with bloodmeal concentration except for the solutions with 60 mg of pepsin where it appeared to be constant (Figure 45 and Figure 46). For the lower concentrations of bloodmeal, reaction rate appeared to be independent of pepsin concentration. This suggests that bloodmeal concentration was limiting reaction rate, i.e. there were more pepsin molecules than places to react on bloodmeal, with the exception of the lower concentration of pepsin.

For pH 3, pepsin reaction rates were up to 4-5 times lower than rates at pH 1.5 for the higher bloodmeal concentrations and appeared to be independent of bloodmeal concentration and pepsin concentration (Figure 47 and Figure 48).

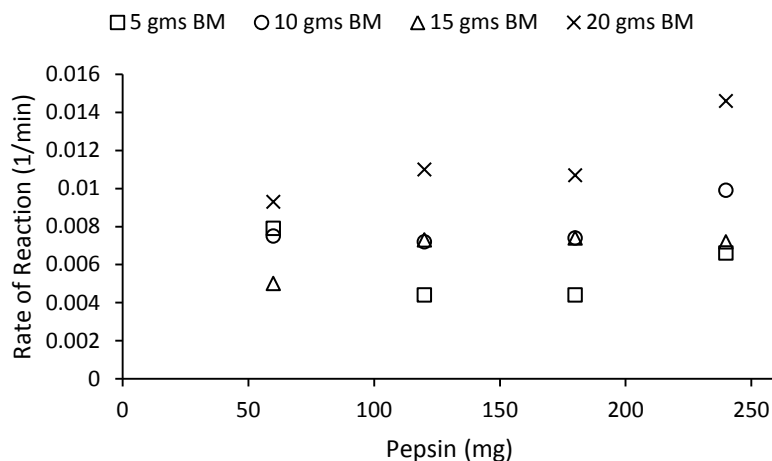


Figure 47 Pepsin rate of reaction at pH 3 vs bloodmeal concentration at different pepsin concentrations.

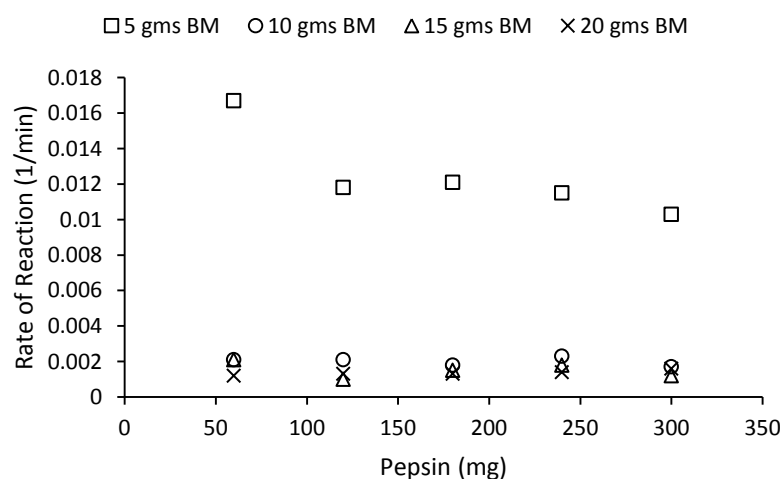


Figure 48 Pepsin rate of reaction at pH 3 vs pepsin concentration at different bloodmeal concentrations.

For both pH 1.5 and pH 3, degree of hydrolysis increased with increasing pepsin concentration, but decreased with increasing bloodmeal concentration for pH 1.5 from 20-25% of peptide bonds broken to 10-15% (Figure 49). For pH 3, it increased with increasing bloodmeal concentration from 1.5-5.5% of bonds broken to 5-7.5% (Figure 50). This can be explained by the length of time the reactions for pH 3 were allowed to run for, which increased from 700 minutes to 5000 minutes (when it was observed that the hydrolysis reaction was very slow) (Figure 43 and Figure 44). For pH 1.5, all reactions ran for the same period of time (~700 minutes) (Figure 41 and Figure 42). For a fair comparison to pepsin at pH 1.5, the degree of hydrolysis for pH 3 should be calculated at 700 minutes, in which case the degree of hydrolysis would be much less.

For example, for 20 g bloodmeal at pH 3 at 700 minutes would be between 2-2.1% compared to 5-7.5% at 5000 minutes.

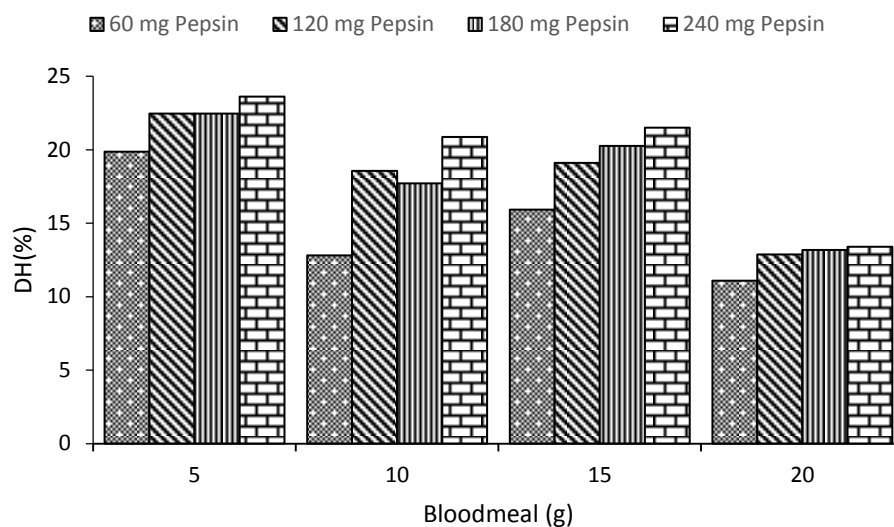


Figure 49 Degree of hydrolysis (%) for different concentrations of pepsin at pH 1.5 for different amounts of bloodmeal.

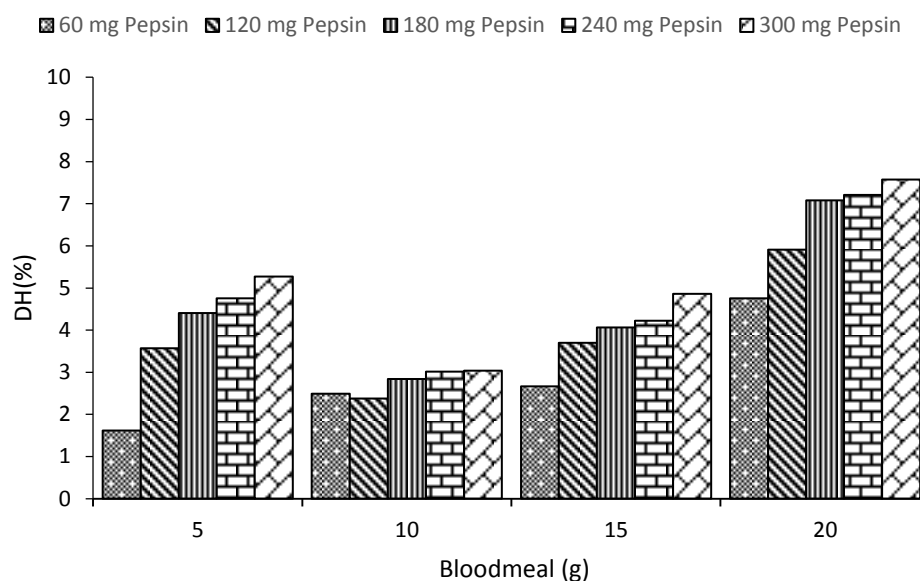


Figure 50 Degree of hydrolysis (%) for different concentrations of pepsin at pH 1.5 for different amounts of bloodmeal.

For pepsin at pH 1.5, mass of bloodmeal solubilized decreased from 75-80% to 60-65% with increasing bloodmeal concentration, but remained relatively constant with respect to the amount of pepsin added (Figure 51). For pH 3, the mass solubilized showed some unusual behaviour with mass solubilized decreasing with increasing pepsin concentration for 10 and 15 g bloodmeal, which is difficult to explain (and the

experiments should be repeated), while for 5 and 20 g it increased, which was expected, but what was unexpected was that the amount solubilized increased with the amount of bloodmeal used (Figure 52).

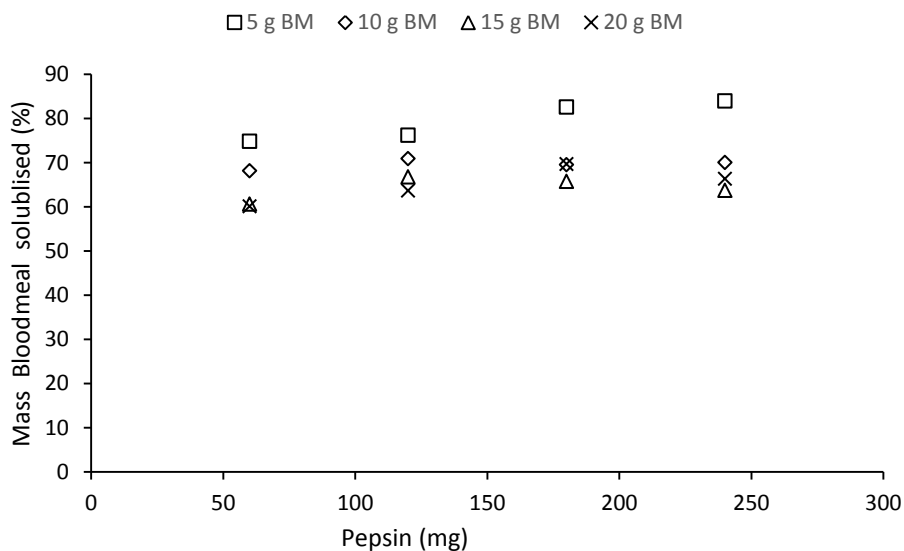


Figure 51 Percentage mass of bloodmeal solubilized for different concentrations of pepsin at pH 1.5 for different amounts of bloodmeal.

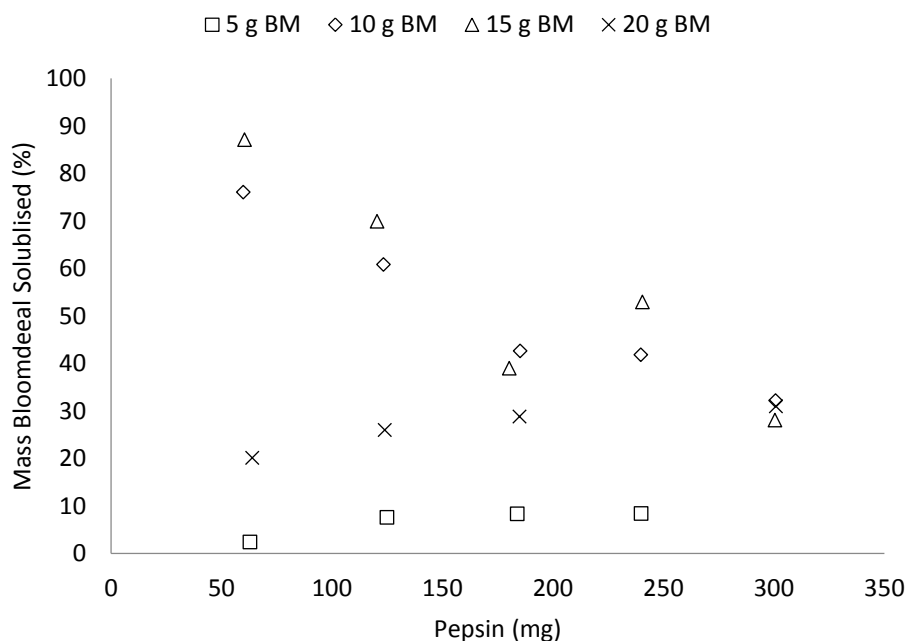


Figure 52 Percentage mass of bloodmeal solubilized for different concentrations of pepsin at pH 3 for different amounts of bloodmeal.

A decrease in mass solubilized and degree of hydrolysis with increasing bloodmeal can be expected as there are more peptide bonds to cleave with the higher bloodmeal concentrations. In addition, there might be hydrolysis products that inhibit further enzyme hydrolysis such as formation of insoluble aggregates, e.g. the haem group which is no longer protected by the hydrophobic pocket in which it normally sits, will aggregate and may take peptides bound to the haem group with it. A similar trend was also observed by Ruan et al (2010) who used pepsin to hydrolyse egg white, where product inhibition was found at higher egg white concentrations. Pepsin preferentially cleaves tyrosine, phenylalanine, leucine, alanine, cysteine and glutamic acid (See section 2.6.2.1), which make up about 45% of the amino acids in bloodmeal (Kramer, Waibel et al. 1978). Under the best conditions (low bloodmeal and high pepsin) and pH 1.5, the degree of hydrolysis was 20%, so potentially, if product inhibition is not present, the degree of hydrolysis could go up to 45%.

Samples of hydrolysate from pepsin experiments at pH 1.5 were examined for average molecular weight using gel filtration chromatography. Peptides ranged between ~20 kDa to 60-70 kDa (Haemoglobin is a 64 kDa tetramer) (Figure 53). No definite trends could be observed with regard to peptide size with pepsin concentration with the exception that it appeared that peptide size decreased slightly. Some issues were encountered using the AKTA FPLC with the baseline shifting on the inline UV spectrophotometer, as well as air bubbles becoming entrained in the detector for some samples which impacted on the quality of the results.

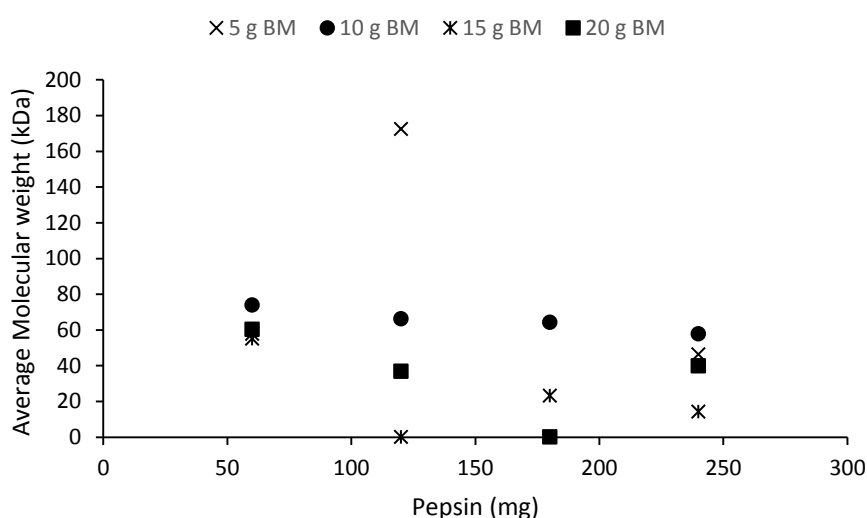
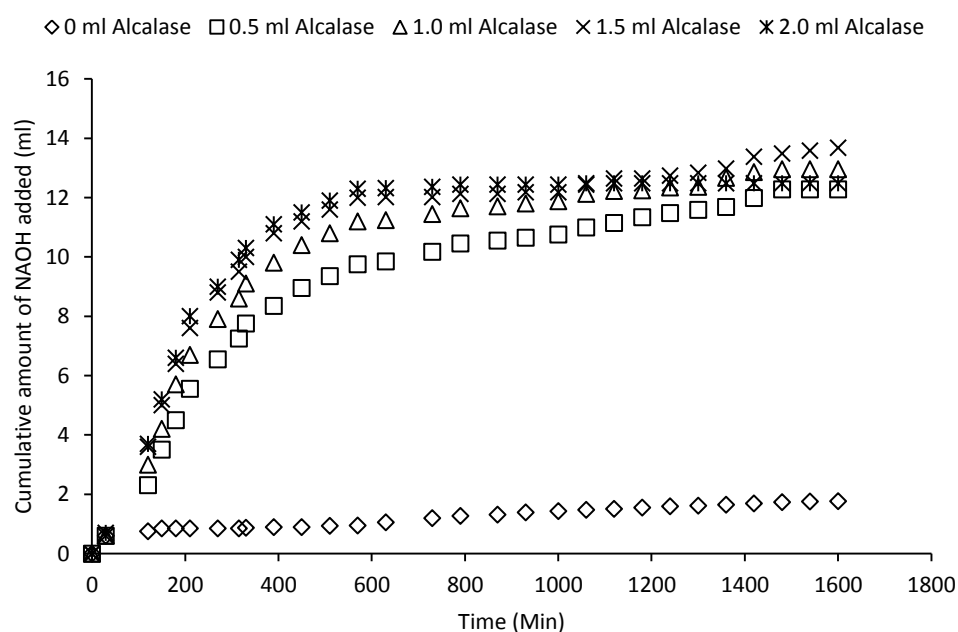


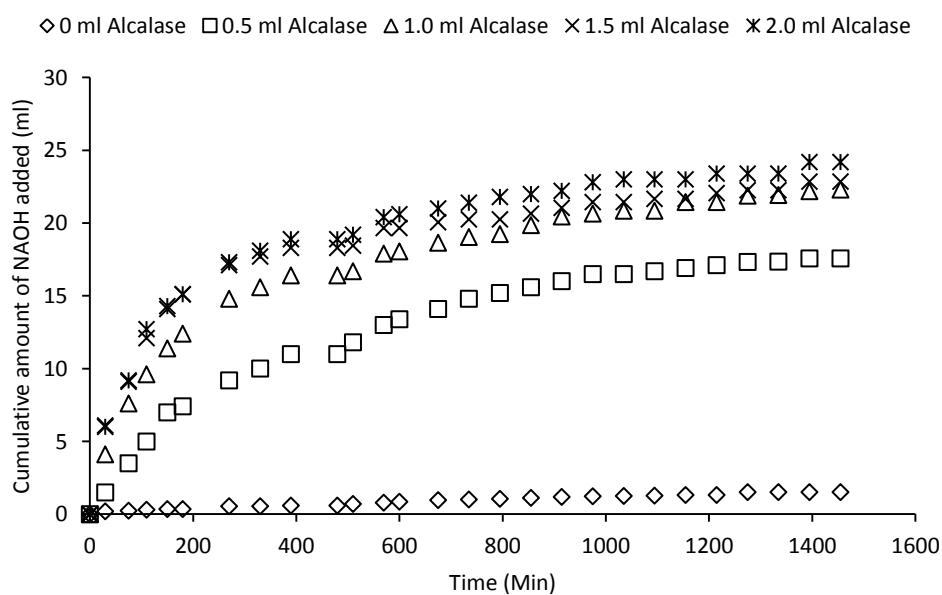
Figure 53 Average molecular weight of pepsin hydrolysate vs pepsin concentration.

4.1.2 Alcalase

Similar to pepsin, the amount of base required to maintain pH at 8 during the alcalase hydrolysis experiments increased with bloodmeal and enzyme concentrations (Figure 54 and Figure 55). Unlike pepsin, however, was that rate of reaction increased with enzyme concentration but decreased slightly with bloodmeal concentration (Figure 56 and Figure 57), whereas pepsin rate of reaction was constant with enzyme concentration and increased with bloodmeal concentration (Figure 45 and Figure 46).

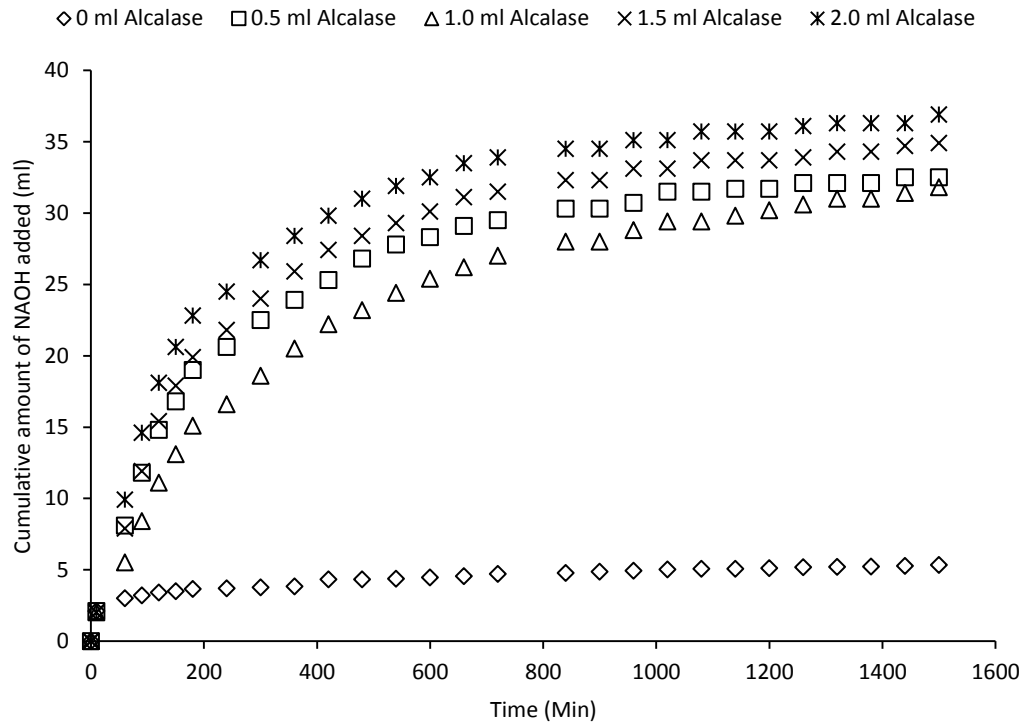


a

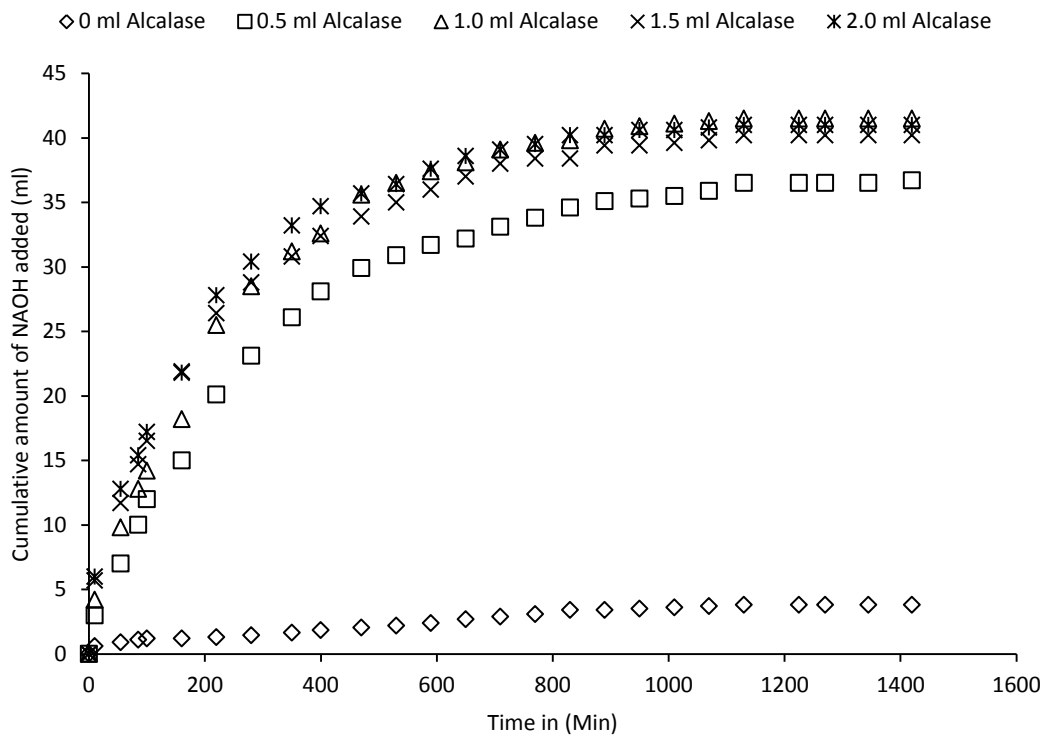


b

Figure 54 Bloodmeal hydrolysis with for a) 5 g bloodmeal and b) 10 g bloodmeal, at pH 8 and 60°C for different amounts of alcalase in 200 ml.



a



b

Figure 55 Bloodmeal hydrolysis with for a) 15 g bloodmeal and b) 20 g bloodmeal, at pH 8 and 60°C for different amounts of alcalase in 200 ml.

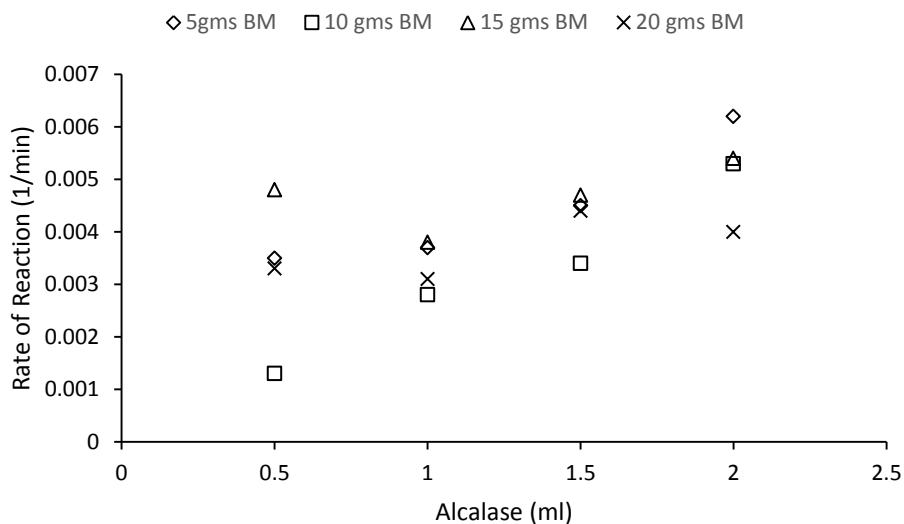


Figure 56 Change in rate of reaction with increase in alcalase concentration

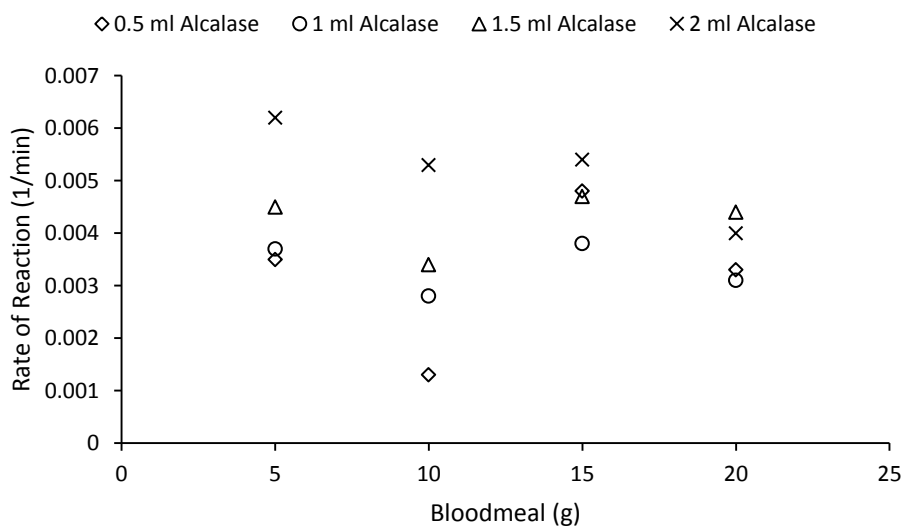


Figure 57 Change in rate of reaction with increasing bloodmeal concentration

Alcalase activity is listed as 2.997 units per ml (Alcalase is supplied in a liquid) whereas pepsin activity is listed as 469 units per mg. Therefore it is likely that alcalase was already at saturation in terms of substrate, hence the increase in rate of reaction with increasing enzyme. The decrease in activity due to the increase in bloodmeal could be due to a lower concentration of alcalase on the bloodmeal surface because of the greater number of bloodmeal particles. In the case of pepsin, which has a much high activity, it did not matter if there was a lower concentration of enzyme on the bloodmeal surface.

Similarly to pepsin, degree of hydrolysis increased with increasing alcalase concentration, but decreased with increasing bloodmeal concentration (Figure 58). The degree of hydrolysis obtained with alcalase was much higher at between 35-40% at 5 g of bloodmeal to 25-30% at 20 g of bloodmeal, compared to 25-10% for pepsin at pH 1.5. Mass of bloodmeal solubilized was slightly less than that of pepsin at between 57 to 75% (Figure 59), compared to 60-80% for pepsin (Figure 51), and decreased with increasing bloodmeal, but appeared to remain constant with increasing alcalase addition.

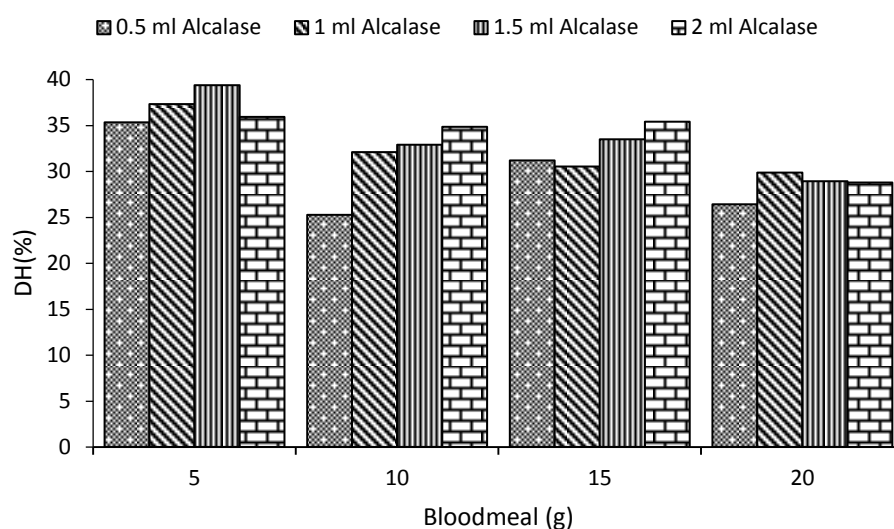


Figure 58 Degree of hydrolysis for alcalase with varying concentration of bloodmeal and enzyme

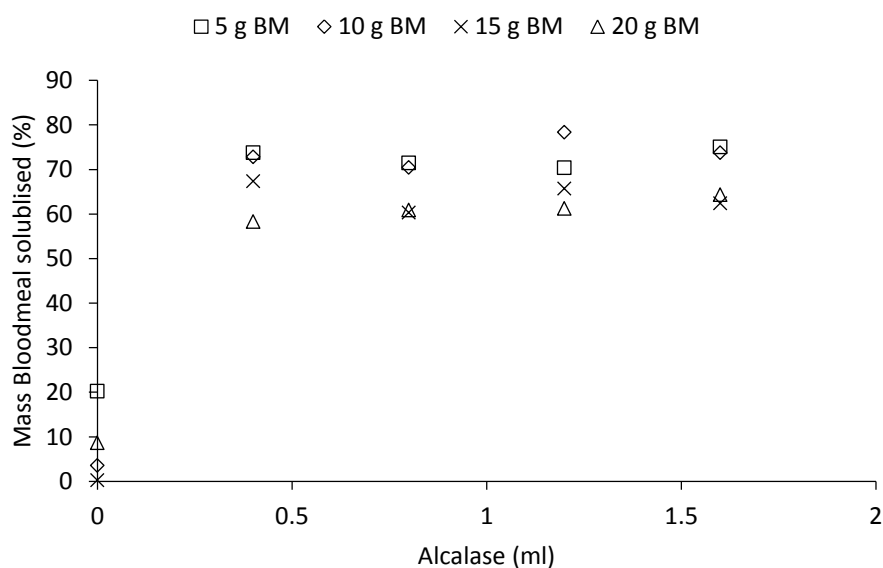


Figure 59 Mass of bloodmeal solubilized (%) vs. volume of alcalase added at different bloodmeal concentrations.

Average molecular weight of the hydrolysate obtained from alcalase was much lower than that from pepsin, at between 2 to 12 kDa for alcalase compared to 20 kDa to 60-70 kDa for pepsin.

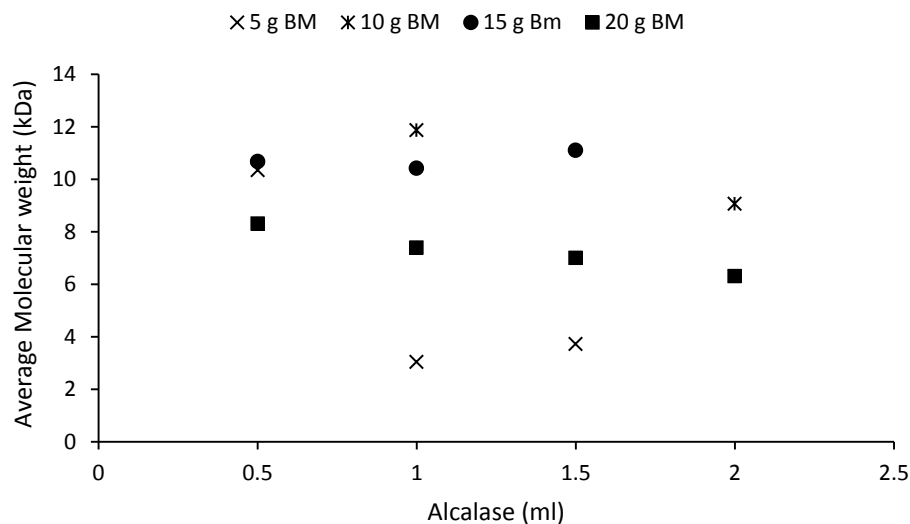


Figure 60 Average molecular weight for alcalase treated hydrolysate at different concentrations of alcalase and bloodmeal concentration.

Alcalase appears to be much more suitable for cleaving bloodmeal into smaller peptides and gives a much higher degree of hydrolysis, although pepsin gives a slightly higher yield of soluble peptides. Alcalase hydrolysis has the advantage of not requiring much of a pH change in the solution compared to pepsin, which requires pH 1.5, which means much lower acid and base addition to change pH and neutralize it afterwards, which is beneficial if the hydrolysate is to be used in NTP or for adsorption onto bentonite clay. A disadvantage of alcalase is the solution needs to be heated to 60°C if the reaction is started using powders. However this is beneficial, if bloodmeal hydrolysis was implemented in a rendering plant, as alcalase could be added straight after bloodmeal coagulation when the solution is at 90°C (or with minimal cooling – alcalase has been used at temperatures between 60 to 90°C), and before the centrifugation step (see section 2.3). In addition, the higher temperature for alcalase probably aided the extent of hydrolysis because the bloodmeal proteins would be more flexible at 60°C compared to at 37°C for pepsin.

4.1.3 Crystallinity of unhydrolysed bloodmeal and hydrolysate

4.1.3.1 *Unhydrolysed bloodmeal*

XRD was carried out on unhydrolysed bloodmeal that had been recovered and dried from each hydrolysis experiment for alcalase and pepsin at pH 1.5. This was to see if the hydrolysis reaction caused any structural change to bloodmeal. Percentage crystallinity was calculated and compared with untreated bloodmeal.

Bloodmeal has an average crystallinity of 26% (Table 18), and showed a little variability between samples with crystallinities ranging between 25 and 27%. The unhydrolysed bloodmeal from each alcalase experiment had a slight decrease in crystallinity with increasing alcalase, but a slight increase in crystallinity with increasing bloodmeal (Figure 61). This follows the same trend as observed for degree of hydrolysis (Figure 58), suggesting the change in crystallinity is related to the degree of hydrolysis, i.e. the greater the degree of hydrolysis the greater the reduction in crystallinity (Figure 62). Similar behaviour was also seen with pepsin treated bloodmeal, but to a lesser extent, presumably because the degree of hydrolysis for pepsin was lower (Figure 49).

Table 18 Average crystallinity of untreated bloodmeal

Sample	Crystallinity
BM 1	25.7
BM 2	26.8
BM 3	27.3
BM 4	26.3
BM 5	25.6
BM 6	25.6
BM 7	25.0
BM 8	25.8
BM 9	25.5
BM 10	26.5
Average	26.0
Std. Dev.	0.65

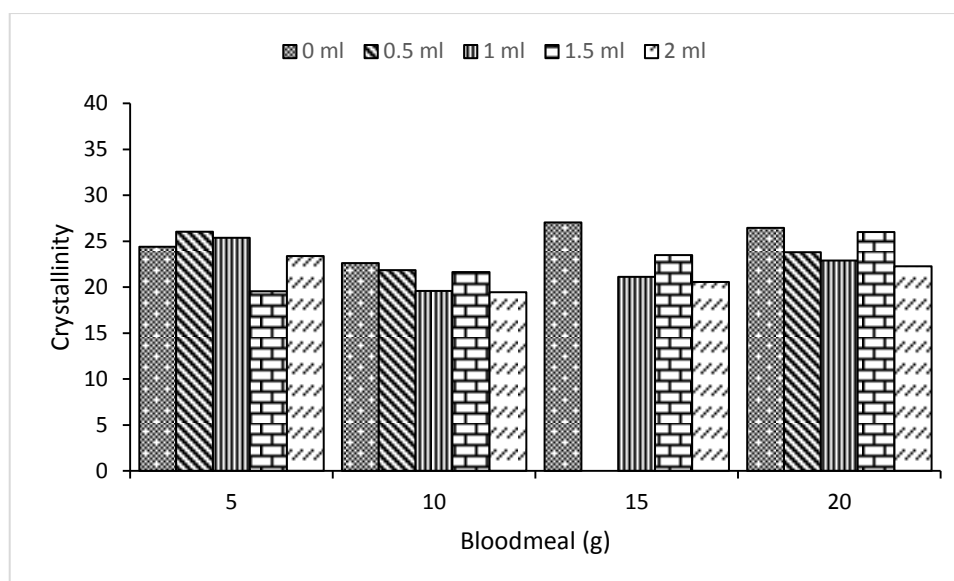


Figure 61 Crystallinity of unhydrolysed bloodmeal from the alcalase experiments (missing data point due to insufficient material).

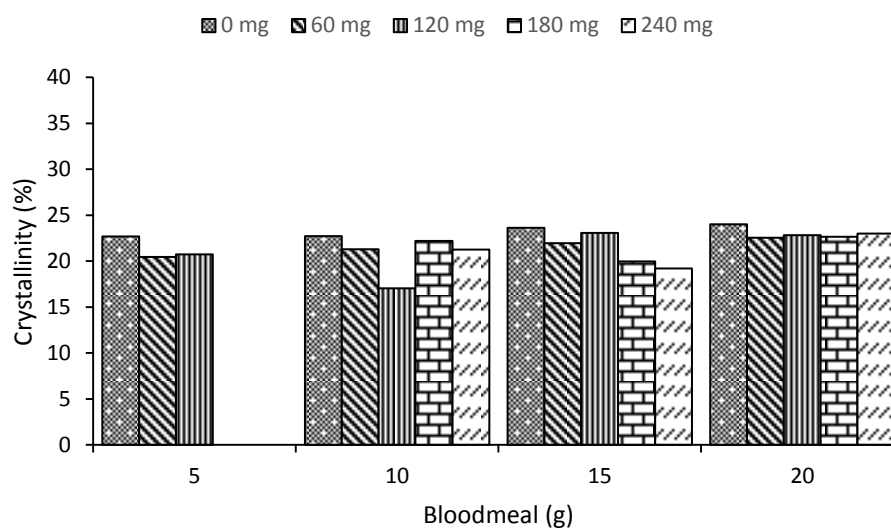


Figure 62 Crystallinity of unhydrolysed bloodmeal from the pepsin experiments (missing data point due to insufficient material).

The reason why the change in crystallinity was not larger could be explained by assuming that the crystalline regions (regions with α -helices and β sheets) are distributed evenly throughout the bloodmeal particle. If hydrolysis is taking place uniformly around the particle, and the particle size is decreasing evenly, a large effect on crystallinity would not be seen.

The samples which are not present in Figure 61 and Figure 62 were not presented due to there being insufficient unhydrolysed bloodmeal from those particular experiments to fill up the XRD sample holder, resulting in anomalous results.

Further studies could be carried out on the unhydrolysed bloodmeal using conventional FTIR spectroscopy, and look for any changes in α -helices and β sheets within the material. If there were significant changes, this could be examined further for spatial variation using Synchrotron light source (Bier, Verbeek et al. 2013) (if funding could be obtained).

When the XRD traces were examined, a slight change in the intensity of the XRD plots could be seen (Figure 63) with a reduction in the peak at 10 2θ and the sharp peak at 20 2θ with unhydrolysed bloodmeal from the 0.5 ml alcalase experiment. This could suggest some physical change in the bloodmeal structure, resulting in lower peaks, or it also could be an artifact of the powdered unhydrolysed bloodmeal, which appeared to be not as fine as bloodmeal itself.

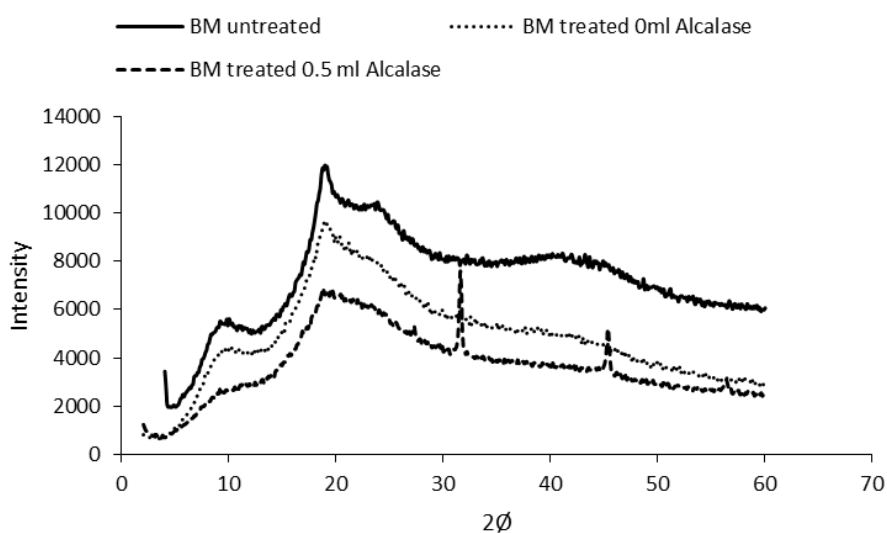


Figure 63 XRD trace for treated and untreated bloodmeal

The very sharp peak in Figure 63 pointed out with an arrow is salt, which was formed from neutralising the samples and adjusting pH during the hydrolysis reaction.

4.1.3.2 Hydrolysate

The crystallinity of the hydrolysate was unable to be found. The hydrolysate formed a film when it was dried which was highly hygroscopic. When it was brought out from oven to be ground into a powder for XRD, it formed a paste. Liquid hydrolysate samples were also tried on the XRD, but no results were able to be obtained, possibly because the protein concentration was not high enough in the liquid. Another approach taken involved drying the hydrolysate in the XRD sample holder, but this resulted in

leakage of the hydrolysate, and what was left formed too thin a layer for XRD to be successfully carried out.

In future, the hydrolysate samples could be dried in custom made containers, directly in the XRD under heating, and track the changes in the XRD trace with evaporation of water, until a film is formed.

Alternatively freeze drying the hydrolysate could also be helpful, but as the hydrolysate is highly hygroscopic, it would still adsorb water, unless kept within a humidity controlled environment.

4.1.4 Acid hydrolysis

Acid hydrolysis of bloodmeal was carried out using different strengths of nitric acid. In this case, solubilisation was measured by UV absorbance of centrifuged samples at 280 nm (for protein) and 580 nm (for the haem group). This was compared to a calibration curve made from protein hydrolysate of known solids content. 2M nitric acid gave the highest protein concentrations in solution with about 92% of the bloodmeal being solubilised at 5 g bloodmeal concentrations and 36% of the bloodmeal being solubilised at 20 g bloodmeal concentrations. Solubilisation occurred relatively quickly within 100-200 minutes.

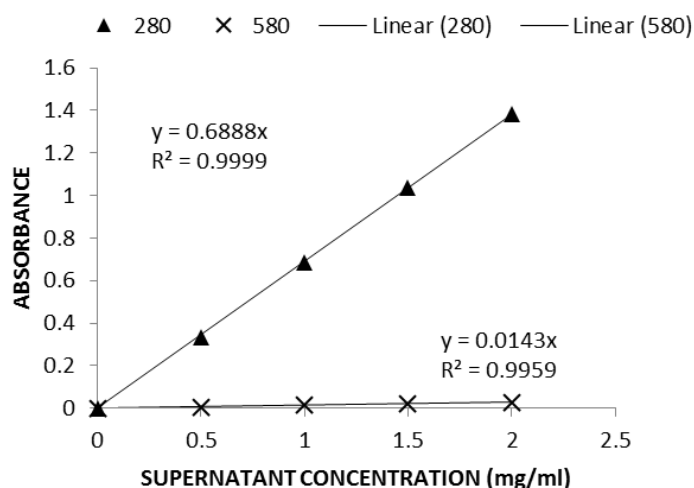


Figure 64 Calibration curve for Protein concentration

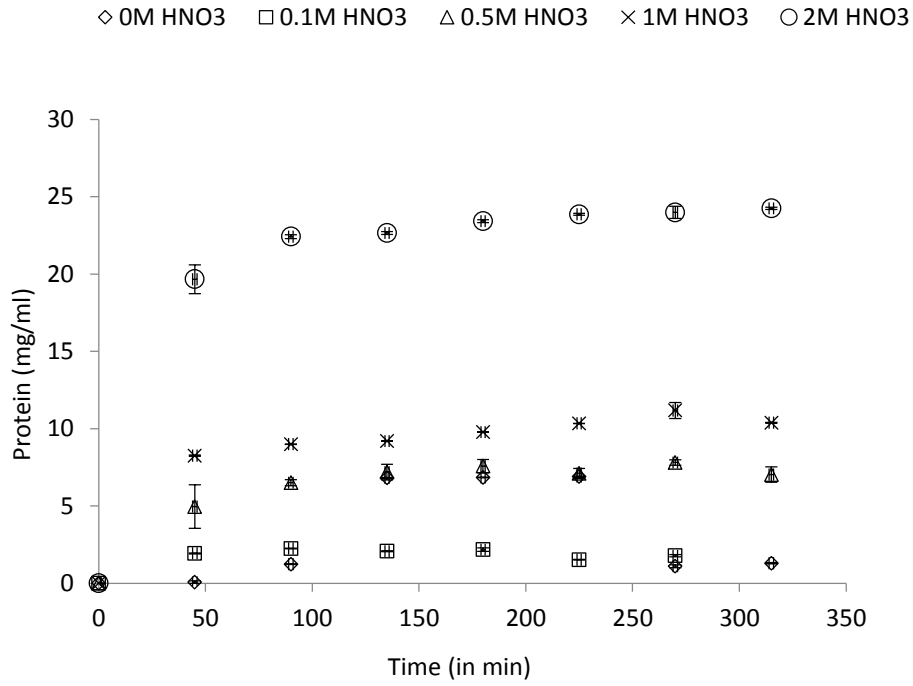


Figure 65 Protein Concentration in solution with varied amount of acid with 5gms BM

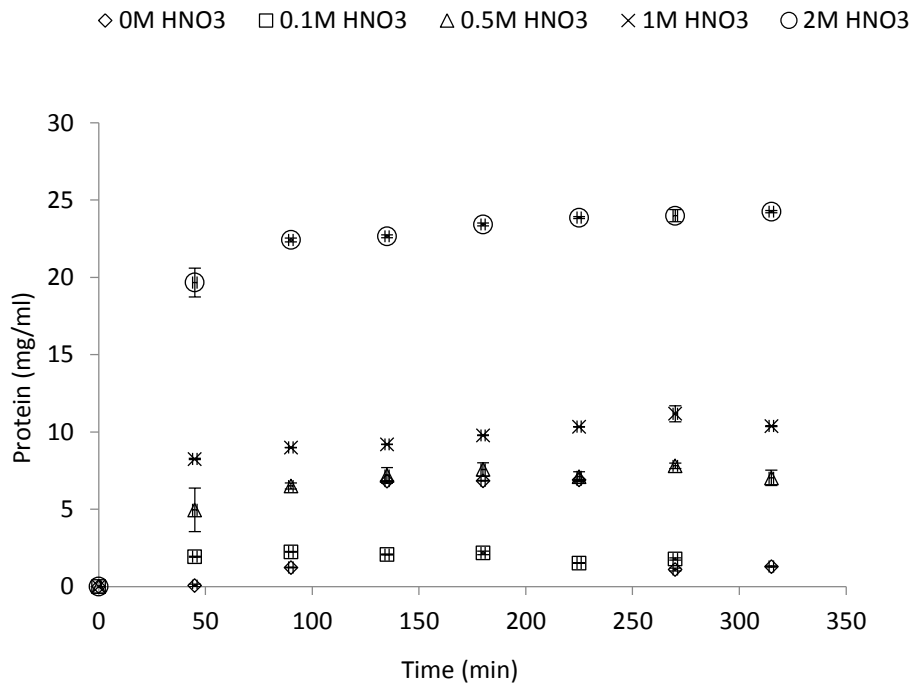


Figure 66 Protein Concentration in solution with varied amount of acid with 10gms BM

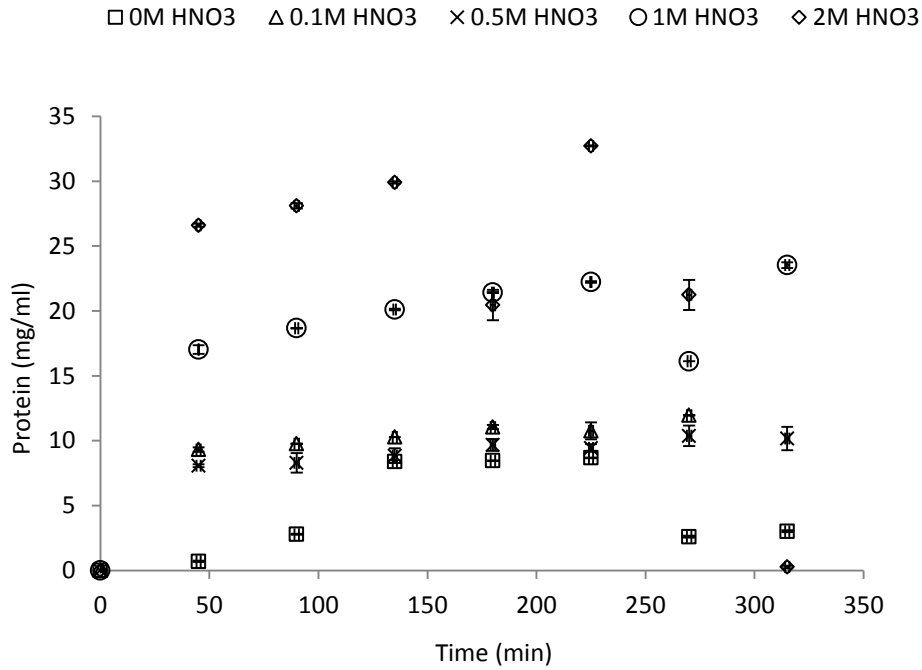


Figure 67 Protein Concentration in solution with varied amount of acid with 15gms BM

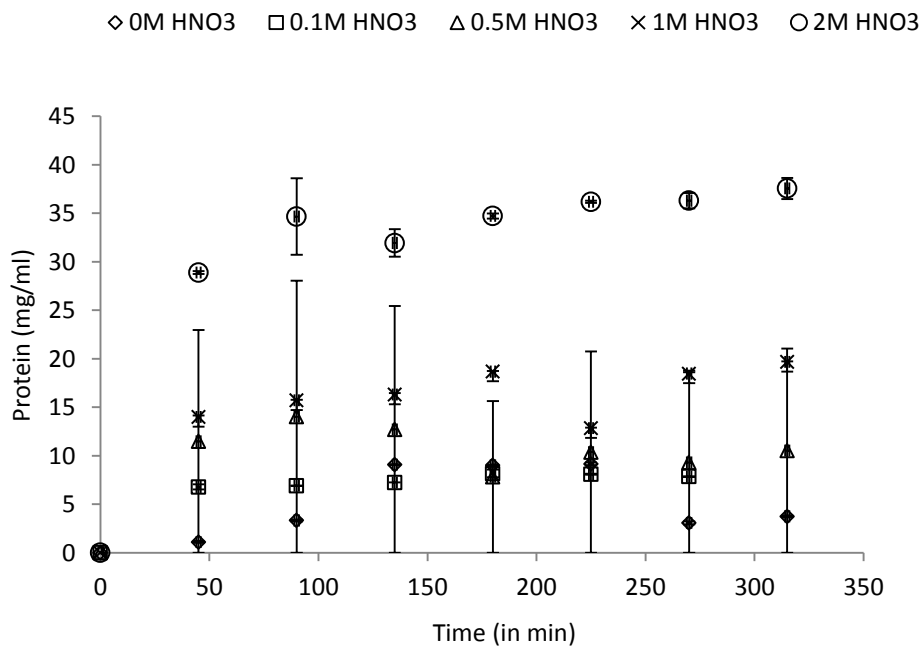


Figure 68 Protein Concentration in solution with varied amount of acid with 20gms BM

While centrifugation of 1 ml samples at 20,000 rpm was able to separate the unhydrolysed bloodmeal from the supernatant, large scale centrifugation of samples at 4000 rpm in 400 ml bottles for an hour was unable to separate the unhydrolysed bloodmeal, which had become a fine particulate which would not settle out. Another

issue was the amount of base required to neutralize the acid, resulting in the production of high amounts of salt in the hydrolysate, which would be detrimental for using as the hydrolysate in NTP or for adsorption to the bentonite clay. The salt would interfere with protein-protein ionic interactions in NTP or increase the hydrophobic interaction in NTP, and would reduce protein adsorption onto the bentonite clay. Enzyme hydrolysis, while taking longer, gave greater hydrolysis yields, with less salt production. Therefore, no further investigation was carried out for acid hydrolysis.

4.2 Large scale hydrolysis

The large scale hydrolysis experiments were carried out by first treating bloodmeal with alcalase. The solution was centrifuged and the pellet recovered and dried. Bloodmeal hydrolysed was calculated to be 78% (Table 19) which was better than that obtained for the small scale hydrolysis experiments which ranged between 57 to 78% (Figure 59). The pellet had a solids content of 19%, while the supernatant containing the hydrolysate was calculated by mass balance and oven drying to have a solids content of 6.5%. There was some loss of water due to evaporation during the hydrolysis which took place over 8 hours, but some of this was made up by water added when adding base to maintain pH at 8 during the reaction.

Table 19 Results from large scale alcalase hydrolysis

Starting water (g)	30000
Bloodmeal added (total) (g dry weight)	2250
Wet pellet weight (g)	2590
Pellet solids content (%)	18.9
Unhydrolysed bloodmeal (g dry weight)	489
Bloodmeal hydrolysed (%)	78.3
Supernatant mass (g)	27177.7
Solids in supernatant (g)	1760.6
Solids content (%)	6.5

10 L of hydrolysate was removed (Alcalase hydrolysate) and trypsin added to the remaining 20 L. This was allowed to run for 8 hours before the solution was split into two, one half retained (Trypsin hydrolysate), pH adjusted to 1.5 and pepsin added to the other half. The reaction was allowed to run for another 8 hours before being neutralised (Pepsin hydrolysate).

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 69). 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.

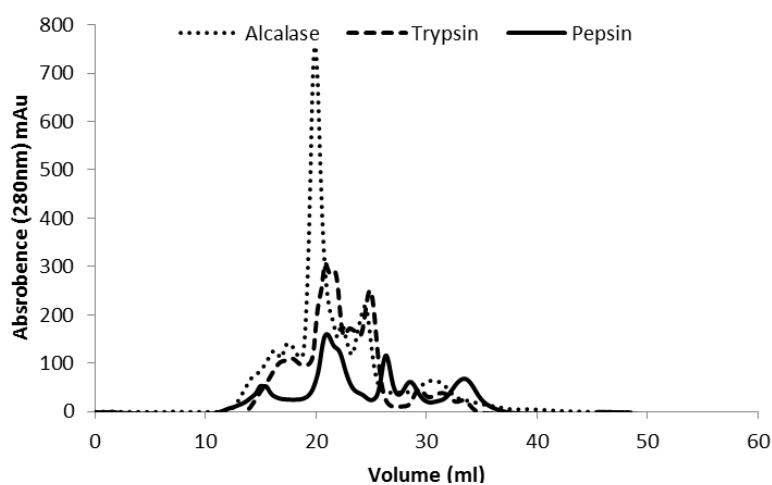


Figure 69 Gel filtration chromatography of large scale hydrolysis using alcalase, trypsin and pepsin

Table 20 Average molecular weight of hydrolysate for large scale hydrolysis using alcalase, trypsin and pepsin.

Hydrolysate	Average molecular distribution (KDa)
Alcalase	8.9
Trypsin	5.5
Pepsin	8.8

Solids contents of the hydrolysates ranged between 2.8% for trypsin and 6.5% for alcalase (Table 21). The trypsin hydrolysate % solids was very low, which could be due to some of the hydrolysate precipitating out, or an error in the measurement. An error in the measurement could be more likely because the UV absorbance measured for each hydrolysate showed only a slight change, suggesting the concentrations were similar. To verify these results, a total nitrogen measurement on the hydrolysates would be useful.

Table 21 Hydrolysate solids content

Hydrolysate	Unconcentrated		Concentrated
	% solids	UV absorbance (280nm) (1/5 dilution)	% solids
Alcalase	6.45	2.24	45.5
Trypsin	2.80	2.17	60.5
Pepsin	5.00	2.12	46.6

Half of the hydrolysate from each was used for intercalation with sodium bentonite and the other half was concentrated to give a final % solids shown in Table 21. The concentrated hydrolysate was used in NTP.

4.3 Sodium bentonite clay adsorption

Each of the unconcentrated hydrolysates from the previous section were mixed with sodium bentonite clay and left for absorption to occur overnight. UV absorbance was measured before and after absorption. There was only a slight change absorbance for alcalase and trypsin hydrolysates, and no change in absorbance for pepsin hydrolysate (Table 22). This amounted to 127 mg hydrolysate per g clay for alcalase hydrolysate, and 46 for trypsin and no adsorption for pepsin. Because the UV absorbance for the starting hydrolysate solutions did not show much change, but the % solids from Table 21 was quite different, the % solids used for trypsin before adsorption was calculated based on UV absorbance. If the measured % solids was used from Table 21 was used, the adsorption for trypsin would be even lower at 18.5 mg per g clay. From previous research using meat rendering plant stickwater and gelatin, sodium bentonite could adsorb between 180 to 240 mg per g clay (Shamsuddin 2013), which is comparable to the alcalase hydrolysate adsorption of 127 mg per g clay. Increasing concentrations of salt in the hydrolysate from pH maintenance and pH adjustment (especially for pepsin

which involved lowering the solution pH to 1.5 for the reaction and neutralising it afterwards) would reduce protein adsorption, explaining the low adsorption for trypsin, and no adsorption for pepsin hydrolysate.

Table 22 Absorption results

	Alcalase	Trypsin	Pepsin
Solution (g)	5126	4260	4316
Sodium bentonite (g)	144	117	120
UV absorbance before (280nm) (1/5 dilution)	2.24	2.17	2.12
UV absorbance after (280nm) (1/5 dilution)	2.11	2.12	2.12
Solids before (%)	6.45	6.23	6.11
Calculated solids after (%)	6.07	6.11	6.11
Change in mass (g)	18.34	5.40	0.00
Absorption (mg/g clay)	127.34	46.13	0.00

XRD analysis of the alcalase and trypsin hydrolysate treated bentonite clays showed a peak at around 6.3 2θ close to the peak for sodium bentonite (Figure 70). This peak was very slight for trypsin clay, but this could be due to the XRD result showing a baseline shift and overall intensity not being high compared to the other XRDs. The bentonite peak for alcalase showed a shift to the left (Figure 70) and a corresponding decrease in basal spacing (Table 23) compared to sodium bentonite, indicating that protein intercalation between the clay layers had occurred. Trypsin treated bentonite showed only a slight increase basal spacing, so protein adsorption was likely to have been around the clay particle only with no intercalation.

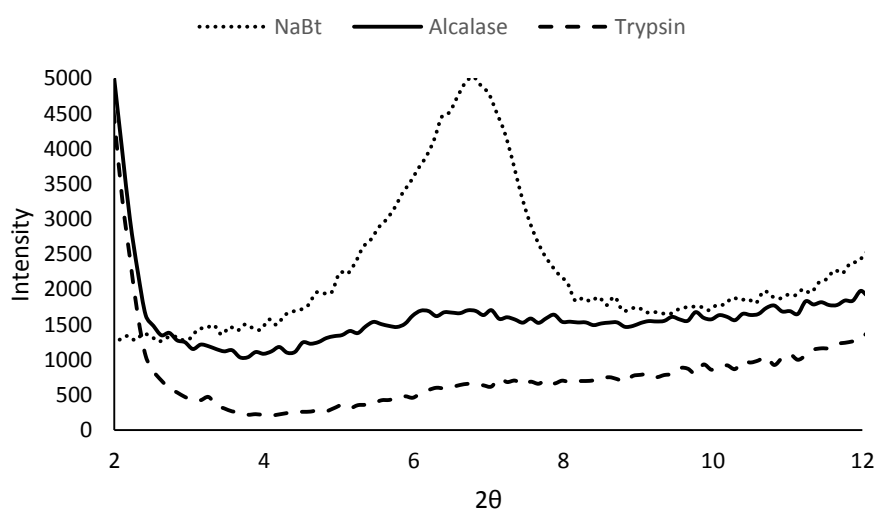


Figure 70 XRD graph showing the sodium benonite peak around 6.3 2θ for untreated and alcalase and trypsin hydrolysate treated bentonite

Table 23 Comparison of hydrolysate molecular weight, sodium bentonite adsorption, and basal spacing calculated from XRD

	Sodium bentonite			
	Untreated	Alcalase	Trypsin	Pepsin
Hydrolysate molecular weight (kDa)	-	8.9	5.5	8.8
Adsorption (mg/g clay)	-	127.34	46.13	0.00
Basal spacing (Å)	13.2	14.2	13.6	-

Only the alcalase adsorption on to sodium bentonite was successful, while high salt concentrations in the trypsin and pepsin hydrolysates reduced or prevented adsorption. If enzyme hydrolysis was used in future experiments to produce peptides for clay modification, the hydrolysate solution would need to be desalted, for example using dialysis or ultrafiltration or nanofiltration with a low molecular weight cut-off (e.g. 1-2 kDa) so the peptides could be retained, or the hydrolysis carried out to reduce salt concentration. One possible method of hydrolysis that could be explored is using high pressure and heat (Bressler 2010), for example in an autoclave.

Future bentonite adsorption experiments would also need to explore the effect of solution pH and hydrolysate concentration on adsorption, to try and increase peptide adsorption.

4.4 NTP with hydrolysate and NTP composites

4.4.1 Extrusion and injection moulding

4.4.1.1 NTP and hydrolysate

In the initial preparation of NTP and hydrolysate mixtures for extrusion, there was an issue of foaming within the blender when the additives were mixed in with the hydrolysate with increasing hydrolysate concentrations (This also happens sometimes when TEG is added). This might be due to some interactions of the additives with the hydrolysate, e.g. between the sodium dodecyl sulphate and the hydrolysate, causing foaming. The foams were not stable because when the foamed material was packed and sealed in zip lock bags and kept in the freezer overnight, the foam had collapsed and the samples appeared to be normal.

With increasing hydrolysate and the material was increasingly harder to extrude with the extrudate coming out in small lengths. This might be due to evaporation of water

resulting in material coming out in chunks. This was supported by the specific mechanical energy (SME) results (Figure 71) which increased with increasing hydrolysate. Pepsin and trypsin hydrolysates gave higher SME than alcalase at the higher hydrolysate levels.

The higher SME could be caused by the slight reduction in water content in the extrudate (Figure 72) 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.

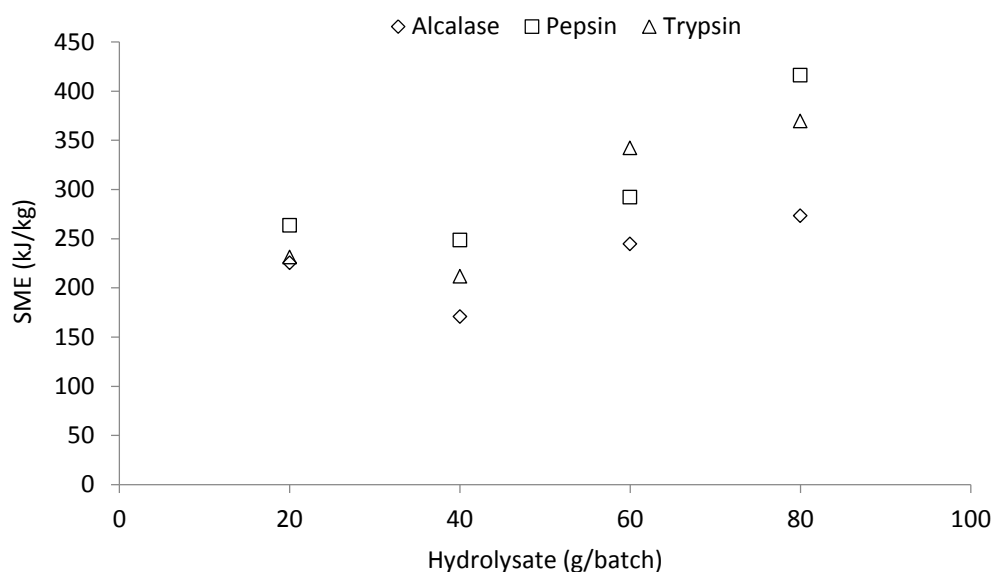


Figure 71 Specific mechanical energy required to extrude NTP with different amounts of alcalase, pepsin and trypsin hydrolysate.

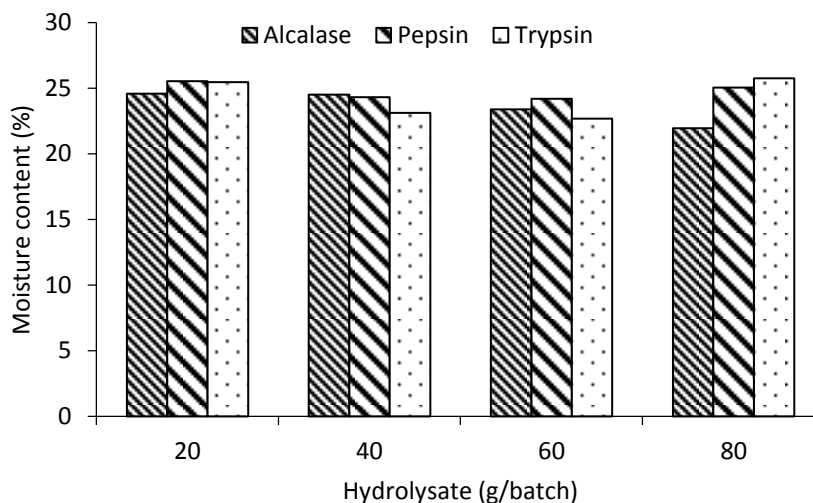


Figure 72 Moisture content (% by weight) of the extrudate. of NTP with different amounts of alcalase, pepsin and trypsin hydrolysate.

4.4.1.2 NTP and hydrolysate treated bentonite clay

No issues were encountered with preparing and extruding the NTP and bentonite clay mixtures. SME showed small change with increasing clay content, but treating the clay with hydrolysate increased the SME, with the biggest increase for pepsin treated clay, followed by trypsin, then alcalase (Figure 73). Water content in the extrudate increased with extent of hydrolysis (alcalase, followed by trypsin and pepsin) and only showed a marginal increase with clay content (Figure 20). Seeing as protein adsorption for trypsin and pepsin treated clay, this could be due to the increased salt content in the hydrolysate, some of which would have been carried over into the clay pellet when the clay was recovered from the hydrolysate.

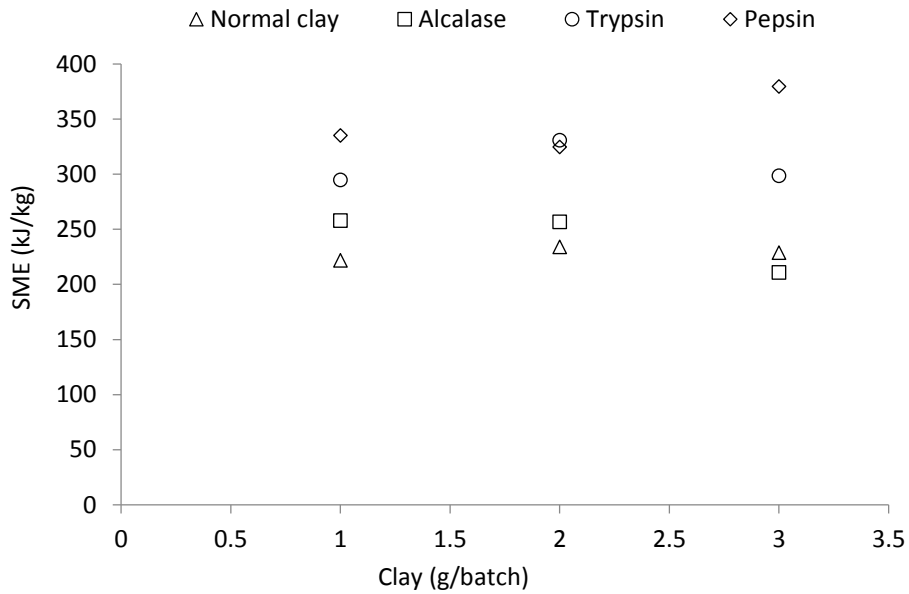


Figure 73 Specific mechanical energy required to extrude NTP with different amounts of treated bentonite clay

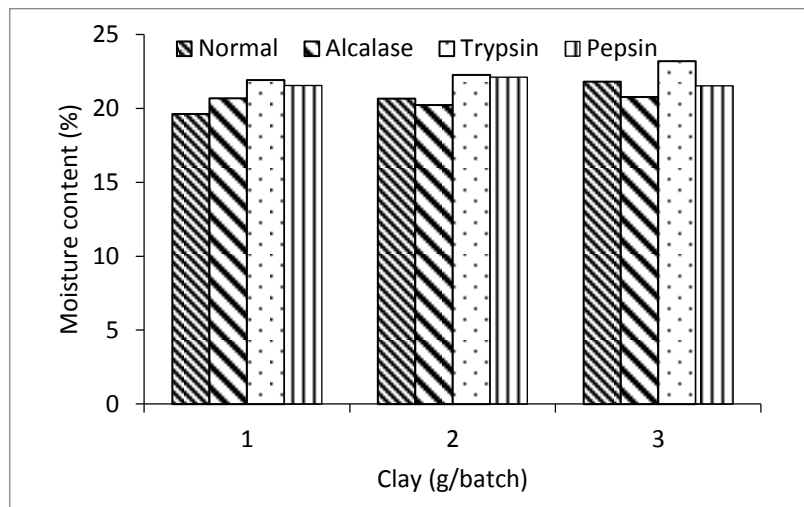


Figure 74 Moisture content (% by weight) of the extrudate of NTP with different amounts of treated bentonite clay.

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.

4.4.1.3 Injection moulding

All the extrudates were able to be injection moulded easily with the exception of NTP with 20 g alcalase hydrolysate. On this sample, the injection moulder blocked and only one partial tensile specimen was produced.

4.4.2 NTP/hydrolysate mechanical properties

Increasing hydrolysate concentration in NTP lowered tensile strength and secant modulus (Figure 75 and Figure 76), 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). Toughness (Figure 78) and impact strength (Figure 79) marginally decreased with increasing hydrolysate while strain at break was 0.01 (Figure 77) indicating an extremely brittle material.

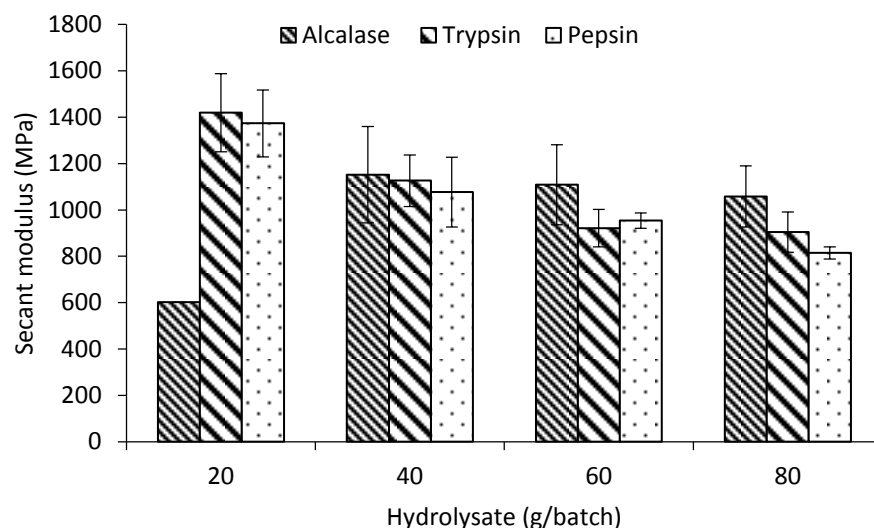


Figure 75 Secant modulus for tensile specimens of NTP with varying hydrolysate

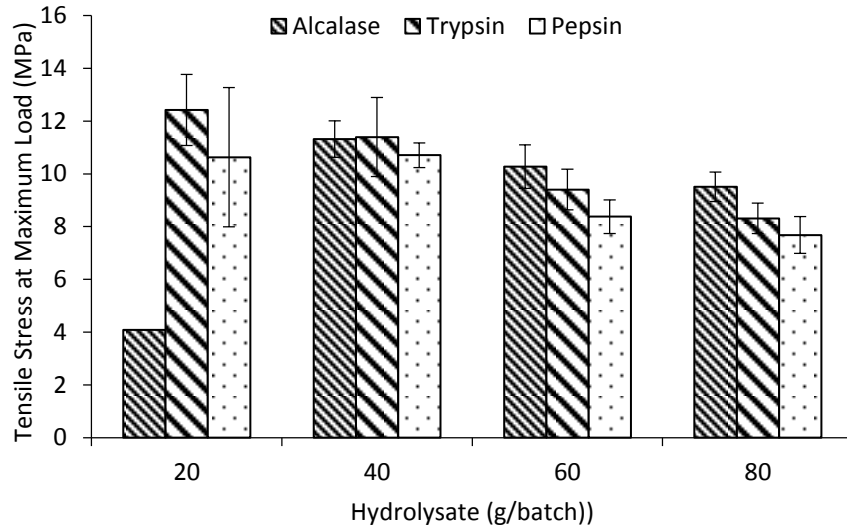


Figure 76 Tensile stress at maximum load for tensile specimens of NTP with varying hydrolysate

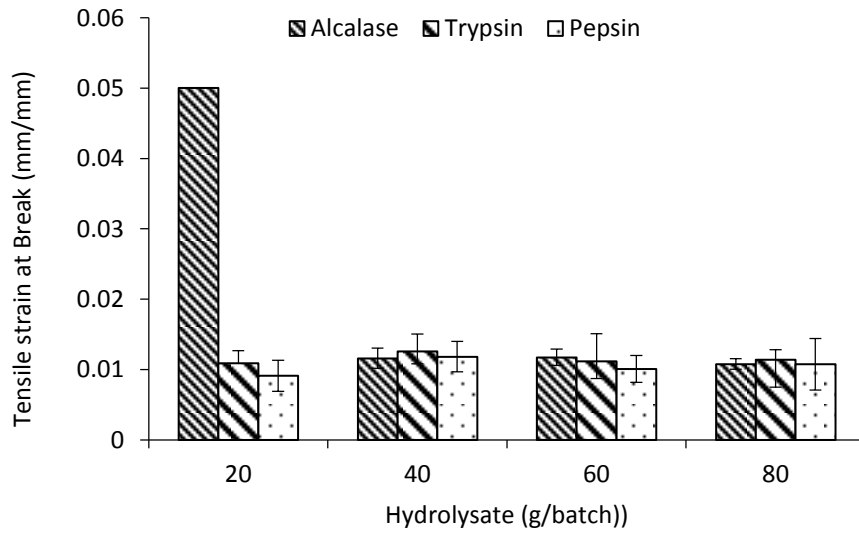


Figure 77 Strain at break at maximum load for tensile specimens of NTP with varying hydrolysate

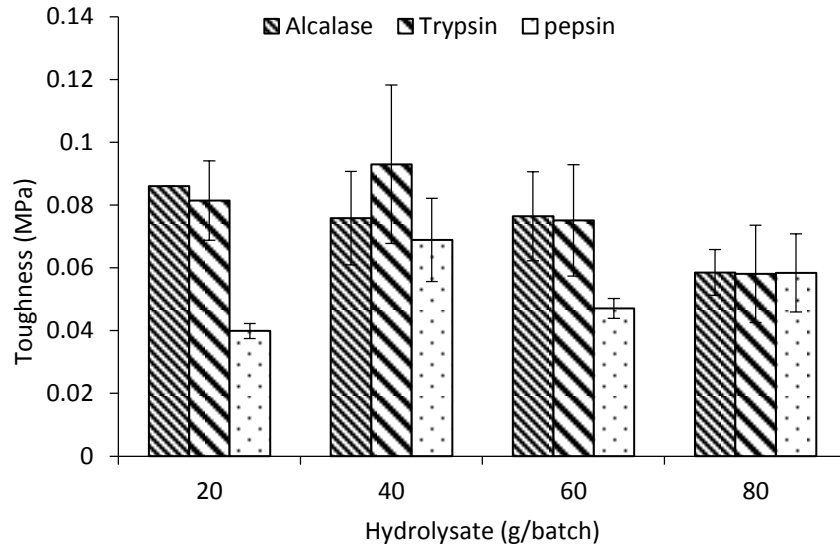


Figure 78 Toughness for tensile specimens of NTP with varying hydrolysate

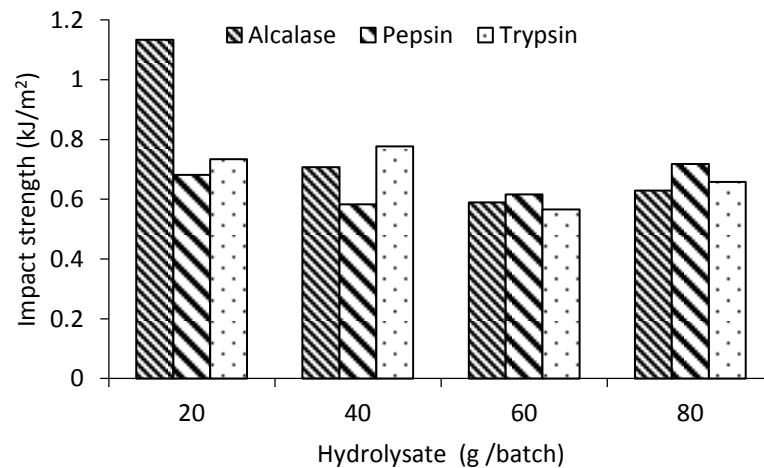


Figure 79 Impact strength for tensile specimens of NTP with varying hydrolysate

4.4.3 NTP/hydrolysate thermal properties

4.4.3.1 Glass transition temperature

Glass transition temperature decreased with increasing hydrolysate (Figure 80), signified by a shift in the peak in Tan Delta vs. temperature (Figure 81), with the exception of alcalase which appeared to remain constant at 78-79°C (Figure 80). All of the samples had a higher glass transition temperature (T_g) than NTP (61°C)(Shamsuddin 2013) The reduction in T_g would be consistent with a reduction in

average protein chain length with hydrolysate addition, with the exception of alcalase where little change was observed. With decreasing glass transition temperature, the material should show a decrease in viscosity and be easier to extrude, but an increase in SME was observed which suggests other interactions, such as salt, were important in SME, rather than average protein chain length.

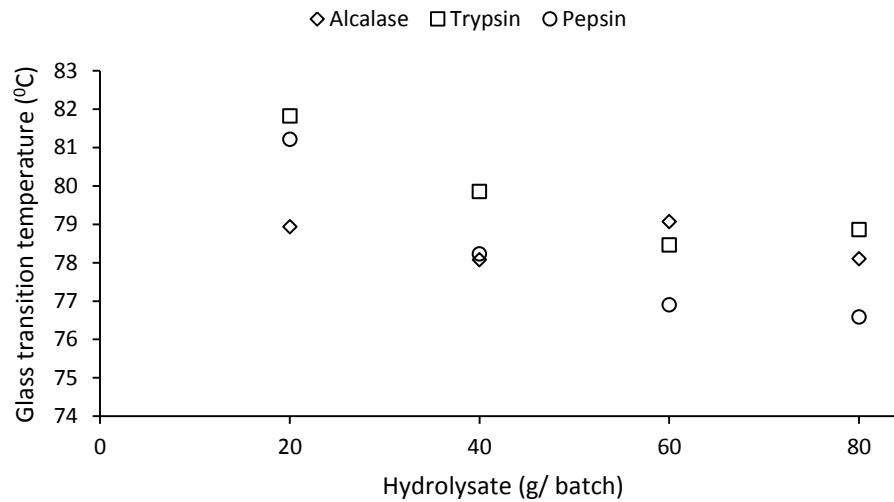


Figure 80 Glass transition temperature in NTP with varying hydrolysate

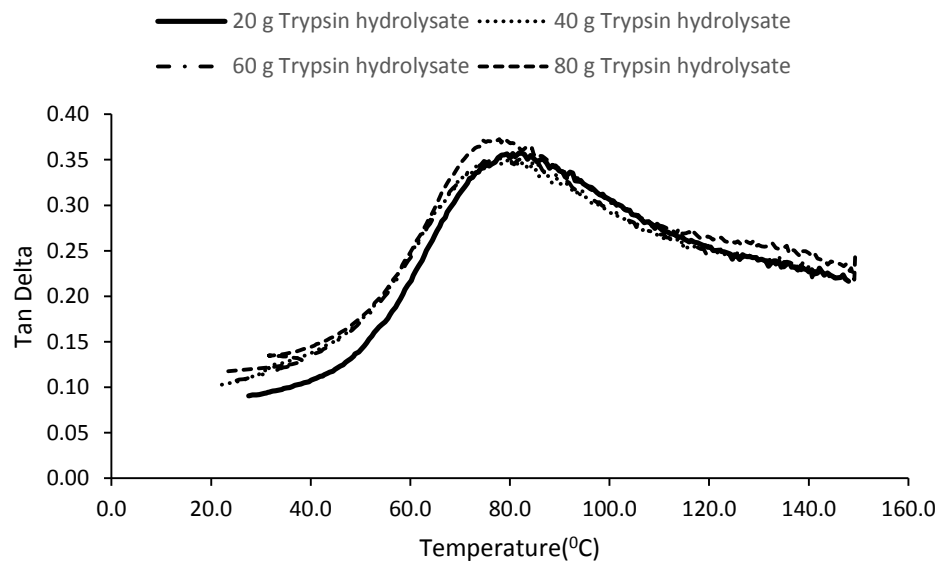


Figure 81 Glass transition temperature with varying pepsin hydrolysate in NTP

When tensile strength and secant modulus was compared against glass transition temperature for each NTP/hydrolysate sample, it appears that both form a linear relationship with glass transition temperature. When compared for each type of

hydrolysate, NTP with pepsin and trypsin increased initially and plateaued for tensile strength, suggesting that beyond a certain T_g , tensile strength becomes independent of T_g . Secant modulus formed a linear relationship with glass transition temperature for trypsin and pepsin.

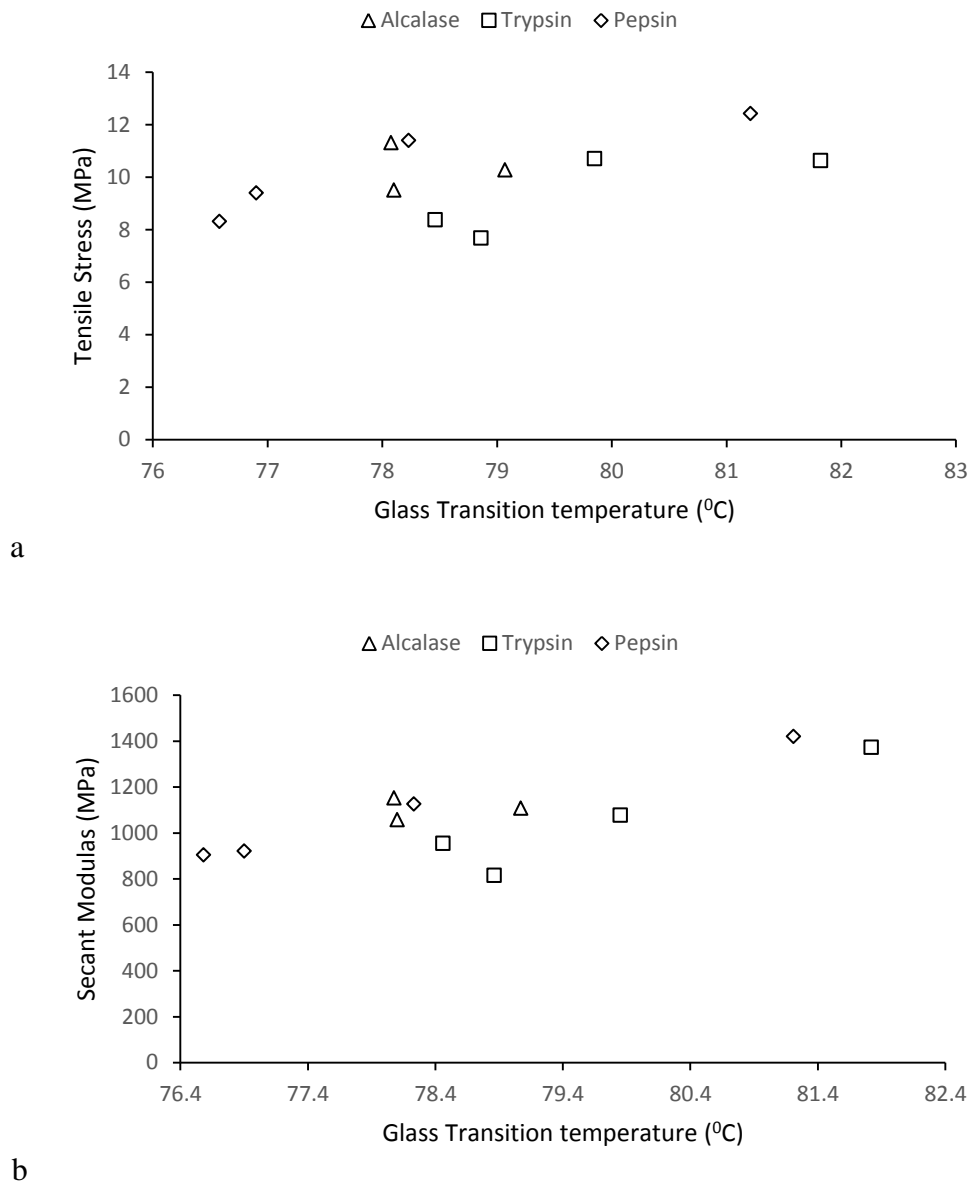


Figure 82 A plot of a) tensile strength and b) secant modulus against glass transition temperatures for NTP the different hydrolysates

4.4.3.2 Thermostability

The thermal degradation of samples were taking place in six stages, first from 20 to 150°C where bound water evaporates, 150 to 230°C where the low molecular weight peptides and urea are vaporized, 230 to 400°C which could correspond to cleavage of S-S, O-N and O-O bonds, after from 400°C to 800°C with removal of high molecular weight compounds, leaving the ash (Shamsuddin 2013). Increasing hydrolysate reduced thermal stability of NTP in the 450 to 800°C region for types of hydrolysate. NTP with pepsin hydrolysate also appeared less thermo stable than NTP with alcalase, which would be consistent with the reduced protein chain length of pepsin. Ash was consistent at about 5%.

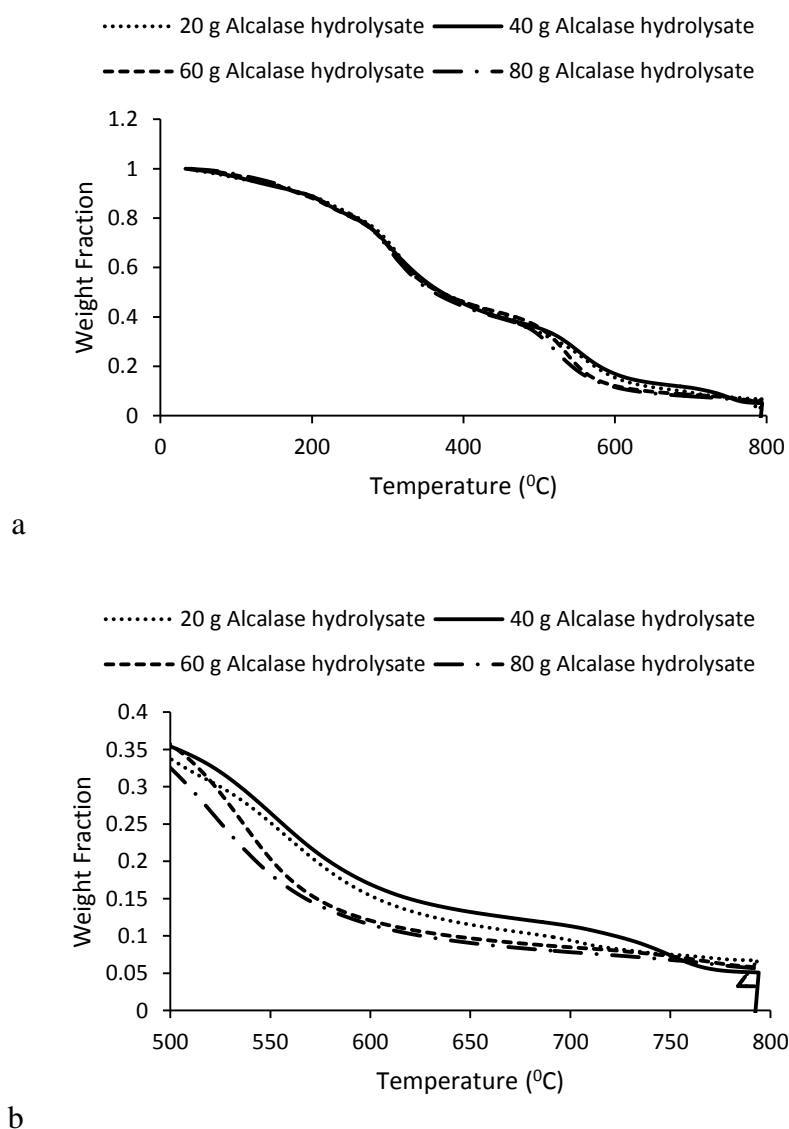
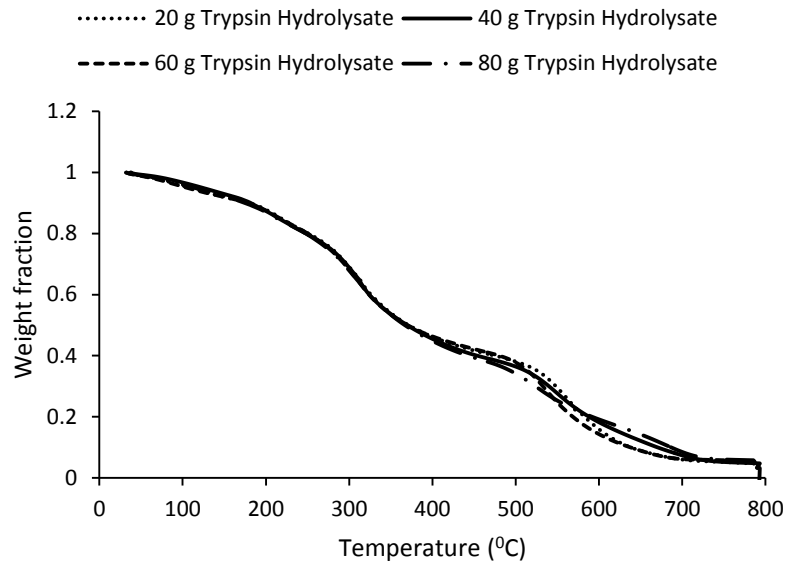
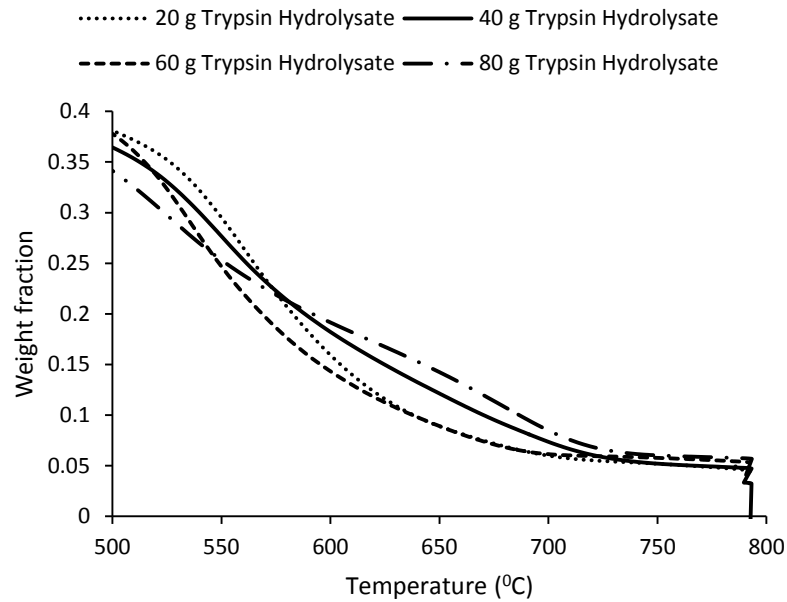


Figure 83 TGA of NTP with alcalase hydrolysate.a) over the full temperature range and b) zoomed in on the 500 to 800°C range

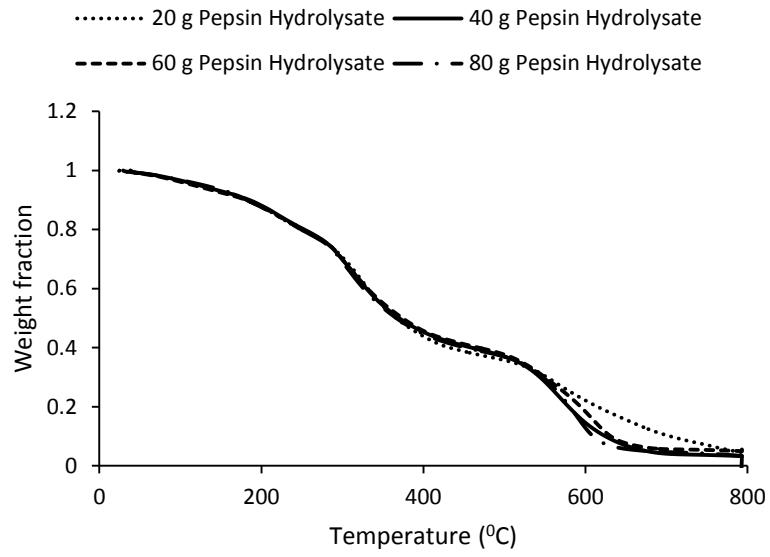


a

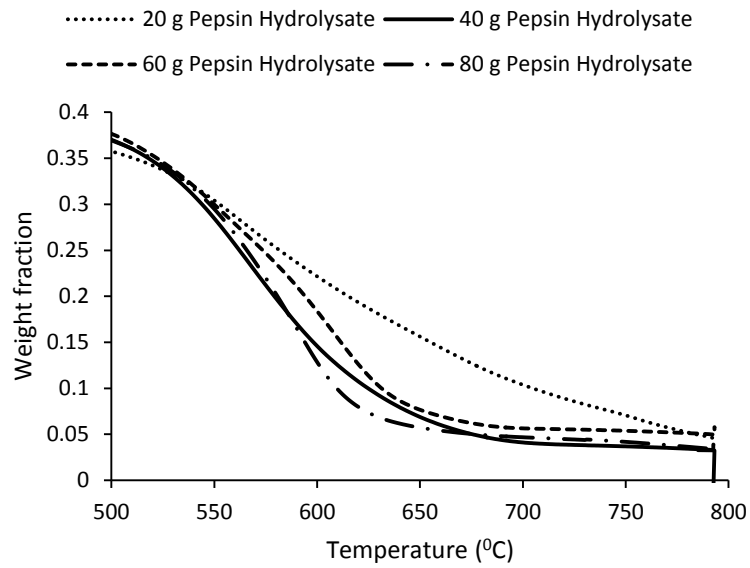


b

Figure 84 TGA of NTP with trypsin hydrolysate.a) over the full temperature range and b) zoomed in on the 500 to 800°C range



a



b

Figure 85 TGA of NTP with pepsin hydrolysate.a) over the full temperature range and b) zoomed in on the 500 to 800°C range

4.4.4 NTP/hydrolysate crystallinity

From XRD of solid NTP samples, NTP crystallinity decreased with increasing hydrolysate (Figure 86). Normal NTP typically has a crystallinity of around 26% (Bier et al 2014). This reduction in crystallinity could be attributed to the peptides present in the hydrolysate, which could be disrupting or diluting the crystalline regions by increasing the amount of amorphous material in NTP. This reduction in crystallinity could also contribute to the reduction in secant modulus and tensile strength of the NTP samples.

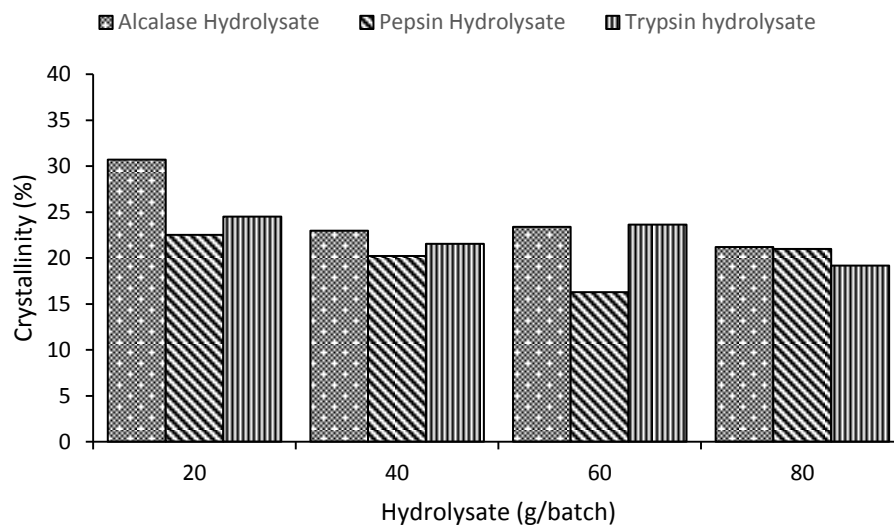


Figure 86 Crystallinity of NTP samples with varying amounts of hydrolysate

4.4.5 NTP/clay mechanical properties

NTP composites with alcalase treated bentonite generally had the highest modulus compared to NTP with other untreated or treated clay (Figure 87). NTP with pepsin and trypsin treated clay showed a higher modulus at 3 g clay, and NTP with trypsin treated clay generally had the lowest modulus. Tensile strength marginally increased with increasing clay with pepsin treated clay at 3 g giving the highest tensile strength (Figure 88). While alcalase treated clay had the most protein adsorbed, and showed some evidence of intercalation, modifying the bentonite surface with peptides from hydrolysis had a catastrophic effect on toughness (Figure 90) and strain at break (Figure 89) making what appeared to be a very brittle material, but which inexplicably had similar or higher impact strength than than NTP with the untreated or other treated clays (Figure 91). Conventional NTP in comparison has a tensile strength of around 9 MPa, a modulus of, toughness of 3.2 MPa and a strain at break of 0.7 (Shamsuddin 2013). The trypsin and pepsin treated clays which had little or no adsorption would have been expected to have a similar effect on composite mechanical properties as the untreated bentonite clay. There was some variability which could be due to salt content in the clay from the hydrolysate solution.

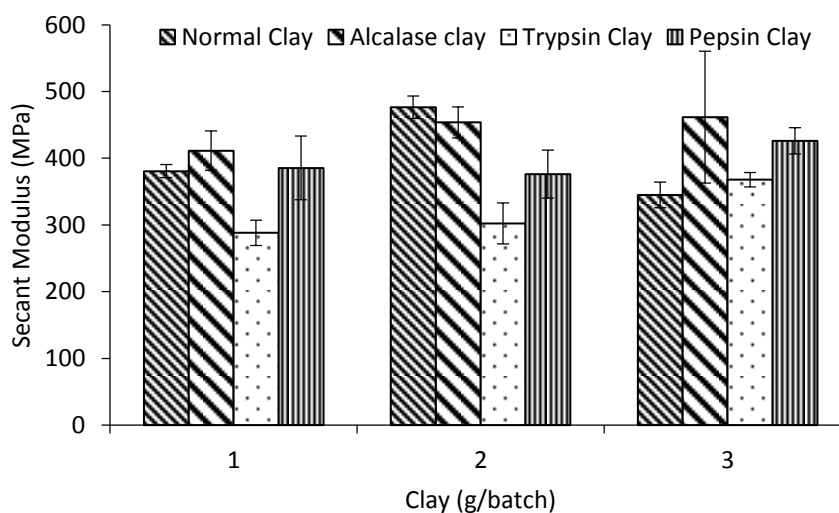


Figure 87 Secant modulus for tensile specimens of NTP with varying clay

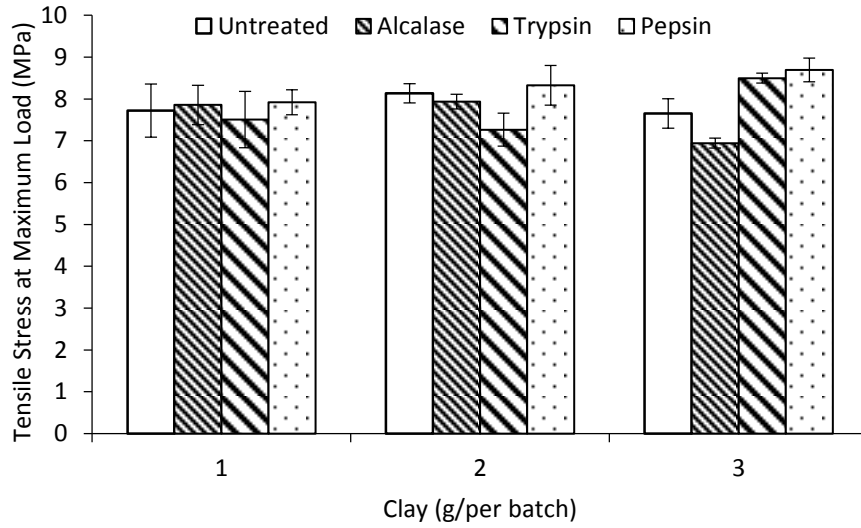


Figure 88 Tensile stress at maximum load for tensile specimens of NTP with varying clay

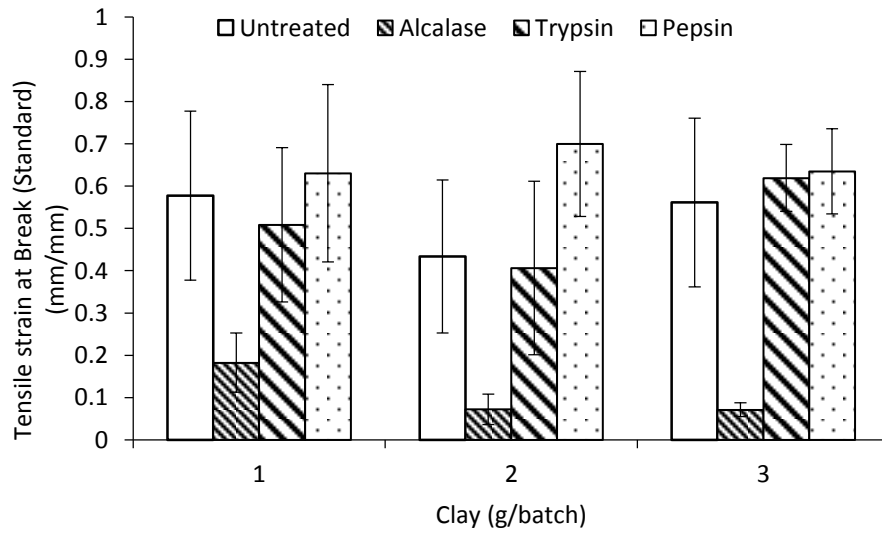


Figure 89 Strain at break at maximum load for tensile specimens of NTP with varying clay

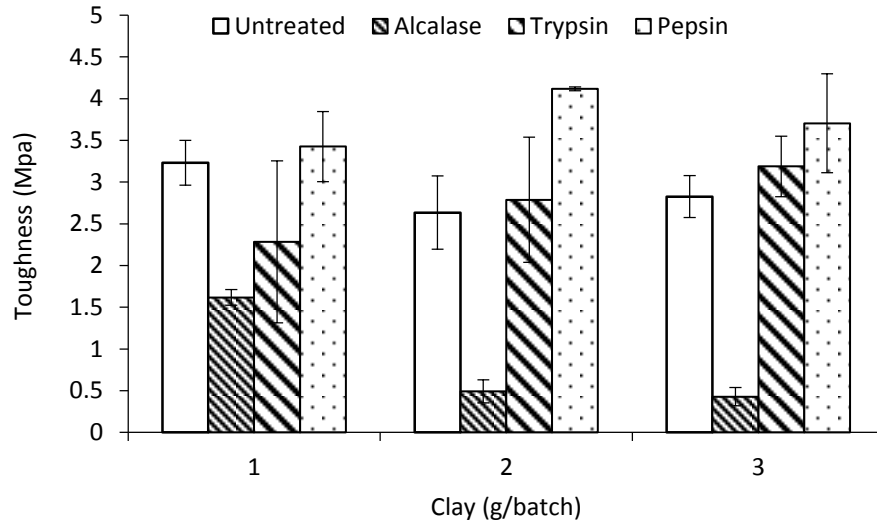


Figure 90 Toughness for tensile specimens of NTP with varying clay

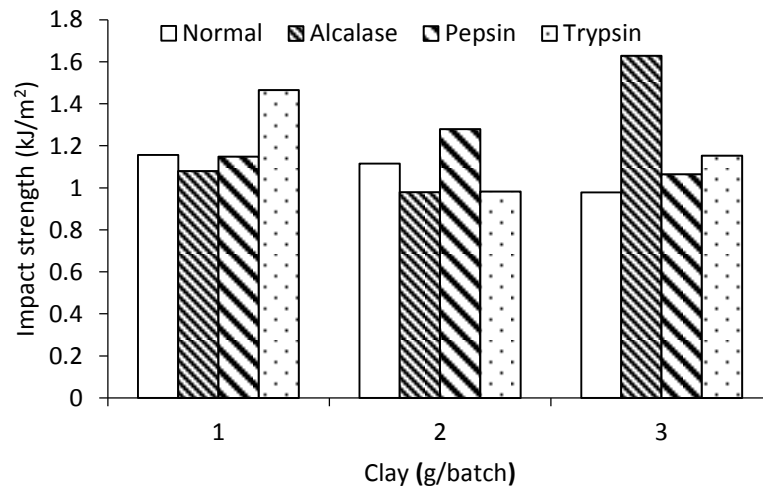


Figure 91 Impact strength for tensile specimens of NTP with varying clay

4.4.6 NTP/hydrolysate thermal properties

4.4.6.1 Glass transition temperature

Glass transition temperature remained constant with increasing clay for all clay types at around 70°C, except for trypsin treated clay, which had the highest T_g at around 83°C. All of the samples had a higher glass transition temperature (T_g) than NTP (61°C) (Shamsuddin 2013) (Figure 92). Clay addition probably was not high enough to make a significant change. A similar trend was reported with bentonite clay treated with stickwater, with little or no difference observed between untreated and treated clay.

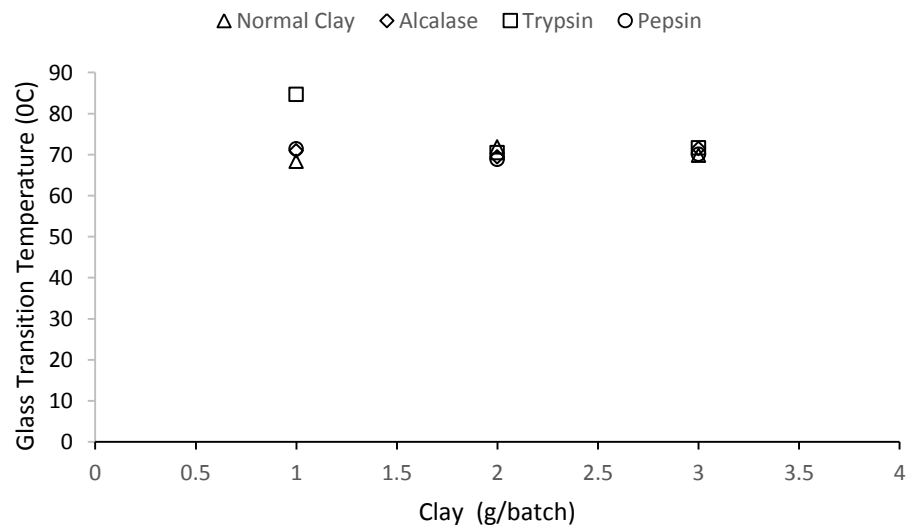


Figure 92 Glass transition temperature in NTP with varying clay

When tensile strength and secant modulus was compared against glass transition temperature for each NTP/clay sample, it appears that both mechanical properties showed little change, although secant modulus was more scattered. The trends observed would be due to the glass transition temperature showing very little change (Figure 93).

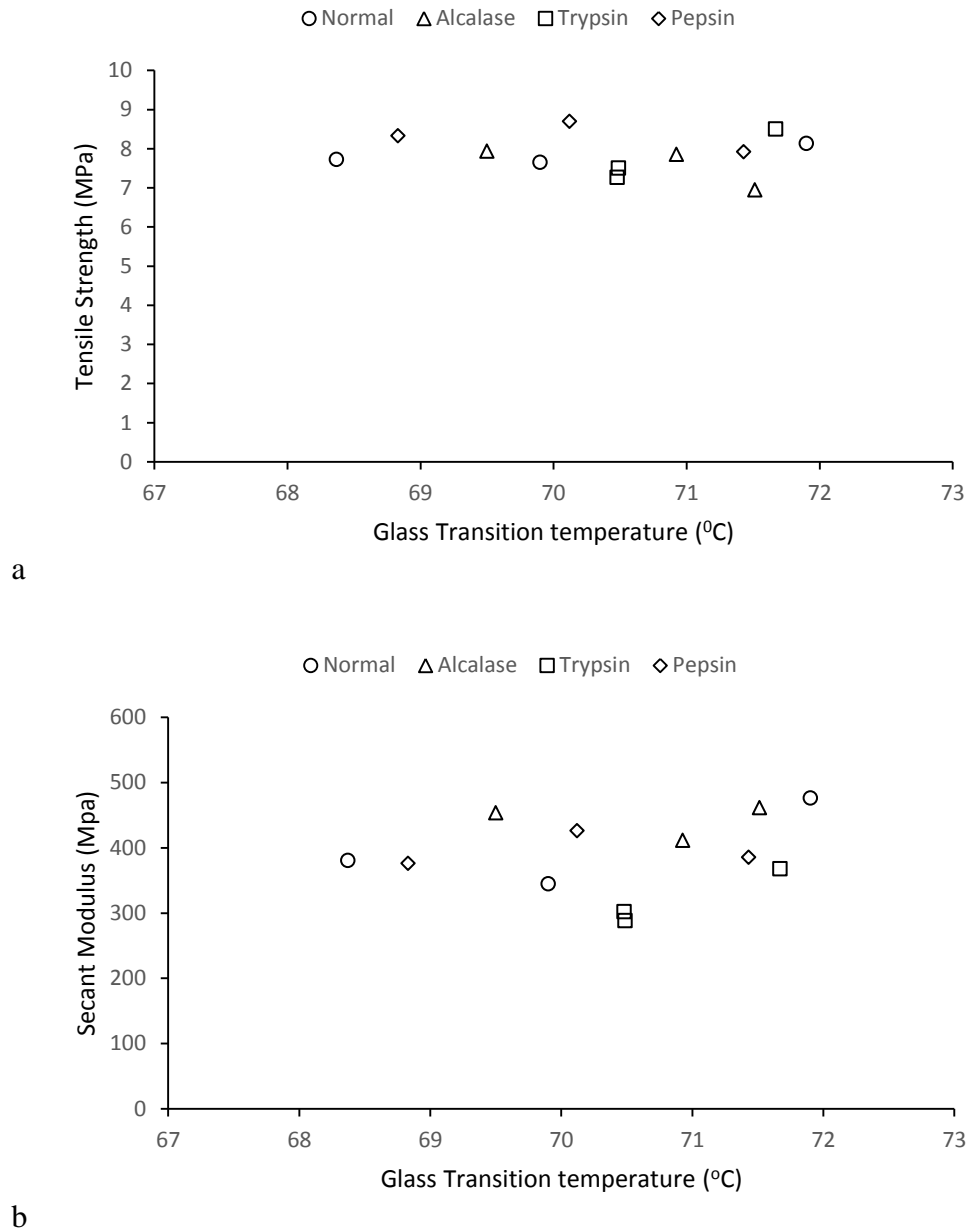
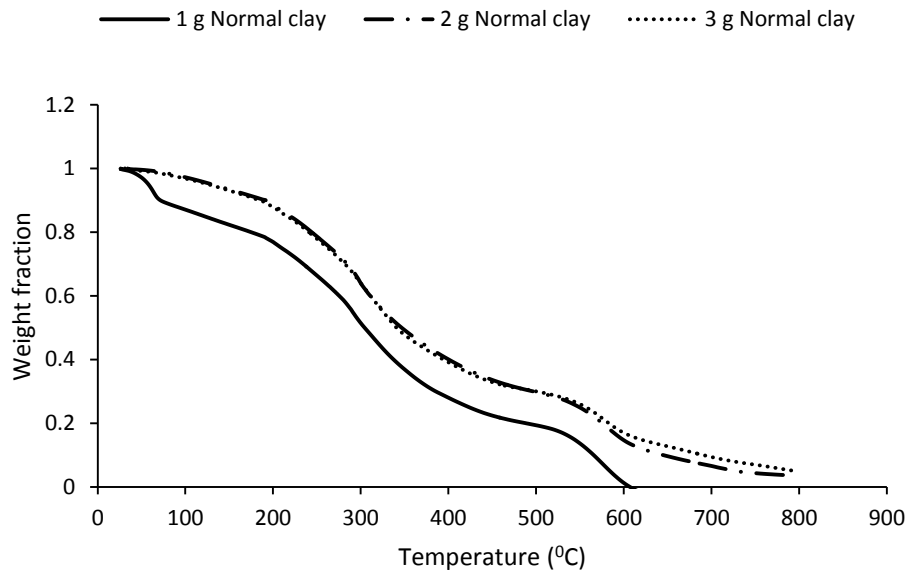


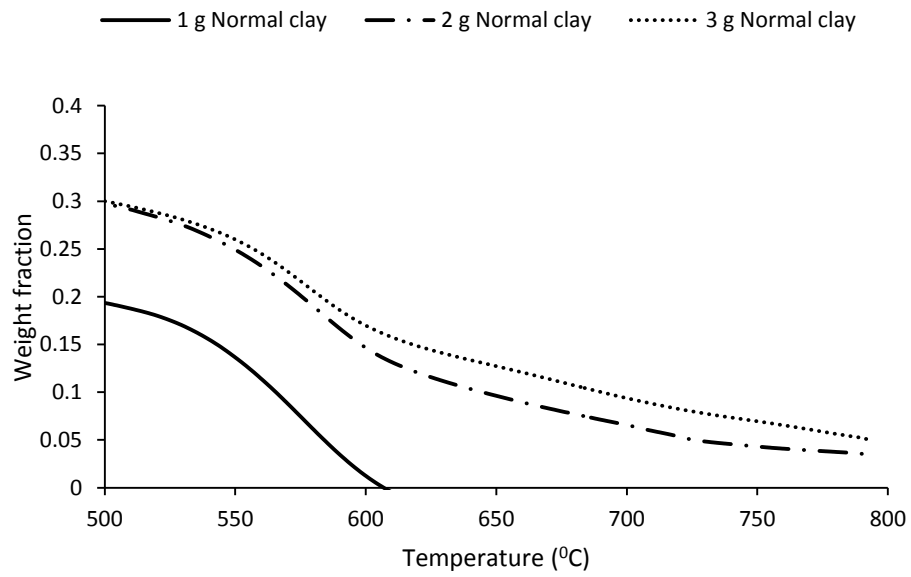
Figure 93 A plot of a) tensile strength and b) secant modulus against glass transition temperatures for NTP the different hydrolysates

4.4.6.2 Thermostability

Increasing clay increased thermal stability of the NTP composites in the 500 to 650°C region, but also resulted in a more rapid decline, suggesting the clay at higher concentrations was accelerating thermal decomposition. There appeared to be little difference in thermostability between the untreated and treated clay types (Figure 94, Figure 95, Figure 96, Figure 97). All samples had a similar ash content of around 4%.

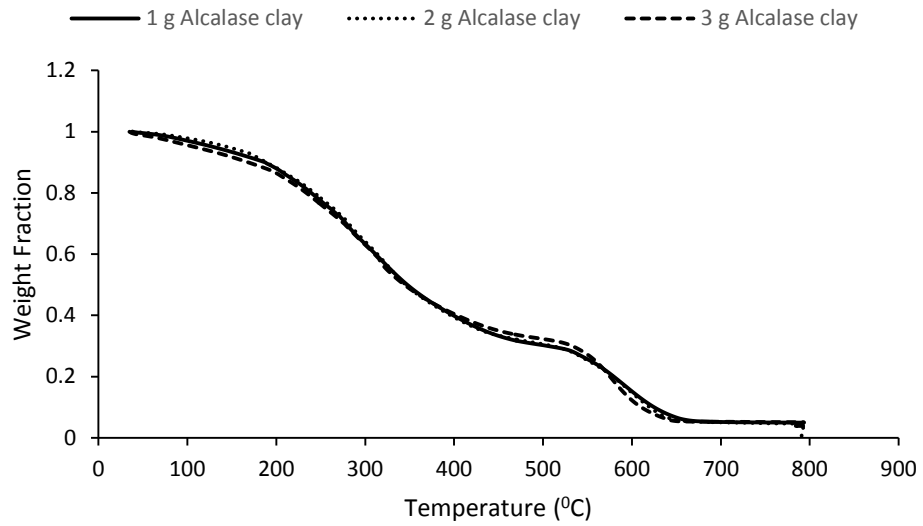


a

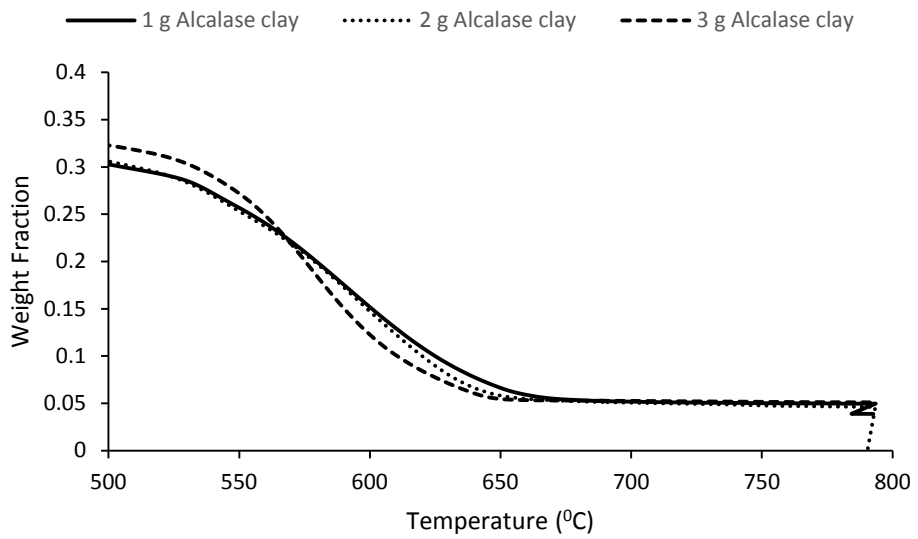


b

Figure 94 TGA of NTP with Normal clay a) over the full temperature range and b) zoomed in on the 500 to 800°C range



a



b

Figure 95 TGA of NTP with Alcalase clay.a) over the full temperature range and b) zoomed in on the 500 to 800°C range

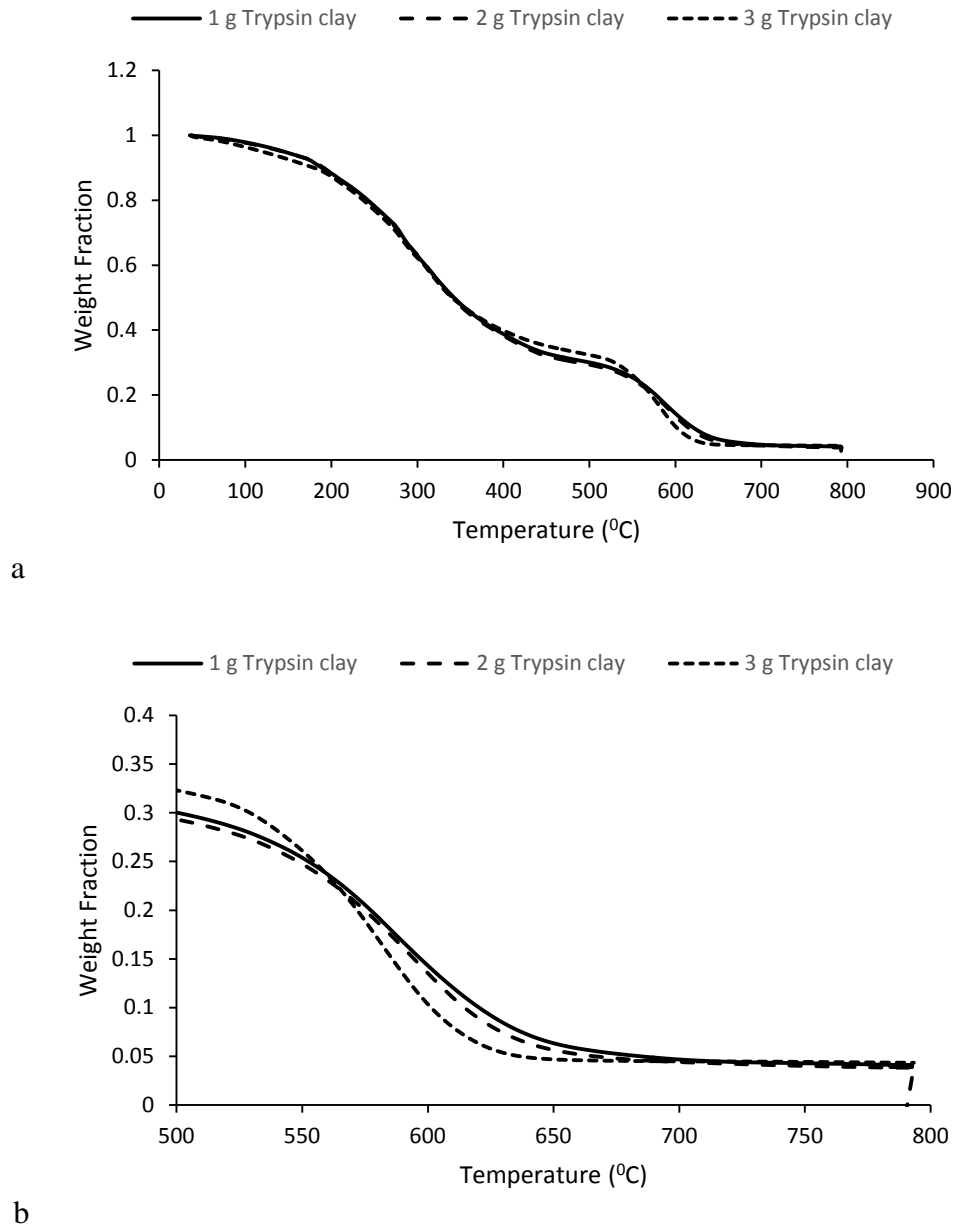
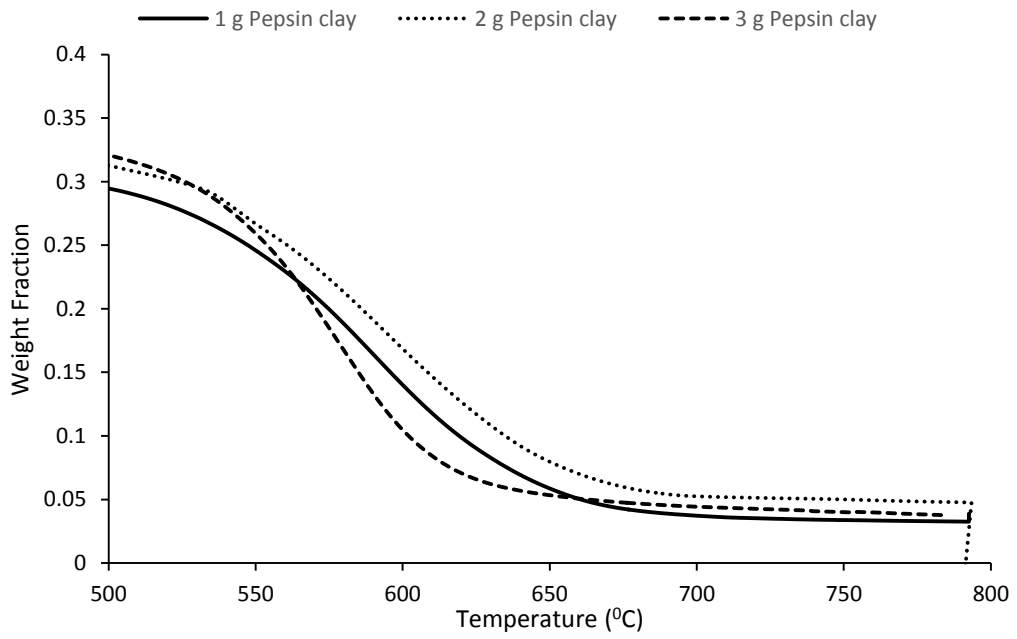
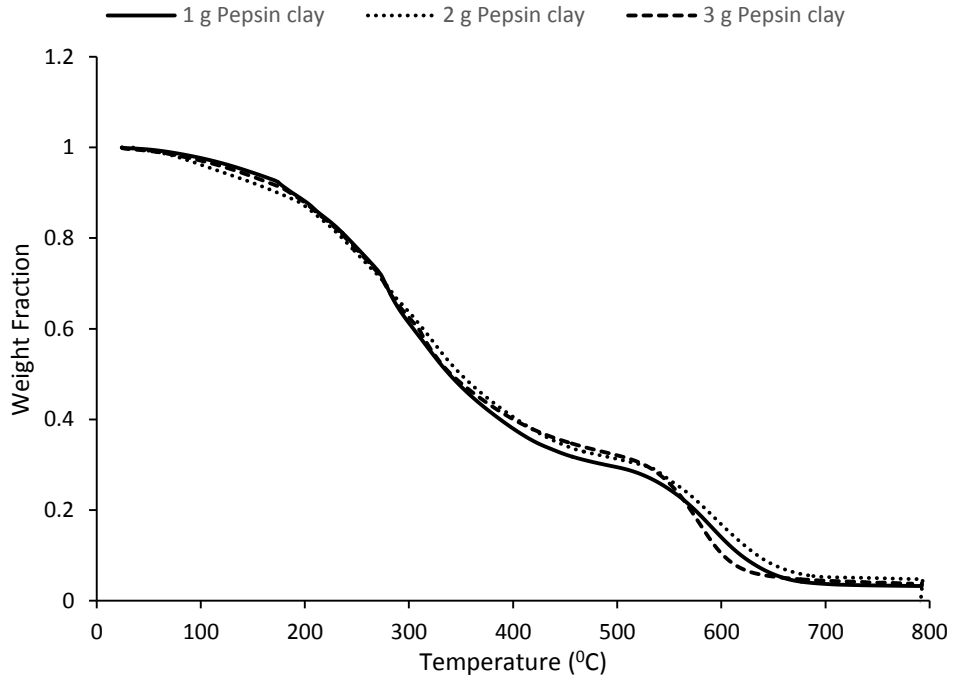


Figure 96 TGA of NTP with trypsin clay.a) over the full temperature range and b) zoomed in on the 500 to 800°C range



b.

Figure 97 TGA of NTP with pepsin clay.a) over the full temperature range and b) zoomed in on the 500 to 800°C range

4.4.6.3 NTP/clay crystallinity

NTP with treated clay generally had a lower crystallinity than NTP with untreated clay (Figure 98), except for 3 g clay per batch where alcalase and trypsin treated clay had a higher crystallinity. All NTP composites had a lower crystallinity compared to normal NTP which has a crystallinity of 26% (Bier et al 2014). This was contrary to what was observed with NTP composites with stickwater and gelatin treated clay where crystallinity in all samples increased (Shamsuddin 2013).

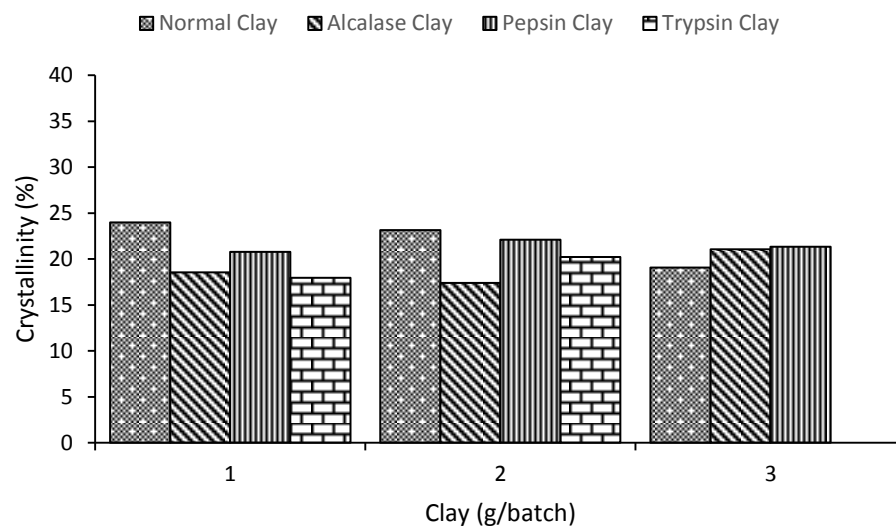


Figure 98 Crystallinity of NTP composites with different clay content and clay type

4.4.7 SEM Analysis

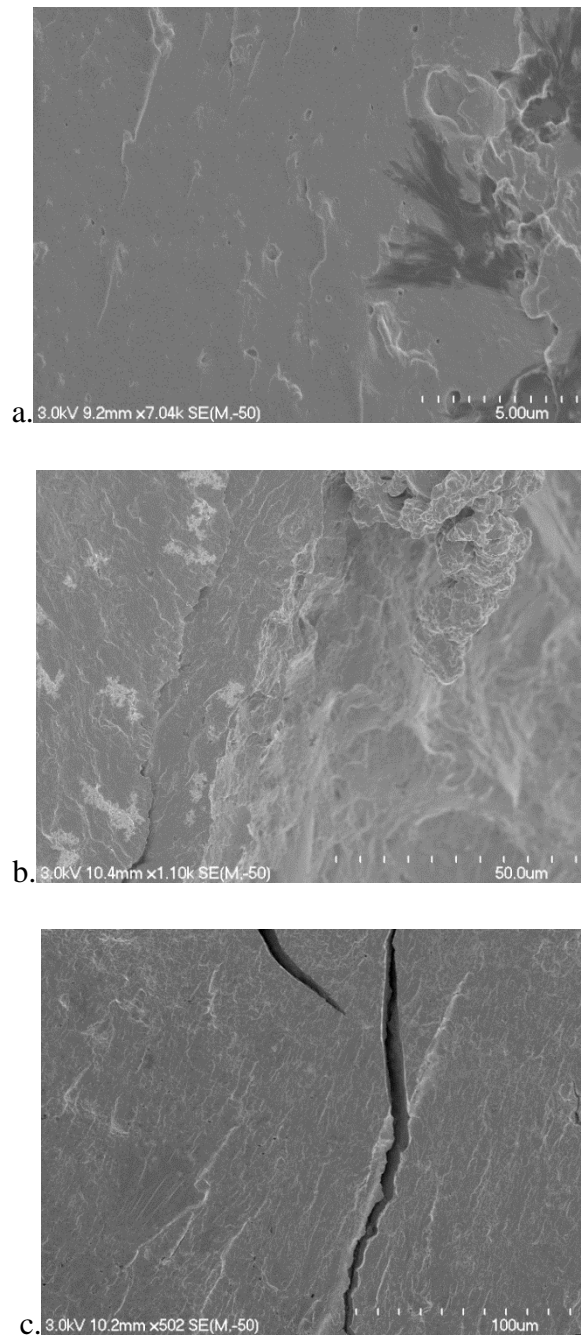


Figure 99 Examples for SEM pics (a. A sample from clay hydrolysate; (b) Sample with trypsin clay; (c. Sample with hydrolysate)

The SEM images for all samples looked the same, and no information could be deduced from the images, except hydrolysate samples showing brittle fractures as seen in Figure 99. All other images for samples were put in the Appendix.

Chapter 5 Conclusions and Recommendations

5.1 Conclusions

The aim of this research was to produce a bloodmeal hydrolysate that could be used in NTP as a substitute for TEG, and also used to treat sodium bentonite clay to improve its properties as a filler in NTP.

Initial enzymatic hydrolysis trials were carried out on bloodmeal using pepsin, alcalase and nitric acid. The following was found:

- Bloodmeal could be readily hydrolysed.
- Pepsin hydrolysis was faster at pH 1.5 than pH 3, taking 12 hours to complete compared to 36 hours for pH 3. Pepsin rate of reaction increased with increase in bloodmeal concentration, but only slightly with increasing pepsin concentration, suggesting pepsin was not saturated with substrate. Degree of hydrolysis was around 20-25% for pH 1.5 and 7-10% for pH 3, hydrolysis yield was between 60-83% for pH 1.5 and average molecular weight of the peptides between 20 to 80 kDa.
- Alcalase hydrolysis was slower than pepsin hydrolysis (at pH 1.5), but degree of hydrolysis was higher at 25-40%, hydrolysis yield between 70-80%, and average molecular weight of the peptides was 2 to 12 kDa.
- Degree of hydrolysis for both pepsin and alcalase increased with increasing enzyme concentration but decreased with increasing bloodmeal concentration.
- XRD analysis on the unhydrolysed bloodmeal showed that crystallinity decreased slightly with increasing enzyme concentration. XRD could not be carried out on the hydrolysates as they were hygroscopic.
- Acid hydrolysis was rapid and completed within 100 to 200 minutes, mass solubilised was around 90% for lower concentrations of bloodmeal down to 40% for higher concentrations of bloodmeal. The resulting hydrolysate could not be easily separated from the unhydrolysed bloodmeal, and required large amounts of base to neutralise.

Large scale hydrolysis was carried out on bloodmeal first with alcalase, then with trypsin and then with pepsin.

- 80% of the bloodmeal was hydrolysed with alcalase giving a hydrolysate solids content of 6.5%, and an average molecular weight of 8.9 kDa for alcalase.

- Trypsin hydrolysate gave a molecular weight of 5.5 kDa while pepsin hydrolysate molecular weight went back up to 8.8 kDa, likely due to the molecular weight of the pepsin added.

Half of each hydrolysate was used for protein adsorption onto sodium bentonite clay at pH 7.

- Alcalase hydrolysate gave the best adsorption at 127 mg protein per g clay and basal spacing of the sodium bentonite increased from 13.2 Å to 14.2 Å.
- Trypsin hydrolysate only gave an adsorption of 46 mg per g clay while pepsin hydrolysate did not adsorb. There was only a slight increase of 0.4 Å in basal spacing for sodium bentonite treated with trypsin hydrolysate.
- The poor adsorption for trypsin and pepsin was due to the salt formed during pH maintenance during hydrolysis and pH adjustment afterwards by adding acid or base.
- Salt would need to be removed from the hydrolysate to improve adsorption onto clay.

NTP was made with hydrolysate as a plasticiser and hydrolysate treated clay as a filler.

- Specific mechanical energy required to extrude NTP with hydrolysate increased with increasing hydrolysate content, and also increased with extent of hydrolysis, i.e. trypsin hydrolysate gave greater SME than alcalase, and pepsin hydrolysate gave greater SME than trypsin, which could be due to the increasing salt content in the hydrolysate.
- Specific mechanical energy required to extrude NTP with modified clay was independent of clay concentration, but increased with extent of hydrolysis, again possibly due to increasing salt content.
- Tensile strength, secant modulus, crystallinity, thermostability and glass transition temperature decreased with increasing hydrolysate content in NTP, likely due to the shorter average protein chain length, indicative of some plasticisation. Glass transition temperature did not change for NTP with alcalase hydrolysate. Toughness, strain at break, and impact strength were low indicating a very brittle material.
- Highly variable results were obtained for the NTP with treated and untreated clay as a filler, but generally gave lower mechanical properties than

conventional NTP. Alcalase treated clay was particularly detrimental on NTP composite strain at break and toughness. Thermostability of the composites increased within the 450-600°C with increasing clay concentration for all treated clays, but showed a much more rapid decrease in mass loss.

5.2 Recommendations for future work

The effect of salt in the hydrolysate on NTP and NTP composite processability and mechanical properties would need to be assessed to determine if it was the salt or protein peptides that contributed to the changes in mechanical properties. The hydrolysate could be desalted using dialysis or nano-filtration and then used in NTP. Alternative hydrolysis techniques could be used such as high pressure and heat, such as using an autoclave.

While hydrolysis gave average molecular weights of peptides around 8 kDa or smaller, the peptides would need to be made much smaller to be of equivalent size to common plasticisers. Hydrolysates could be fractionated to isolate to the smaller molecular weight peptides for use. The order in which hydrolysis is carried out (currently alcalase, trypsin, then pepsin) could be examined as well to see if this would reduce peptide size.

While pH monitoring and adjustment is useful for following the rate of hydrolysis, it contributes to increased salt concentration in the hydrolysate. A strongly buffered solution might help in keeping pH constant while reducing salt formation.

If alcalase hydrolysis is continued on the large scale, hydrolysis could be carried out in the rendering plant after the blood has been coagulated and before it has been centrifuged, as alcalase can operate at 90°C, the same temperature of the coagulated blood solution.

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Appendices

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