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Hydrolysed Protein as a Renewable Plasticiser for Renewable Materials

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TANVI SURATKAR

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Abstract

Novatein Thermoplastic (NTP) is a bloodmeal based plastic developed by the University of Waikato by mixing bloodmeal (by-product of slaughterhouses) with water, sodium dodecyl sulfate, sodium sulfite and urea, and plasticiser so it can be extruded and injection moulded. The aim of this research was to produce a low molecular weight bloodmeal with low salt content to replace tri-ethylene glycol as a plasticiser for NTP.

Bloodmeal was hydrolysed using alcalase, enzyme mixture of chymotrypsin and trypsin, and papain which was later ultrafiltered to obtain low molecular weight peptides of 5 -12 kDa. All the three enzymes provided low degree of hydrolysis while chymotrypsin/trypsin provided high yield of 65% followed by alcalase with 45% yield and papain with 23% yield. The amount of enzyme, bloodmeal concentration and pH governed the rate of hydrolysis. By ultrafiltering the hydrolysate, a low salt content was achieved.

Large scale hydrolysis was carried out to produce hydrolysate for addition in NTP as a substitute for triethylene glycol. The hydrolysate was ultrafiltered to produce peptides of low molecular weight (less than 10 kDa) in permeate and retentate. Extrusion of mixtures with hydrolysate produced extrudates with lower specific mechanical energy than standard NTP, and the extrudate had a good appearance visually. Tensile strength, impact strength, secant modulus, energy at break, and glass transition temperature, and thermal stability generally decreased with increasing the amount of hydrolysate added. A similar trend was observed for its impact strength but overall, the impact strength improved.

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Chapter 1

Introduction

1.1 Background

The role of plastics has increased in human life due to its flexibility and wide range of applications. From simple packaging to complex engineering, plastic products can be seen almost everywhere. Its worldwide production has surpassed that of steel and is still growing. In fact, the production of plastics had increased twenty times than that 50 years ago [1] and was estimated to cross 300 million tons by 2015 [2].

The word ‘plastic’ originated from the Greek word “plastikos” meaning ‘able to be moulded into different shapes’. Chemically, plastics are amorphous, long chain polymer molecules made up of inorganic and organic raw materials like carbon, hydrogen, oxygen, silicon, nitrogen and chloride, along with other materials like additives, stabilizers, colorants, processing aids, etc. [3, 4].

The polymer molecules for conventional plastics normally originated from the petroleum by-products. Plastic production is the second largest application of petroleum, first being energy [2]. Plastics have wide applications and are mainly used in packaging of food, chemicals, pharmaceuticals, cosmetics and detergents due to their availability and low cost and has therefore replaced almost every natural material [3, 4]. These petroplastics have good mechanical performances such as tensile and tear strength, good barrier to oxygen, carbon dioxide, anhydride and aroma compounds, heat sealability and so on [4]. But like a coin has two sides, petroleum plastics, though it has lots of uses, are in fact, hazardous to the environment.

1.2 Petroplastics – A Nuisance

Petroleum plastics are resistant to microbial attacks; this is because since its initial production and use, nature could not produce enzymes that could decompose these materials quickly [3]. At the same time, some plastic packaging materials can be contaminated by foodstuffs and biological substances and thus recycling such materials is not a feasible option [4]. Due to the lack of rapid decomposition, plastic wastes have been accumulating ever since their first use and has been the primary cause of worry. Many landfill sites were being filled with these light wastes. Even in the oceans, there has been accumulation of these petroplastics. The conventional plastics are able to withstand ocean environments and hence take decades to centuries to decompose. During the long term study of 22 years in the Atlantic Ocean, Law, Moret-Ferguson [5] witnessed that the largest plastic sample collected in a single tow (1069 pieces) was almost equivalent to 580,000 pieces' km^{-2}.

1.3 Bioplastics – The Solution

To overcome the environmental problems, substitution for petroplastics has started. These substituted materials originate from renewable, biodegradable or compostable resources, hence the product is known as bio-based plastics, or **bioplastics**. The worldwide production of bioplastics is estimated to increase at an annual rate of up to 30% in near decade to reach around 3.5 million tonnes in 2020 [2].

Many different bioplastics are being synthesised from different renewable sources: starch, cellulose, protein (soy, zein, and animal protein), polyhydroxyalkanoates and poly(lactic acids). Different sources are also being utilised such as modified vegetable oils (polyurethane), bio-ethanol (polyethylene), bio-butanol (polypropylene) and biomass (poly(ethylene terephthalate)) [2].

Biodegradability and compostability are important attributes when synthesizing biopolymers. Biodegradability depends on the chemical structure of the materials; particularly, the type of chemical bond which

determines whether or not and over what time frame the microbes can degrade the material. Compostability explains the time required for composting the disposal of packages in the soil. When biopolymers are degraded, they produce carbon dioxide, water and inorganic compounds without toxic residues [4].

NTP, abbreviated form of Novatein Thermoplastic, is a biopolymer made out of bloodmeal, water and additives like plasticiser, urea, SDS and sodium sulfite. This thermoplastic is a novo plastic produced by Aduro Biopolymers, a joint venture between the Wallace Corporation and the University of Waikato. NTP is biodegradable as well as compostable and loses half its mass after 12 weeks. Due to this compostablility, NTP has potential uses as pegs, plant pots, and in meat processing applications such as pithing rods, weasand clips, and rectal plugs.

Tri ethylene glycol (TEG) is used as a plasticiser for NTP, and is manufactured from ethylene, a petroleum product. While TEG is biodegradable, it mainly comes from a non-renewable resource, therefore the aim of this thesis is to produce a hydrolysate plasticiser with low molecular weight and low salt content so that it can be used in NTP.

Chapter 2

Literature Review

2.1 Introduction

The importance of biopolymers has increased owing to the environmental issues related to the petroleum-based polymers. Several biodegradable plastics are in use such as those derived from natural renewable sources like proteins and also polylactic acid and polyhydroxyalkanoates. This literature review summarises NTP, and elaborates on its backbone which are proteins. It also provides information on some bioplastics made from different sources. Lastly, it gives an insight on the different additives added into the proteins that affects the properties of the final product which is the bioplastic.

2.2 NTP

NTP, or Novatein Thermoplastic, is a biopolymer made out of bloodmeal, water and additives like plasticiser, urea, SDS and sodium sulfite. This thermoplastic is a novo plastic produced by Aduro Biopolymers, a joint venture between the Wallace Corporation and the University of Waikato. NTP is biodegradable as well as compostable losing half its mass after 12 weeks. Due to this compostablility, NTP can be used as pegs, pithing rods, weasand clips, rectal plugs and plant pots.

In an abattoir industry, large amounts of blood is collected which is treated as a by-product [6]. This blood obtained from bovine family contains about 80.9% water, 17.3% protein, 0.23% fat, 0.07% carbohydrate and 0.62% minerals [7]. Using different drying methods either for whole blood (vat or conventional, fast conventional and spray-drying) or for coagulated blood (flash and ring-dried), bloodmeal is produced which is often used as animal feed due to its high protein (86.96 to 100.41%) and high lysine (7.55 to 10.23%) content [6, 8]. Based on the different drying conditions, the amino acid content of the bloodmeal differs by few percentage [8]. The bloodmeal used in this research has been produced by steam coagulation, centrifuging

of whole blood at 90°C which separates the solid products from the water followed by rotary drum drying at 140°C. **Table 1** contains the amino acid contents of dry blood (bloodmeal). Other than animal feed, bloodmeal can also act as a substitute for making plastics due to its high protein content since proteins are long chained biological polymers.

Bloodmeal alone has a glass transition temperature (T_g) of 219.85°C which is too high to be used as a material for thermoplastic. Adding additives like water, urea, sodium sulfite and sodium dodecyl sulphate, the T_g of modified bloodmeal drops from **219.85°C to 80.15°C** at which it can be extruded for plastic processing [9].

Table 1: Amino acid contents of bloodmeal [10]

Amino acid content	Bloodmeal (%CP)
Alanine	7.82
Arginine	4.38-4.91
Asparagine	4.67
Aspartic acid	6.20
Cysteine	0.11-1.92
Glutamine	4.32
Glutamic acid	6.38
Glycine	3.86-4.95
Histidine	5.57-6.61
Isoleucine	1.18-2.54
Isoleucine	11.4-14.8
Lysine	8.25-10.7
Methionine	0.01-1.17
Phenylalanine	5.83-8.20
Proline	6.29
Serine	4.49
Threonine	3.95-7.06
Tryptophan	1.30-3.89
Tyrosine	2.86
Valine	8.21-10.45

%CP = % crude protein

Second to bloodmeal, water is the main constituent in thermoplastics. Water along with polar additives like TEG (triethylene glycol) are often added as a

plasticiser to reduce the intermolecular interactions between the polymer (protein) chains and to increase the flexibility of the overall product [11]. Intermolecular interactions occur due to hydrogen bonding between the protein chains which hold these chains together making it rigid. Urea and sodium dodecylsulfate (SDS) are used to disrupt the hydrogen bonding and hydrophobic interactions while sodium sulfite breaks the disulphide bonds between protein chains [12].

Depending on the amount of these additives, the mechanical properties of NTP varies. For example, NTP having 60 pph_{bm} (parts per hundred bloodmeal) water, 1 pph_{bm} SDS, 1 pph_{bm} sodium sulfite and 20 pph_{bm} urea has a tensile strength of 8 MPa when conditioned to almost 10% moisture while it is 1.4 MPa when unconditioned. Similarly, the Young's modulus is 320 MPa and toughness 1.64 $m^{1/2}$ when conditioned while it is much lower (11 MPa and 0.38 $m^{1/2}$, respectively) when unconditioned [13].

Though bloodmeal can be used to produce plastics, it does have a foul smell and dark colour which lowers its potential applications. The dark colour arises from the heme group present in blood. By chemical oxidative reaction, the heme group in the blood gets degraded producing decoloured and deodoured bloodmeal. Decolourisation and deodorisation of the bloodmeal can be done using hydrogen peroxide or peracetic acid though the latter is more effective than the former [14].

2.3 Proteins

Proteins are a major component of a living cell existing almost everywhere in skin, tissue, organs, blood, hormones and even within cells in the form of receptors and enzymes [15]. For example, blood which is composed of plasma and cellular material, has proteins such as albumins, immunoglobulins, α- and β-globulins and fibrinogen in the plasma component [7]. Proteins constitute more than 50% of the dry weight of the cell. Cells and thus living beings are ultimately highly dependent on proteins to function. The following flow chart highlights the different functions of proteins and hence their importance [16].

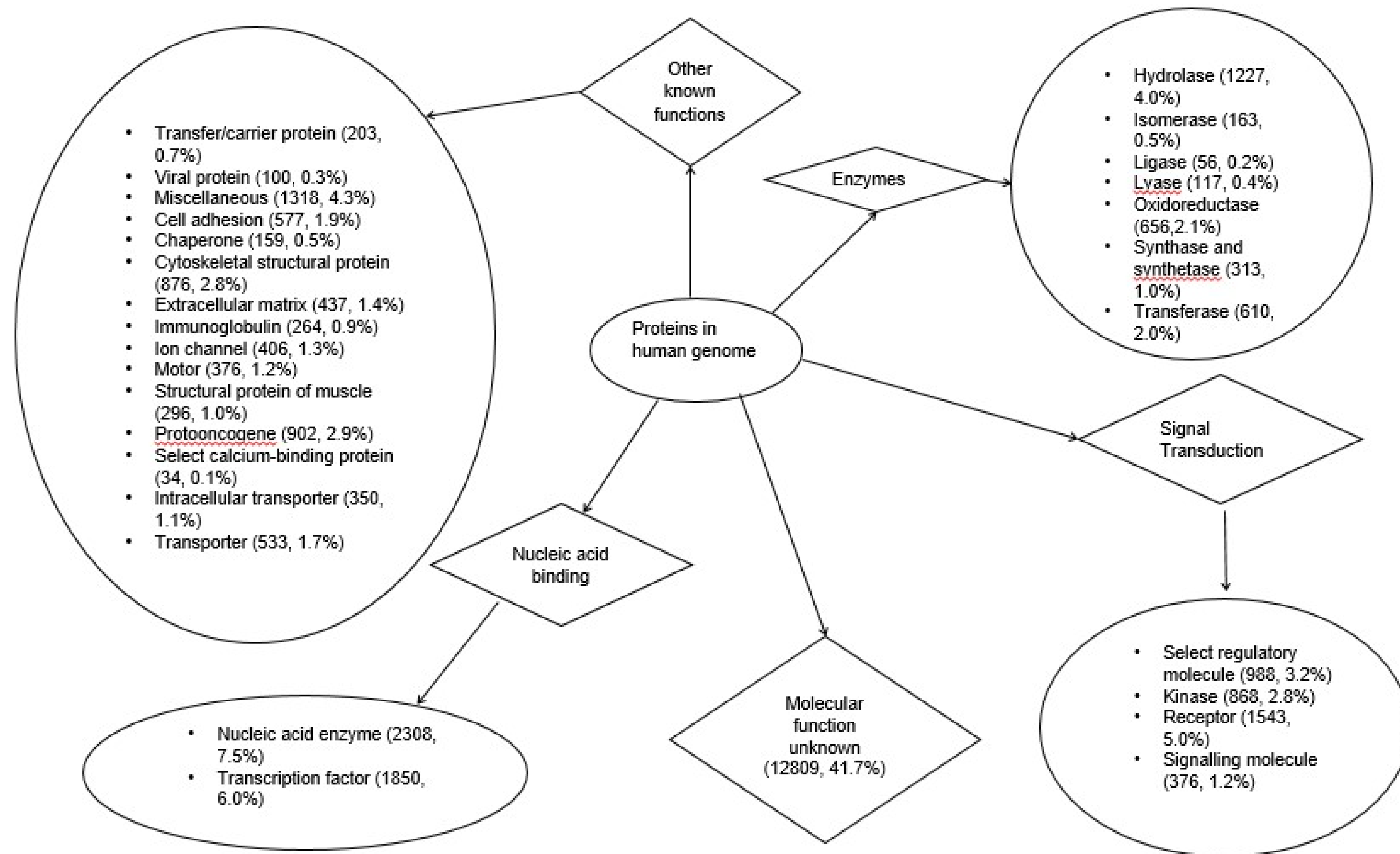


Figure 1: Protein genome, as of 2001 [16]

2.3.1 Structure

The construction of proteins is very complex. A single protein molecule is made up of sequence of smaller molecules called amino acids. In nature, there are about 20 different and important amino acids whose different combinations generate different types of proteins with varying properties. In addition to these 20, there are few post-translationally modified proteins that are equally important for smooth functioning of the cells.

Table 2: Amino acids and their molecular weights [15]

Name	Abbreviations	Molecular weight (g/mol)
Alanine	Ala (A)	89
Asparagine	Asn (N)	132
Cysteine	Cys (C)	121
Glutamine	Gln (Q)	146
Glycine	Gly (G)	75
Isoleucine	Ile (I)	131
Leucine	Leu (L)	131
Methionine	Met (M)	149
Phenylalanine	Phe (F)	165
Proline	Pro (P)	115
Serine	Ser (S)	105
Threonine	Thr (T)	119
Tryptophan	Try (W)	204
Tyrosine	Tyr (Y)	181
Valine	Val (V)	117
Arginine	Arg (R)	174
Histidine	His (H)	155
Lysine	Lys (K)	146
Aspartic acid	Asp (D)	133
Glutamic acid	Glu (E)	147

Table 3: Some less common but essential amino acids

Name	Found in abundance	References
Selenocysteine	Glutathione peroxidase	[17]
Ornithine	Urea cycle	[18]
Hydroxyproline	Collagen	[19]
Hydroxylysine		

The amino acid sequence of a protein is considered to be the simplest sequence hence the **primary structure (1°)**. Though it is the simplest sequence, it is an important structure of a protein molecule since the position of amino acids in the molecule determine its further complex structures. The amino acids in the primary structure are joined together by peptide (amide) bonds – a condensation reaction with an elimination of water molecule [16]. These peptide bonds are enzyme catalysed and are mostly catalysed at the ribosome during translation of mRNA (mature RNA product) [20].

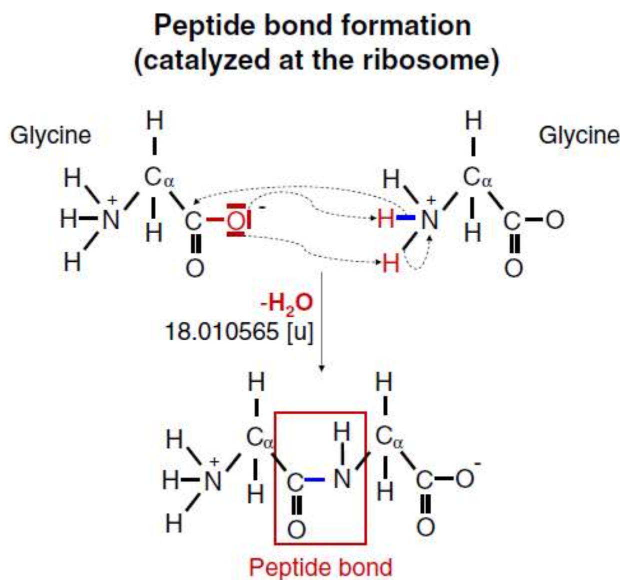


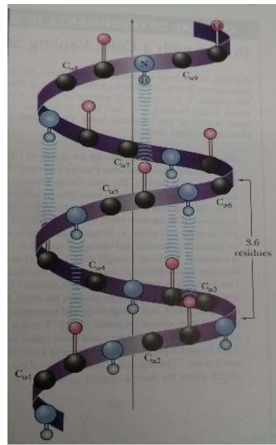
Figure 2: Peptide bond formation [20]

The primary structure of amino acid sequence is able to fold or arrange itself into a specific characteristic segments due to hydrogen bonding between the side chains of the residues; this segments are termed as the **secondary structure (2°)**. The hydrogen bonding between the side chains allows the sequence to take up two conformations: α-helix or β-pleated sheets [16].

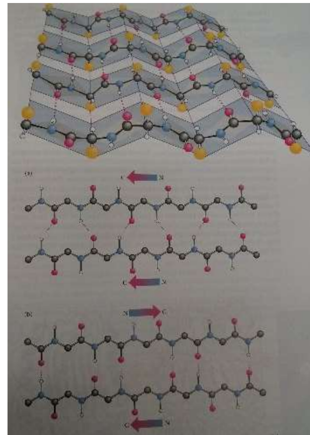
Of the two, *α*-helix is the "key secondary structure" (**Figure 3A)**. Along the helix axis, each amino acid residue covers 1.5 Å (0.15 nm) and within each turn, resides 3.6 amino acids; therefore, each residue travels 5.4 Å (0.54 nm, 3.6 X 1.5 Å) along the helix axis per turn. The total travelling distance is referred to as translation distance or 'pitch' of the helix. To attain overall stability in the helix, the carboxyl terminal of the side chain residues is hydrogen bonded to the N-terminal residues four groups away from the chain [16].

β-pleated sheets (also known the pleated sheet or the β-structure) are observed as a cooperative formation of hydrogen bonds. The peptide strands are H-bonded in such a way that the backbone appears in a zigzag pattern with α-carbons falling at the folds of the pleats. The pleated sheets exist in both parallel and antiparallel forms. Parallel *β*-pleated sheet has their adjacent side chains running in the same directions whereas, the adjacent strands run in the opposite directions in antiparallel pleated sheet (**Figure 3B**). The distance between the residues for parallel sheets is 0.325 nm while that for antiparallel sheets is 0.347 nm. The H-bond thus formed in these structures are interstrand instead of intrastrand.

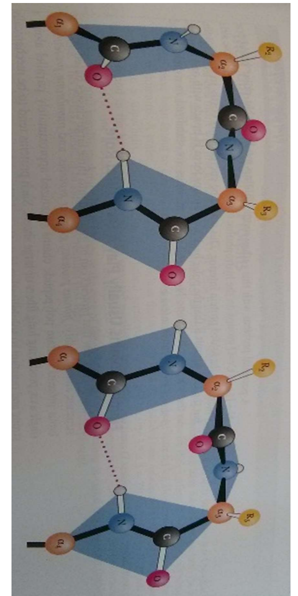
To be able to fold in a compact, globular structure so that it can fit in a cell, some polypeptide chains are required to bend, turn and reorient themselves. Such protein structures are termed as *β*-turn (tight turn or *β*-bend) **(Figure 3C)**. In this type, a tight loop is formed in the peptide chain. This loop arises from hydrogen bonding between carboxyl oxygen of one residue, and amide proton of the residue three positions down the chain. The H-bond between such residues provide stability to the *β*-turn. Two major types of *β*-turns are found in protein molecules namely, type I and type II, though there are few types that are less common but also found in the polypeptide chain. It is the *β*-turn that allows the peptide chain to reverse its direction. Proline, to some extent, also forces the formation of the *β*-turn due to its cyclic structure and fixed φ angle [16].



A. Secondary Structure: α-helix



B. Secondary Structure: β-pleated sheets



C. Secondary Structure: β-turn. Type I (top) and Type II (bottom)

Figure 3: Secondary structure of proteins [16]

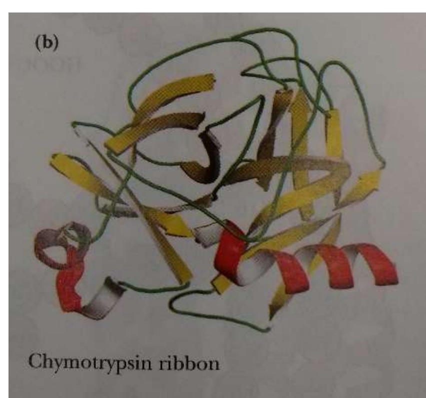
Garrett and Grisham [16] defines **tertiary structure (3°)** (**Figure 4A**) as “the arrangement of all atoms of a single polypeptide chain in three-dimensional space”. It is this arrangement in the chain that helps protein to fold in a compact structure. The polypeptide chain usually contains the mixture of hydrophobic and hydrophilic amino acids, where hydrophilic residues tend to hydrogen bond with water while hydrophobic amino acids tend to be unstable in water. This mixture of hydrophilic and hydrophobic amino acids allows polypeptide chains to form compact structure in such a way that all hydrophobic residues lie on one side of the structure while hydrophilic on the other. The tertiary structure helps in categorizing proteins in three groups: fibrous, globular and membrane proteins.

The polypeptide chains in *fibrous proteins* are organized approximately parallel along the single axis forming long fibres or large sheets that are mechanically strong and resistance to solubilisation. These proteins have a structural role in nature. Examples are collagen and keratin.

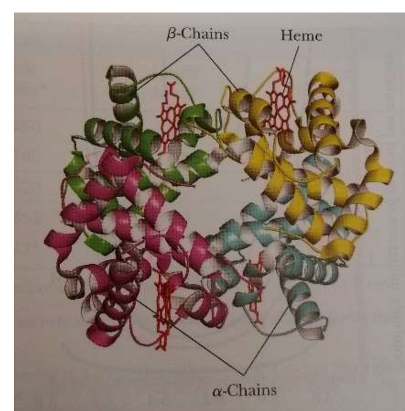
Globular proteins, known for their spherical structure, are most common protein present in nature. The vast number of these proteins provide numerous functions from binding, catalysis and regulation, to transport, immunity, cellular signalling and much more. It is the different structures adopted by the polypeptide chains that provide vast numbers of globular proteins. Example is bovine ribonuclease A.

Membrane proteins are often closely associated with membrane systems of cells. These proteins have their hydrophobic amino acid side chains oriented outward so that nonpolar phase can interact within membranes. On the basis of this, these membrane proteins are insoluble in aqueous solutions but soluble in solutions of detergents. Membrane proteins normally have few hydrophilic amino acids.

Tertiary structure is mostly one of the constituents in a protein molecule, termed as a subunit of the protein. Two or more subunits interact together to form a more complex structure called **quaternary structure (4°)**. Protein molecules containing this structure are normally termed as oligomers. Oligomers contains two or more subunits whereas polymer contain many subunits, all folded in quaternary structure. Proteins found in quaternary structure are often seem to be symmetrical. The most common example of quaternary structure is haemoglobin (**Figure 4B**) [16].



A. Tertiary Structure of chymotrypsin



B. Quaternary Structure of haemoglobin containing two α- and two β-chains

Figure 4: Tertiary and quaternary structures of protein, respectively [16]

2.3.2 Interactions

The primary, secondary, tertiary and quaternary structures of protein are able to form due to certain chemical interactions between the amino acid residues. These interactions help the residues in the peptide bond to achieve more complex higher conformations. Interactions such as covalent bonding, hydrogen bonding, hydrophobic interactions, ionic interactions and van der Waals forces help polypeptides at different levels to achieve desired and stable conformation [16].

The most common type of bonding interactions in any polypeptides are **covalent bonds** which include peptide bonds, joining two amino acids together (**Figure 2**) and disulphide bonds. The latter type of bonding interaction is amino acid specific and occurs between two cysteines in the endoplasmic reticulum of the cell, due to its oxidative environment, either co- or post-translationally. Disulphide bonds or disulphide bridges are catalysed by protein disulphide isomerase, enzyme that catalyses disulphide bonds from either the reduced form or randomly joined disulphides. The formation of these bonds are due to thiol/disulphide exchange where a thiolate anion of one cysteine reacts when a disulphide bond is close to it [21]. The following figure (**Figure 5**) illustrates this reaction.

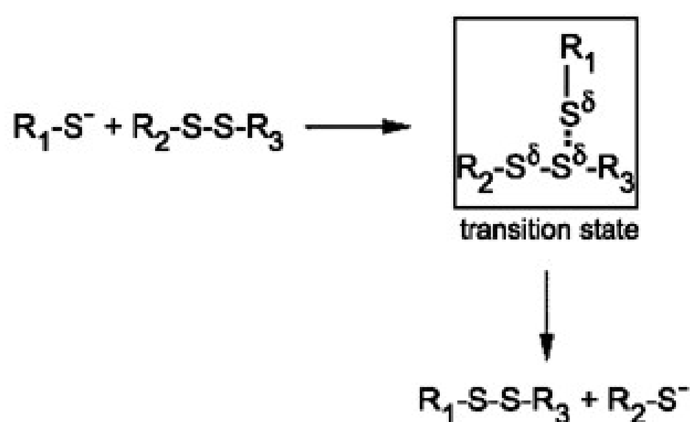


Figure 5: Disulphide bonding between cysteines [21]

The second simplest interaction, other than peptide bonds, that occurs within the protein structure is **hydrogen bonding**. As the name suggests, hydrogen bonding forms a bond of hydrogen atom between two electronegative atoms (nitrogen or oxygen) of amino acid side chains. Being

a simplest form of interaction, it is the most common interaction found in protein structures and their environment. It is due to this hydrogen bonding that *α*-helices and *β*-sheets are able to form along with other complex structures [16]. Hydrogen bonding is also a major contributor of interactions occurring in a polymer. Polymer, both synthetic and natural, are composed of many -OH and -NH groups which result in intermolecular and intramolecular interactions between plasticisers and polymer. The strength of the hydrogen bonding between any polymer depends on the distance between the H bonds and its effect on plasticisation. The more closely the H bonds are in the polymer, the less plasticiser will penetrate within the chains and the more distance there is between polymer chains, the easier it will be for plasticiser to penetrate through the chains [22].

Hydrophobic interactions between nonpolar side chains and nonpolar solutes occur to avoid interactions with polar solvent such as water. As the name suggests, the protein side-chains containing hydrophobic R groups tend to be unstable in water and thus forms a cluster of hydrophobic amino acids in order to minimise its interaction with water. The cluster formation is driven by entropy and is the main stimulus for protein folding [16].

Ionic interactions are either electrostatic interactions between opposite charges or repulsions between like charges. There are few amino acids that are either positively charged like lysine, arginine and histidine or are negatively charged such as aspartate and glutamate. Along with these amino acids, the N-terminal and C-terminal residues of a protein or polypeptide chain also carry a charge (positive and negative, respectively) due to them being in an ionized state. The charges on the side chain of these residues allows the polypeptide chains to take up a particular structure. In a given structure, the charged residues are normally situated on the protein surface so that they can interact with water molecules [16].

Van der Waals forces are found almost everywhere just like hydrogen bonds. These interactions include both attractive and repulsive forces, with attractive forces forming as a result of instantaneous dipole-induced dipole interactions that arise due to fluctuations in the electron charge. Fluctuations

occur because the electron charge is distributed with the adjacent non-bonded atoms [16].

2.3.3 Post-translational modification and its effect

In a cell, once a gene has been transcripted into RNA, the final RNA produced (known as mRNA) is translated into chains of amino acids for its functions (**Table 2**). During translation, certain amino acids get modified either co- or post-translationally resulting in specific but unique amino acids (other than 20 common amino acids; total number of amino acids that occur naturally are 300 [20]). These post-translational modifications have a great impact on the structure and thus, on the functions of the protein. One such example is collagen.

Collagen is the main constituent of bone, skin, cartilage, tendons and tooth and is mostly found in triple helical structure. Collagen is translated from mRNA as a precursor procollagen having additional peptide chains that solubilises the molecule for transportation out of the cell to its destination and to avoid unwanted fibril formation. Once the final protein has reached its destination, these additional chains are removed providing final product – collagen. Collagen undergoes many post-translational modifications forming hydroxyproline, hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine. These modifications are enzyme-catalysed and are catalysed by the enzymes prolyl hydroxylase, lysyl hydroxylase, galactosyltransferase and glucosyltransferase, respectively. The former two modifications occur on the amino acids proline and lysine respectively, while the latter two modifications occur after the lysine has been hydroxylated [19].

Hydroxyproline is the one of three important amino acids other than glycine and proline that provides structure of collagen and is found in all types of collagen. Though it is important for the structure, hydroxyproline is not directly incorporated into the amino acid chains of collagen. It is the proline on the main chain that gets hydroxylated forming either 3-hydroxyproline or 4-hydroxyproline [19]. Hydroxylysine plays an important role in cross-linking

of collagen fibrils and is found in abundance in type II human collagen as compared to other two collagen types, types I and III [23]. Both the enzymes prolyl hydroxylase and lysyl hydroxylase has similar cofactors, co-substrates and reaction mechanism. After lysine has been hydroxylated, the hydroxylysine undergoes further modification forming galactosylhydroxylysine through transfer of galactose from UDP-galactose to hydroxylysine and, glucosylgalactosylhydroxylysine through transfer of glucose from UDP-glucose to galactosylhydroxylysine [19].

Though most of these protein modifications occur enzymatically, there are few amino acids or proteins that get altered without the use of enzymes. Such alterations are known as non-enzymatic modification and include glycation, acylation of sulphur atom in cysteine, tyrosine nitration, deamidation of asparagine and glutamine to name a few. Most of these modifications are a result of reactive oxygen species present in the environment [20]. In a human cell, non-enzymatic modifications of amino acids, in particular proteins, have a desired consequences resulting in neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease [24].

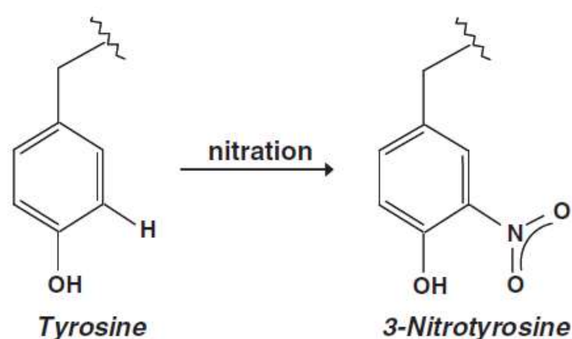


Figure 6: Tyrosine Nitration, example of non-enzymatic modification [20]

Of these non-enzymatic modification, glycation occurring in a cell is of major concern especially in diabetic patients. Glycation has two types – Maillard reaction and caramelisation, of which Maillard reaction plays an important role especially in food. The chemistry of Maillard reaction is complex and involves many end products depending on the physiological pH. The reaction mostly occurs between a reducing sugar and a free amino group of amino acid or protein forming N-substituted glycosilamine that rearranges

to form the Amadori rearrangement product (ARP) whose degradation depends on the pH of the system. The following chart summarises the different end-products obtained through Maillard reaction [25].

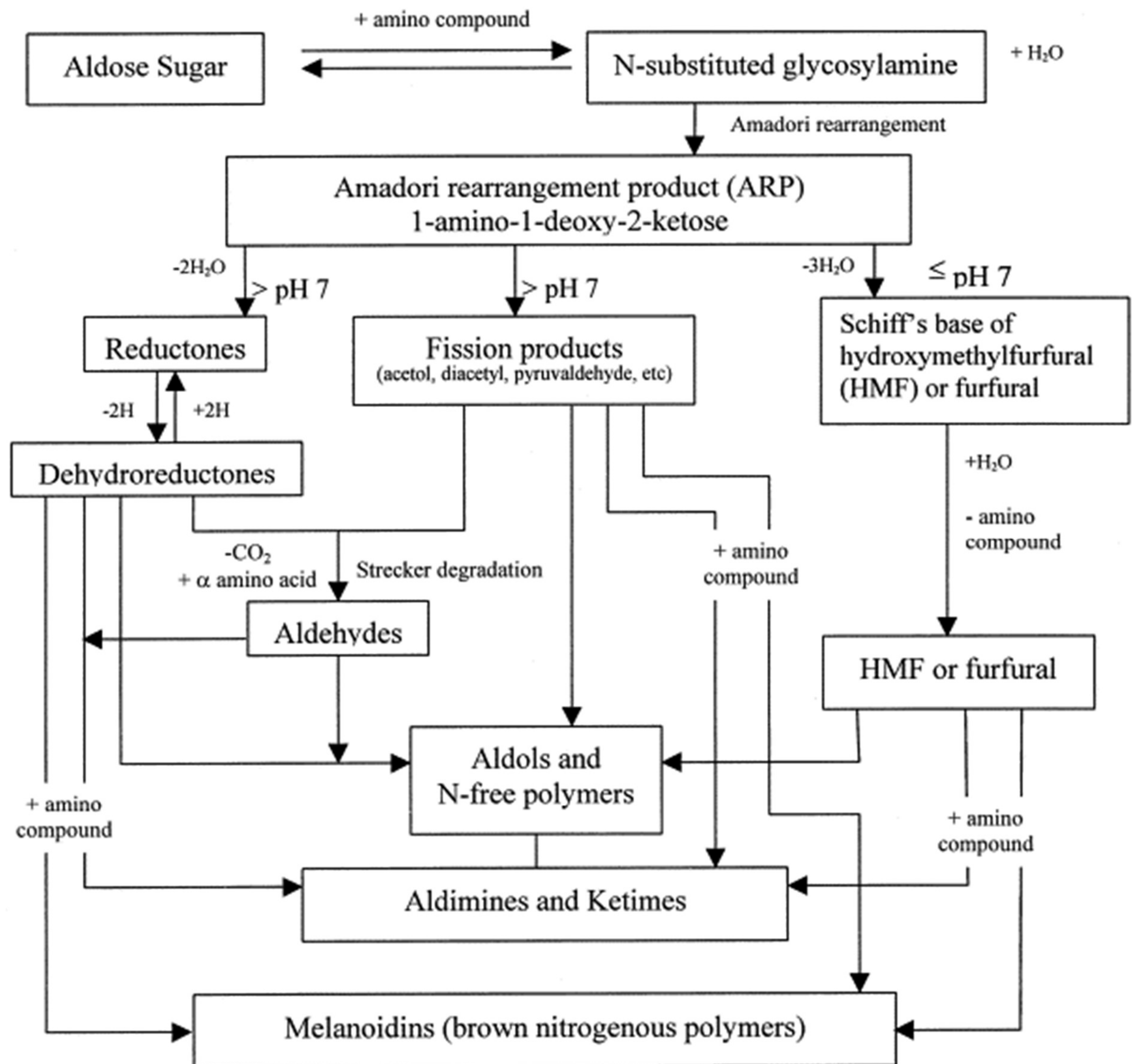


Figure 7: Maillard reaction and its end products [25]

2.3.4 Protein Hydrolysis

As mentioned above, proteins have wide range of applications – from the source of energy to transporting molecules into and out of the cell/membrane. Proteins, or smaller fragments of polypeptide chain, has its applications in biochemistry, food science, microbiology, clinical and diagnostic studies [26]. Proteins are a useful biomarker in detecting various disorders and diseases like cancer. The amino acid composition of the small peptide fragments is typically required for analysis and nutritive value of

food. These peptide fragments are only possible through hydrolysis. Hydrolysis, which means breakdown of matter releasing water, can be done using enzymes, acid or base with acid hydrolysis being the most common process for obtaining fragments followed by alkaline hydrolysis. Breakdown of molecules by enzymes is a seldom applied method due to the high cost of enzymes required for hydrolysis. In recent years, research has been done to hydrolyse biomolecules through the use of microwave irradiation, heat and even water at subcritical temperature.

While selecting biological means for hydrolysis, many enzymes have been looked through especially the ones which are highly active at neutral pH. Enzymes like pronase, chymotrypsin and papain were found to be highly active around neutral pH.

2.3.4.1 Pronase

Pronase (molecular mass between 15 and 27 kDa) is the mixture of different proteolytic enzymes produced by the bacterium *Streptomyces griseus* K-1. Being a mixture, this enzyme is known to contain at least ten proteolytic components – five serine-type proteases, two Zn^{2+} endopeptidases, two Zn^{2+}-leucine aminopeptidases and one Zn^{2+} carboxypeptidase. Due these different proteolytic components, pronase has very broad enzyme specificity and is normally the choice of enzyme when complete degradation of protein is required. The optimal pH activity of pronase is at pH 7-8 but individual components of the enzyme has shown activity over wide pH ranges. The enzyme also has an optimal temperature at 40°C and its specific activity can reach up to about 7000 PU/g (protease units per gram). Due to this high enzyme activity and broad specificity, pronase can hydrolyse many peptide, amide and ester bonds [27].

2.3.4.2 Chymotrypsin

A digestive enzyme, chymotrypsin is a 25kDa serine protease that is found in the pancreas [28]. Being a digestive enzyme, chymotrypsin helps in catalysing peptide bonds present in proteins. The enzyme has a specificity for aromatic amino acids (tyrosine, phenylalanine or tryptophan) and leucine

present at the C-terminal [29, 30]. It can also hydrolyse peptide bonds within other amino acids as well but to a lesser extent [29].

The synthesis of chymotrypsin is in the form of long single polypeptide chain of 245 amino acids called chymotrypsinogen. Chymotrypsinogen is inactive and its activation to chymotrypsin is a complex process containing three steps: initial cleavage by trypsin (forming π-chymotrypsin) and subsequent two cleavages by chymotrypsin itself (δ-chymotrypsin and α-chymotrypsin). Of these three chains, α-chymotrypsin is the active molecule of the enzyme [29, 30]. Production of inactive chymotrypsinogen reduces the premature hydrolysis of chymotrypsin as well as other enzymes [29]. Though the activation of chymotrypsin results in three polypeptide chains, these chains are strongly held together by disulphide bonds.

The mechanism of chymotrypsin (**Figure 8**) is complex. It is the first enzyme that has been studied extensively and thus often serves as a model for other enzymes in general. The optimum pH of this enzyme is in the range of 7.5 and 9.0 with temperature optimal at 50°C [28, 29]. Being a serine protease, the enzyme has serine (Ser 195) at its active site. Serine often works with two other amino acids, histidine (His 57) and aspartic acid (Asp 102), forming a catalytic triad.

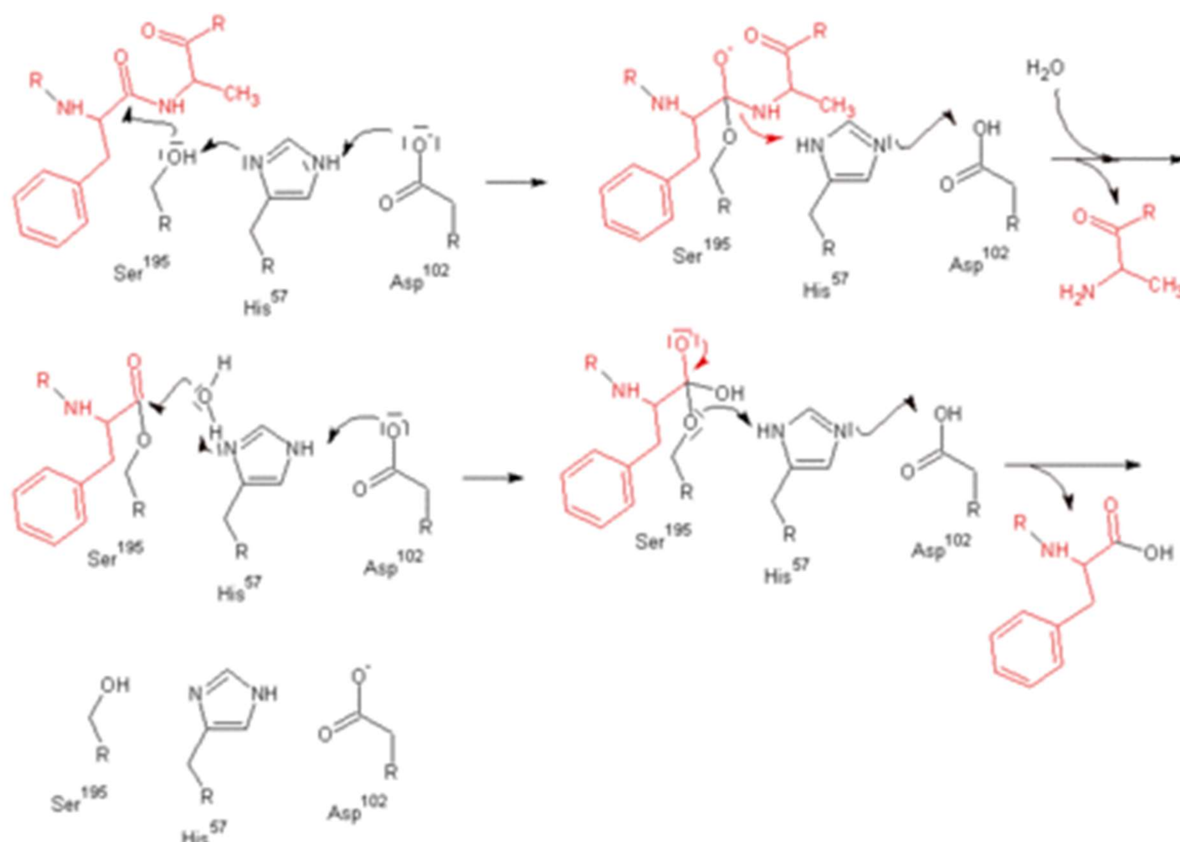


Figure 8: Chymotrypsin mechanism [31]

2.3.4.3 Papain

A 23.4 kDa cysteine protease from the papaya latex, papain has its use as a meat tenderizer. Papain can act as an endopeptidase, exopeptidase, amidase and esterase which explain its use in tenderizing meat. Due to this broad specificity, it is one of the most used enzyme in the food industry. It cleaves basic amino acids, glycine and leucine residues but prefers amino acids with hydrophobic side chains more [28, 30].

Papain is a simple enzyme having 212 amino acid residues with majority of these residues being hydrophobic. The activity of this enzyme lies between pH 6.0 and 7.0 whereas its optimum temperature is at 65°C. At the active site of the papain lies three amino acid residues which helps in the catalysis: cysteine (Cys 25), histidine (His 159) and arginine (Arg 175). Arginine is not involved in the catalysis of the substrates but is important in keeping the histidine in its stabilized imidazole form. Cys 25 and His 159 perform the actual catalysis in the following manner [28, 30]:

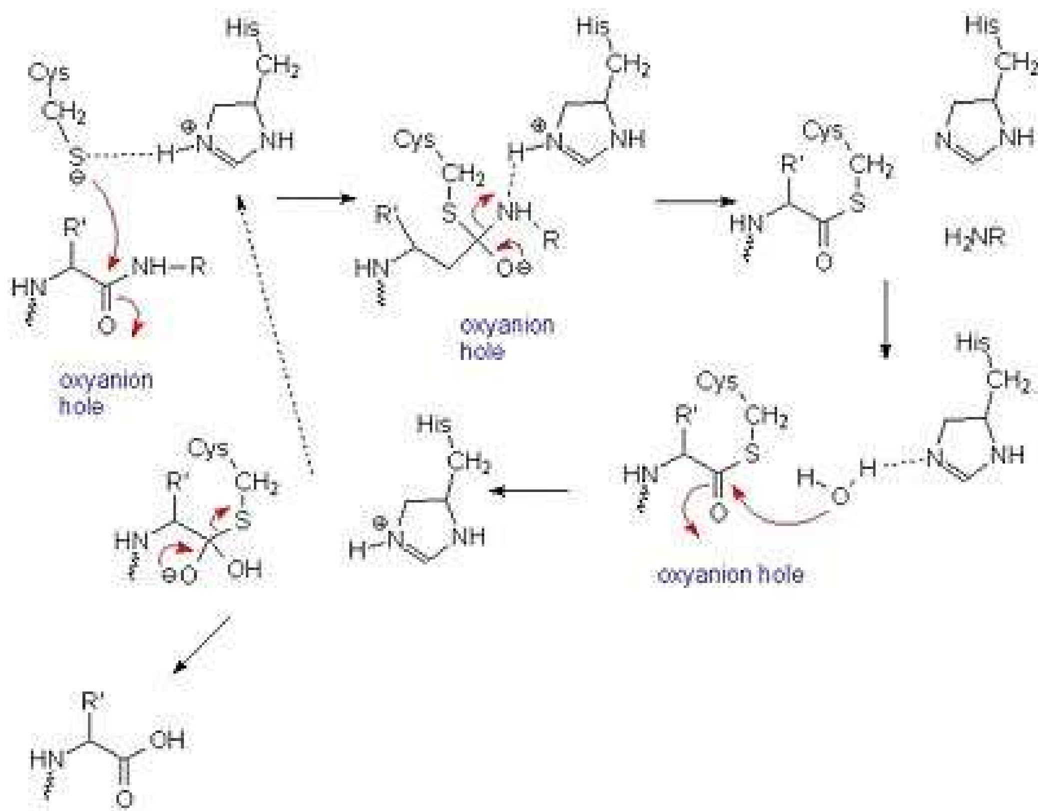


Figure 9: Papain mechanism [30]

2.3.5 Proteins used for plastics

Though plastic made out of bloodmeal is a fairly new approach, plastics made out of proteins is not new. The protein and protein hydrolysate contain active functional groups on the side chain of the amino acids and the end group of each protein chains: amines (-NH_2), carboxyls (-COOH), sulfhydryls (-SH), hydroxyl (-OH) and carbonyls (-CHO). These abundant functional groups are the one that modifies to produce marketable, high-value plastics and biopolymeric materials [32].

Plastics have been successfully made from soy protein [33], whey, casein, gluten, zein, keratin, ovalbumin and many more [34]. Films are also been made from proteins which are obtained from waste products after filleting the fish [35]. Along with proteins, starch [36] and cellulose are also used to make films [34].

The biopolymers are formed by either of the two processes: wet process or dry process. In the wet process, the biopolymer is dispersed or solubilised

in a film-forming solution, called solution-casting, which is later followed by evaporation of the solvent [15]. Solution-casting is the oldest form of biopolymer producing technology and was used initially to produce films and images in photographic industry [37]. In solution-casted protein bioplastics, proteins act as barrier for polymers having oxygen permeability and grease and as a nutritional source for taste [38].

Dry process, on the other hand, depends on the thermoplastic behaviour of proteins and polysaccharides that is being displayed at low moisture contents in compression moulding and extrusion. It is the choice of drying process that commands the type and extent at which the protein thermoplastics can be modified physically and chemically [15].

Extrusion: One of the oldest form of biopolymer processor, other than solution-casting, extrusion has been in the industry of plastics since the 1930's. It converts plastics raw materials into products of uniform density and shape by pushing it through die under controlled conditions [39]. An extruder contains of a hopper, a heated barrel having either a single or twin screw, and a die. Granulated or powdered raw materials are fed into the hopper which are passed through the heated barrel having screws to melt the material later through the die for uniformity. Conditions such as temperature profile, feed rate, screw speed and configuration, screw length-to-diameter ratio and die size determines the thermomechanical properties of the extruded product [38, 40].

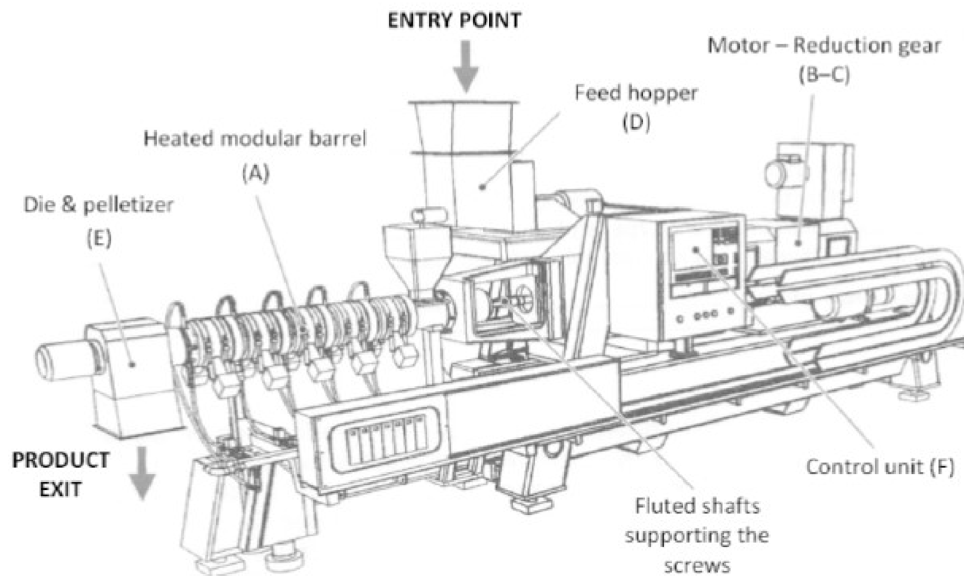


Figure 10: Schematic representation of co-rotating twin screw extruder [40]

Depending on the use, extrusion of the material can be done by different processes. **Cold extrusion** involves gentle mixing of the raw material in the extruder barrel at room temperature without applying any heat. **Hot extrusion**, on the other hand, involves transforming the raw material into the desired product by applying heat and pressure into the barrel. In **steam-induced expansion**, the products get expanded due to water flashing off at the die exit and in **expanded co-extrusion** steam-induced expansion gets combined with filling injection due to which the expanded products get more textures [41].

Injection moulding: Once the product has been extruded for uniformity, it is moulded into desired shape and size by passing it through injection moulding. It is a fast process and follows same procedure as extrusion with the exception of having mould for injection moulding instead of die for extrusion [38, 42]. Injection moulding process has six main stages:

Stage 1: Clamping where the mould is clamped under pressure;

Stage 2: Injection where the granules are fed into the hopper and injected into the barrel where the material is heated to melting point;

Stage 3: Dwelling where the hydraulic pressure is applied to the molten plastic in the mould;

Stage 4: Cooling where the molten plastic is cooled inside the mould;

Stage 5: Mould opening where the two clamps of the mould are opened exposing the final product; and,

Stage 6: Ejection where the final moulded product is ejected [42]

Figure 11: Injection moulding machine [43]

Compression moulding: In this process, the granulated or powdered plastic raw material is heated in a mould to give desired shape of that mould by applying pressure to that plastic material. Because of its high volume and pressure, this moulding method can be used to mould complex, high strength fibreglass reinforcements [42].

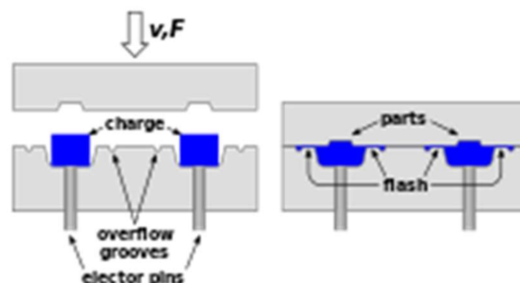


Figure 12: Compression moulding [44]

Compression moulding consists of five main elements all of which are controlled by a control unit. The machine has a high speed press controlled by a hydraulic alignment, a heating and cooling system for the mould, an extruder or plasticising unit to process un- and reinforced thermoplastics, a mould unit and a transfer unit to put the plasticising material into the mould [42].

2.3.6 Use of hydrolysates in producing protein plastics

Dhara and Bhargava [45] had successfully used egg white as a binder for gelcasting (near net shape ceramic forming process) of ceramics. Binders are important additives in slurries or mixers used for ceramic making. Presence of binders, especially organic binders, influence the surface charge on particles, foaming and foam stability, highest attainable solids loading, green strength and dimensional control. During ceramic forming, processes using these organic binders in small amounts are acceptable since they provide components with high green density. In other literature using ovalbumin hydrolysate plasticiser in ceramics [46], similar results were observed where alumina suspensions blended with these hydrolysate provided easily mouldable green bodies.

Protein hydrolysate from chrome-tanned leather waste was successfully blended in synthetic polymer, metallocene-based linear-low-density polyethylene (mLLDPE) which enhanced the properties of the synthetic polymer [47]. In 2013, Pei et al. [48] had successfully prepared cellulose/collagen hydrolysate films combining the properties of cellulose and collagen to obtain good compatible biomaterial.

Mekonnen, Mussone [32] produced thermosets by using hydrolysed protein as a curing agent in the diglycidyl ether of bisphenol A (DGEBA) epoxy resin. The protein used as a curing agent was obtained from SRM (specified risk material), proteins that are likely to get attacked by prion proteins (agents that caused BSE (bovine spongiform encephalopathy)).

2.4 Properties of plastic

Though plastics made from biodegradable natural sources are been known for quite a while, their mechanical properties are somewhat inferior to the petroleum-based plastics. The mechanical properties of bioplastics are strongly influenced by the presence of water (known as moisture content) and the type and amount of plasticisers added in the polymer mixture [12]. The influence is generally due to the hydrophilicity generated by inter- and intra-molecular forces between the proteins, water and plasticisers.

2.4.1 Plasticisers

Plasticisers are an important class of low molecular weight compounds in polymer industries. These compounds are non-volatile and generally used as additives in manufacture of plastics [34].

The council of IUPAC (International Union of Pure and Applied Chemistry) defines a plasticiser/plasticizer as "a substance or material incorporated in a material (usually a plastic or elastomer) to increase its flexibility, workability, or distensibility". Improving the flexibility and processability of polymers is the main role of these compounds; these can be achieved by lowering the second order transition temperature, the glass transition temperature (T_g). Along with flexibility and processability, plasticisers can reduce the tension of deformation, hardness, density, viscosity and electrostatic charge of polymer. Other physical properties of polymers are also affected such as degree of crystallinity, optical clarity, electric conductivity, fire behaviour and resistance to biological degradation [34]. According to Mekonnen, Mussone [2], an ideal plasticiser is the one that "lowers the glass transition temperature, is biodegradable, non-volatile and nontoxic and exhibits minimal leaching or migration during use or aging".

With plastic industry growing in a steady state, so is the demand for plasticisers. In the last decade, the production of plasticisers worldwide was around five million tonnes a year. Due to this large demand, different types of plasticisers are available depending on the need for plastic. As plasticisers are high boiling point liquids with molecular weights between 300 and 600 g/mol, these compounds can easily occupy intermolecular spaces between polymer chains reducing secondary forces among the chains. The molecular organization of polymer in three-dimension also gets changed due to reduce in energy required for molecular motion and formation of hydrogen bonding between the chains. As a result, an increase in free volume and molecular mobility is observed. Thus, the chemical structure of the plasticiser, including chemical composition, molecular weight and functional groups, modifies the degree of plasticity of polymers.

Based on their functional properties, plasticisers are classified in three different types [34]:

1) *Internal and External plasticisers*:

Internal plasticisers form an integral part of the polymer molecules as well as part of the final product where it is either co-polymerized into the polymer or reacted with the original polymer. Since these molecules have bulky structures, these plasticisers provide space between polymers so that the polymer chains can move freely and not come in contact with each other. Increasing the space between chains lowers the Tg which reduces the elastic modulus [34]. In bloodmeal based plastics, water and triethylene glycol acts as an internal platiciser that binds the bloodmeal proteins together along with allowing them to move freely [49].

External plasticisers contain low volatility so that when added to polymer, these molecules can interact with polymer chains but not chemically attached to them. Because of no chemical attachment, external plasticisers are easily lost by evaporation, migration or extraction [34]. In bloodmeal bioplastic, urea is added to disrupt protein-protein interaction, but it does tend to migrate out of the plastic [49].

Irrespective of whether the plasticisers are internal or external, a strong temperature dependence of material properties is observed, though this property is more distinctive for internal plasticisers. Having an external plasticiser is advantageous when right substance is selected, based on the desired properties of product.

2) *Primary and Secondary plasticisers*:

Primary plasticisers are the one in which a polymer with a high concentration gets solubilised. In this type of plasticisers, the molecules used are the sole plasticisers in the polymer where they rapidly gel the polymer in the normal processing temperature range and does not get exuded from the plasticised material [34]. In bloodmeal based plastics, water is the primary plasticiser required for extruding and moulding.

Secondary plasticisers are normally blended with primary plasticisers since these secondary plasticisers have low gelation capacity and limited compatibility with the polymer. Blending the secondary plasticiser with primary ones also improve product properties and reduce cost [34]. For instance, linear low-density polyethylene was blended with NTP using maleic anhydride grafted polyethylene (PE-g-MAH) as compatibilizer that improved the mechanical properties of NTP [50].

3) *Water soluble and insoluble plasticisers*:

Plasticisers used in biopolymers can also be divided on the basis of their water solubility since such types of plasticisers and its amount affect the film formation from polymeric aqueous dispersions. Hydrophilic plasticisers when added to the polymer dispersions, gets dissolved in aqueous medium and when added in high concentrations, can lead to an increase in water diffusion in the polymer. Hydrophobic plasticisers, on the other hand, close the micro-voids in the film leading to less water uptake. Having a water insoluble plasticiser in a polymer can also cause phase separation leading to loss in flexibility [34].

To choose an appropriate plasticiser(s) involve some critical factors like low melting point, low volatility, compatibility between components, permanence in the film, amount of plasticiser required, water resistance, chemical and solar radiation, toxicity and cost [15, 34]. Proteins are a polymer containing different units (amino acids) which provide different functions to the polymer. These functional properties arise due to several intermolecular bonds between amino acids located at different positions. Being a polymer of amino acids, there are several hydroxyl groups present; therefore, a plasticiser which can hydrogen bond with amino acids is considered good for protein-based bioplastics. Some of the plasticisers used to make protein bioplastics are given in the table below [15, 34]:

Table 4: Protein-based film and the different plasticiser used [15, 34]

Protein-based films	Plasticisers used
Zein	Oleic and linoleic acids, DATEM (diacetyl tartaric acid ester of monoglycerides), dibutyl tartrate, dibutyl phthalate, glycerol, octanoic acid, palmitic acid and water
Caseinate-pullulan	Water and sorbitol
Whey protein	Glycerol and sorbitol
Whey protein/beeswax emulsion	Glycerol
β-lactoglobulin	Sorbitol, glycerol, PEG (PEG 200 and 400), EG, propylene glycol, sucrose, DEG, TEG
Sunflower protein	Saturated fatty acids
Peanut protein	Glycerin, sorbitol, PEG, PG
Wheat gluten	Glycerin, 1,4-butanediol, glycerol, lactic acid, octanoic acid and water
Feather keratin	Glycerol
Fish mince from Atlantic sardines	Sorbitol, glycerol and sucrose
Fish skin protein	Fatty acids and sucrose esters (FASE)
Water-soluble fish proteins	Glycerol, PEG, EG, sucrose and sorbitol
Fish muscle proteins	Glycerol, PG, DEG and EG
Fish myofibrillar protein	Glycerin and water, sorbitol and sucrose
Gelatin	Glycerol, sorbitol, sucrose, oleic acid, citric acid, tartaric acid, malic acid, PEG of different molecular weights (300, 400, 600, 800, 1500, 4000, 10,000, 20,000), mannitol, EG, DEG, TEG, EA, diethanolamine and TEA
Pigskin gelatin	Glycerol, sorbitol
Bovine gelatin	Fatty acids, sorbitol and glycerol
Soy protein	Glycerol

The plasticisers mentioned above behave differently with different protein polymer. Based on these interactions, three plasticising mechanisms has been proposed [2, 15]:

1) The lubricity theory: "the plasticizer is seen as acting as a lubricant to facilitate mobility of the chain molecules past one another"
2) The gel theory: "considers the disruption of polymer-polymer interactions (hydrogen bonds and van der Waals or ionic forces)"

3) The free volume theory: "considers that the plasticizer increases the free volume and mobility of polymer chains. This theory has been used to understand the effect of plasticizers in lowering the glass transition temperature"

A fourth mechanism has also been proposed which is the coiled spring theory which explains the plasticising effects on a polymer from a tangled macromolecules point of view [15].

Although these mechanisms are widely accepted and used, Shtarkman and Razinskaya (1983, as cited in [2] expressed some limitations. These authors found that the plasticization theories are limited to particular scenarios and have limited predicting capabilities. These mechanisms also lack direct studies. The authors suggest the requirement of compatibility-efficiency-property study that looks at the structure of polymeric system to select a particular plasticiser. The plasticization mechanisms mentioned above were formulated based on the properties of the synthetic plastics especially PVC which might be the reason why these mechanisms will not work on modern bioplastics [2].

2.4.1.1 Effect on plastic properties

The type and amount of plasticisers strongly influence the physical properties of protein films; this is due to the plasticisers' molecular weight and the number and position of hydroxyl groups [34]. Plasticisers having low molecular weights are easily incorporated into the protein matrix and display a more efficient plasticising effect than plasticisers with larger molecular weights. Also, the tensile strength of a material decreases as the concentration of plasticisers is increased. This observation is opposite for elongation of film material where increasing plasticiser concentration increases elongation [51].

Water is considered to be one of the best natural plasticiser for protein since the water molecules can reduce the T_g and increase the free volume of biomaterials. At the same time, the protein molecules and other plasticisers

can absorb water due to hygroscopic properties of these two materials. However, while extruding protein containing excess water, the melt viscosity would decrease which would, in turn, produce low motor torque and specific mechanical energy input. This would result in low product temperature that might reduce the degree of protein transformation and interactions [15].

Other than water, glycerol (Gly) is one of the most common plasticiser used in protein films, and its presence has differing effects on the properties of different protein films. In fish protein films, glycerol presence had reduced opacity, colour and T_g [34]. In whey protein films, increasing glycerol concentrations from 40% to 50% had led to increased percent elongation from 85% to 94% and decreased tensile strength from 8 to 4 MPa. It has also been noticed that when glycerol concentration starts decreasing, tensile strength and elastic modulus starts increasing providing very brittle, difficult-to-process sheets [15]. The following table illustrates different tensile properties of protein films with different glycerol concentrations.

Table 5: Tensile properties of whey protein (WPI) and soy protein (SPI) films with glycerol concentrations and processing method [15]

Film formulation	Formation method	Tensile properties		
		Tensile strength (MPa)	Elastic modulus (MPa)	Elongation (%)
40% Gly-WPI	Compression moulding	8	144	85
50% Gly-WPI	Compression moulding	4	60	94
46% Gly-WPI	Extrusion	4	46.5	127
49% Gly-WPI	Extrusion	3.5	37	121
40% Gly-SPI	Compression moulding	2.6	-	-
50Gly:100SPI	Extrusion	7.1	144	-

Incorporation of fatty acid materials like oleic and linoleic acids as plasticisers in zein based films resulted in flexible sheets of high clarity, low modulus and high elongation and toughness but low tensile strength. The separation of fatty acids normally has an effect on loss of flexibility and

increased water absorption through zein aggregation. Addition of linoleic acid reduces water absorption of the sheets while oleic acid provide relatively tough and water-resistant sheets which has its use in thermoformed packaging trays [34].

Amides have also shown to influence the properties of protein plastics just like polyols. Amides have an advantage over polyols with respect to moisture content and mechanical properties since the latter have high moisture sensitivity and reduce tensile strength and modulus due to the presence of the oxygen molecules. Liu, Tian [52] studied the effects of the soy protein plastics by incorporating different amides. The authors used formamide, acetamide and acrylamide as plasticisers while compression moulding the soy protein plastics. At 0% relative humidity, the formamide plasticized sheets exhibit good transparency with formamide content being 20 phr (part per hundred parts of resin; percent mass ratio of plasticiser to SPI in grams). The glass transition temperature (T_g) of the sheets is similar for all three amide plasticised sheets and is also single due to homogeneity and similarity between the amides and proteins. This homogenous structure leads to good compatibility between the plasticiser and the protein. The plasticiser efficiency of the three amides for SPI were in the following order: formamide > acetamide > acrylamide. Off the three amides selected, formamide is considered to be an effective plasticiser for soy protein plastics due to its smaller molecular weight, less steric hindrance and stronger hydrogen-bonding interaction with soy protein. Presence of amides had also shown to improve optical transmittance, flowability and flexibility of the plastic [52].

2.4.2 Fillers

Filler, as the name suggests, is used to ‘fill’ the plastic compounds. It is an additive that is strictly used to increase the bulk of the plastics material reducing, at the same time, the processing cost of that plastic [53]. Following is the list of most common fillers used in polymers [54]:

Table 6: Fillers used in polymers

Chemical family	Examples
Inorganics	
Oxides	Glass (fibers, spheres, hollow spheres and flakes). MgO, SiO_2, Sb_2O_3, Al_2O_3, and ZnO
Hydroxides	$Al(OH)_3$, and $Mg(OH)_2$
Salts	$CaCO_3$, $BaSO_4$, $CaSO_4$, phosphates, and hydrotalcite
Silicates	Talc, mica, kaolin, wollastonite, montmorillonite, feldspar, and asbestos
Metals	Boron and steel
Organics	
Carbon, graphite	Carbon fibers, graphite fibers and flakes, carbon nanotubes, and carbon black
Natural polymers	Cellulose fibers, wood flour and fibers, flax, cotton, sisal, and starch
Synthetic polymers	Polyamide, polyester, aramid, and polyvinyl alcohol fibers

Though the fillers main objectives are to increase the bulk and reduce the cost, its functions are not limited to only these two properties. The increasing use of multi-functional fillers in the plastic industry not only rises the bulk and reduce the cost but also modifies the properties of overall plastic material [53, 54]. These fillers have shown to affect coefficient of linear thermal expansion, dimensional stability at elevated temperatures, thermal conductivity, electric conductivity, gas permeability, chemical reactivity, colour, moisture pick-up, UV stability, weather resistance, transparency, opacity, flammability etc. of polymers and rubbers [55].

Fillers normally has unfavourable geometrical features, surface area or surface chemical composition [53, 54]. To assess fillers, specific grain distribution curves are the main parameters used but these diameters are often quoted. The abrasive, surface and thermal properties and moisture content are the features that determine the use of filler in a polymer.

Abrasion: Can cause a serious risk when mineral fillers are used. Bruenig test determines the abrasiveness of the filler. The test is conducted in hydrous suspension which makes a precise differentiation possible.

Surface properties: Surface determines the number of potential polymer/filler adhesion points. Large surface gives many adhesion points with better mechanical characteristics but too large a surface area produces dispersion problems or uncontrollable viscosity in processing [53].

Along with surface area, surface energy (stated in mJ/m^2) also influences the polymer/filler interaction and with it the mechanical properties, particularly of the polar plastics [53]. Surface energy is important for wetting and adhesion [56] of the filler material and is defined as;

$$\gamma s = \gamma_s^D + \gamma_s^{SD} [57] \quad (1)$$

Where, γ_S^D is the non-specific or London type forces and γ_S^{SD} the specific component (the contribution of all other types of interactions: dipole–dipole, H-bonding, acid–base and so on) [57]. It is the energy required to form a new surface of the material per unit area in a vacuum [56].

Just like surface area, dispersion problems are produced by high surface energies reducing the mechanical properties. This drawback of filler material can be improved if the material is coated [53].

Thermal properties: Thermal conductivity of filler is much higher than that of plastics (about twenty times higher). With low plastic thermal conductivity, lot of energy is invested to heat the polymer to its processing temperature. Similarly, cooling is slow which affects the length of the moulding cycle for thermoplastics. In contrast, the specific heat of the plastic is much higher whereas that of the filler is about 50%. Due to better thermal properties of filler, using it might improve the productivity in processing [53].

Moisture content: Filler contains water-soluble compounds such as salts of sodium or potassium. Due to its water solubility, these compounds have

high chances of getting affected by outdoor exposure, thereby damaging the performance of polymer compound. If fillers are surface-treated, it is advantageous towards moisture-resistance, reduction in surface energy, reduction in melt viscosity, improvement in dispersion, improvement in processing characteristics, reduction in stabilisers and lubricants and improvement of end-product surface [53].

2.4.2.1 Effect on plastic properties

Fillers are sometimes added in the polymer matrix to change the mechanical characteristics of the material [58]. Often these changes include modulus of the plastic as well as its tensile strength. Overall, the effect of fillers on the properties of plastics are strongly influenced by the interaction within the polymer, between polymer and filler, and within filler particles [55].

The effects are also influenced by the grain size and shape of the filler which governs how strong the properties of polymer composites are. In a typical polymer, the size of less than 40 μm is normally used for general purposes. Finer filler particles of less than 3 μm are used for stronger enhancements in properties whereas, the dimensions ranging up to 100 nm are used for strongest enhancements. The shape (measured by the aspect ratio – ratio of the longest length of particle to its thickness), on the other hand, affects flexural modulus, permeability, and flow behaviour of the filled polymer [59].

Mehrjerdi, Adl-Zarrabi [60] studied the properties of high density polyethylene (HDPE) using combination of two fillers, carbon black and talc. Carbon black, being an economical additive, provides good UV-screening strength for HDPE but it reduces the mechanical strength of the polyethylene especially impact resistance. Talc, on the other hand, seemed to counteract the negative effects of carbon black, at the same time, improves the thermal conductivity and thermo-physical properties of the composites. Addition of talc increased the tensile strength of the material even at higher concentrations.

As mentioned earlier, the flow characteristics of the filled polymer is affected by the presence of fillers. Fillers increase viscosity, interfere with the polymer flow pattern, produce thixotropy, and intensifies machine wear. The viscosity of the filled polymer using rigid spherical fillers can be estimated using Einstein equation:

$$\eta = \eta_s(1 + 2.5\phi) \tag{2}$$

Where η is the viscosity of the filled polymer which is related to η_s, viscosity of the polymer. Φ denotes the filler volume fraction.

The above equation is only applicable where the filler concentration is less; at higher concentrations this equation does not apply as the particles physically start interacting with each other.

2.4.2.2 Surface modifiers

In a typical polymer, fillers on its own, can modify the properties of polymer composites, but their surfaces due to them being charged, often reduces the impact of these properties, with the exception of carbon black. To neutralise these negative effects and also to further improve the properties of polymer, surface modifiers are added in the filler composition. Presence of surface modifiers improves filler production which is beneficial as milling aids. It also improves filtration and reduce hardness development during drying.

Surface modifier have shown to improve the rate of filler incorporation into the polymer thereby reducing the viscosity of the mixture, which in turn could lead to energy saving and faster throughput [56].

Surface modifiers are categorised in two types: coupling and non-coupling. This categorisation is based on the ability of the modifier to interact with the polymer matrix. Non-coupling surface modifiers like saturated fatty acids chemically bond to the filler surface but does not interact with the polymer matrix. Coupling modifiers or coupling agents, on the other hand, bond

chemically to the filler surface as well as show strong interaction with the polymer matrix. The interaction is mainly due to chemical functionality on the matrix which allows such reaction, but sometimes, it is due to the species that are anchored and having long chains that allow them to get entangled with the matrix and co-crystallise it [56].

Surface modifiers can be synthetic chemicals like fatty acids or natural polymers like catecholamine. Lee, Lee [61] attempted to used catecholamine polymers like poly(dopamine) and poly(norepinephrine) as surface modifiers for carbon and glass fibers in fiber-reinforced composites. Presence of these polymers as surface modifiers have shown to increase interfacial shear strength between fibers and polymer matrix, thereby increasing the interlaminar shear strength of fiber-reinforced composites. This is due to the adhesive characteristics of these natural polymers that provide mechanically strong composites.

2.5 Previous work on NTP

Enzyme hydrolysed bloodmeal was successfully incorporated into NTP as a plasticiser. Alcalase hydrolysate produced smaller peptide sizes of 8.9 kDa followed by pepsin hydrolysate with 8.8 kDa and trypsin hydrolysate with 5.5 kDa. The hydrolysis using three enzymes was continuous which led to increasing salt content for pepsin hydrolysate partly due to low pH conditions required for pepsin hydrolysis. Increasing salt content greatly affected specific mechanical energy of the extruded NTP along with increasing hydrolysate content. Salt content also greatly affected the mechanical properties of the thermoplastic producing brittle material [62]. To overcome these problems, bloodmeal had to be hydrolysed under neutral pH. So the aim of my thesis is to hydrolyse bloodmeal using enzymes that are optimal at neutral pH producing less salt content.

Protein chain length also had an effect on the mechanical properties with tensile strength, secant modulus, crystallinity, thermostability and glass transition temperature decreasing with presence of high molecular weight

peptides [62]. So my second aim is to produce hydrolysates with smaller peptide size and incorporate them in to NTP as a plasticiser.

Chapter 3

Methodology

3.1 Overview

The aim of this research is to obtain bloodmeal hydrolysates of low molecular weights and low salt content and incorporate it into NTP as a plasticiser.

Three enzymes are chosen for hydrolysis of bloodmeal: alcalase, chymotrypsin/trypsin and papain. Initial trials will be conducted using chymotrypsin/trypsin and papain as enzymes for hydrolysis of bloodmeal. After obtaining which bloodmeal and enzyme concentrations from these initial trials produce high yield, large scale hydrolysis of bloodmeal will be carried out. For alcalase hydrolysis, the bloodmeal and enzyme concentration are selected from the previous results [62]. To obtain low molecular weight peptides, the hydrolysates obtained will be passed through ultrafiltration column having molecular weight cut-off of 10 kDa. The hydrolysates, retentates and permeates will be concentrated to obtain desired solids content which will be incorporated as a plasticiser.

Different formulations of NTP blend will be made which will be extruded, granulated and injection moulded to obtain bloodmeal thermoplastic. The thermoplastic obtained will be characterised using tensile and impact tester, thermogravimetric analysis, dynamic mechanical analysis and X-ray diffraction.

The equipments and materials used are listed in the following tables (**Table 7** and **Table 8**).

Table 7: List of equipments used in these experiments

Equipments	Supplier
AKTA FPLC	Pharmacia
Filters	Sartorius Stedim Biotech
Glass beakers (400 ml)	Duran
Centrifuges	Sigma Centrifuges
	Sorvall RC 26 Plus
Conditioning chamber	Lloyd Instruments
Conductivity meter	CyberScan 100
Dynamic Mechanical Analyser	Perkin Elmer DMA 8000
Oven	Contherm Thermotec 2000
Electronic balance	Sartorius CP225D
Grinder	Magic bullet
Impact tester	Rayran
Injection moulder	BOY 35A
Magnetic stirrer	Chiltern Scientific MM31
Notch maker	T Verter
Pasteur pipettes (3ml graduated)	Raylab
pH meter	CyberScan 100
Syringes	BD
Tensile tester	Instron 33R 4204
Thermogravimetric Analyser	SDT 2960 Simultaneous DTA-TGA
Tri-blade granulator	Castin Machinery
Twin screw extruder	LabTech Engineering Company Ltd
Ultrafiltration cartridge (MWCO 10 kDa)	Millipore
UV spectrophotometer	Shimadzu UV Pharma Spec
X-ray diffraction	Panalytical Empyrean

Table 8: List of chemicals used

Materials	Grade	Supplier
Bloodmeal	Industrial	Wallace Corporation
Alcalase	Analytical	Calbiochem
Papain	Analytical	Zymus
Trypsin/chymotrypsin	Analytical	Novozymes
Sodium phosphate ($Na_2HPO_4.2H_2O$)	Analytical	Ajax Finechem
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	Analytical	Ajax Finechem
Hydrochloric acid	Analytical	Merck
Sodium hydroxide	Analytical	Scharlau
Urea	Analytical	Merck
Sodium dodecyl sulphate	Technical	Bio-lab
Sodium sulfite	Technical	Merck

3.2 Hydrolysis

3.2.1 Initial hydrolysis trial

Hydrolysis was carried out in a series of five 400ml beakers with different enzyme and bloodmeal concentrations. It was carried out using the Boltac JarStar with G value using the first five impellers for mixing. The impeller speed was kept at 120 rpm and the water bath was kept to maintain the temperature of the hydrolysis reaction. Aluminium foil and a white plastic sheet was used to prevent the sample mixture and water respectively, from evaporation.

Figure 13: Initial bloodmeal hydrolysis trials

In the product information provided for enzyme, papain is set to be stable in the pH range of pH 5.0 – 7.0 with pH 5.0 optimum for haemoglobin. In order to confirm this, hydrolysis of papain was carried out at both pH 5.0 and pH 7.0.

In five beakers with 200 ml distilled water each, 20g bloodmeal was added and the mixture heated to 65°C and pH 7 for the activation of enzyme. Papain was added in the beakers in the following order: 0 ml, 0.5 ml, 1 ml, 1.5 ml and 2 ml, and the reaction was carried out for six hours by maintaining

the pH by adding 1M NaOH after which it was halted by raising the temperature to 80°C for enzyme inactivation. The same procedure was followed with 10 g bloodmeal, pH 7, 20 g bloodmeal, pH 5, and 30 g bloodmeal, pH5. For hydrolysis of 20g and 30g bloodmeal at pH 5.0, the pH of the reaction mixture was maintained by adding 1M HCl and the enzyme amount was doubled for 30 grams of bloodmeal.

Similar procedure was followed using the enzyme mixture chymotrypsin/trypsin. The bloodmeal mixtures (10g and 20g bloodmeal in 200ml distilled water) were heated at 50°C at pH 9.0 and the enzyme added in the following order: 0 mg, 60 mg, 120 mg, 180 mg and 240 mg. The reaction was carried out for 8 hours maintaining the pH using 1M NaOH after which it was halted by cooling the temperature.

3.2.2 Final hydrolysis

3.2.2.1 Hydrolysis by alcalase

In a beaker containing 4 L water, 302 grams of bloodmeal was mixed and heated at 62°C using the water bath. The pH of this bloodmeal mixture was noted as pH 7 and 30 ml alcalase was added. The reaction was mixed using the overhead stirrer at a speed of 120 rpm and was carried out for 8 hours after which the reaction was stopped by keeping the hydrolysis mixture in a refrigerator for deactivation of the enzyme.

3.2.2.2 Hydrolysis by enzyme mixture

Distilled water (21 L) was heated in a 50 L container till the temperature reached up to 50°C. A heating element with a temperature controller was fixed to the container to monitor the temperature using thermocouple and to keep it constant. An overhead stirrer with a speed of 120 rpm was used to mix the reaction mixture. Once the water temperature reached 50°C, bloodmeal (1050 g) was added and the pH raised to pH 9 after which 25.2 g of enzyme mixture was added and the hydrolysis reaction was initiated. The pH was monitored and kept constant using the same procedure as for initial trials using 1M NaOH.

Figure 14: Large scale hydrolysis of bloodmeal using enzyme mixture

3.2.2.3 Hydrolysis by papain

Due to large volume of 53 L, hydrolysis using papain was divided in two equal volumes and was conducted in two batches. In a 50 L container containing 26.5 L distilled water, 1325.3 g of bloodmeal was added and the mixture was heated at 65°C. When the temperature was close to the desired temperature, the pH of the bloodmeal mixture was measured which was neutral and 265 ml papain was added to initiate the hydrolysis. 1M NaOH was used to keep the pH constant which was monitored every 15 minutes for the first two hours and later every 30 minutes for 3 hours. The pH of the last 6th hour was measured before terminating the hydrolysis by raising the temperature to 80°C for 1 hour for enzyme inactivation.

3.3 Centrifugation

The hydrolysis product obtained was centrifuged using a Sigma Laboratory Centrifuge at 4000 rpm for 20 minutes. The supernatant and the pellets obtained after centrifugation were collected separately for further analysis.

Figure 15: Papain hydrolysate before and after centrifugation

3.4 Suction filtration

The supernatant collected from the centrifugation method above was further filtered by suction filtration using 70 mm diameter filter paper. For larger hydrolysis volumes, 150 cm diameter filter paper was used. The filtrate obtained was retained for further analysis while any remaining solid residues left from the centrifugation method was discarded. Of the total hydrolysate obtained, one-third of the bloodmeal hydrolysate was left as it is for concentration while the remaining two-thirds was passed through ultrafiltration column for separation of low molecular weights from the larger one.

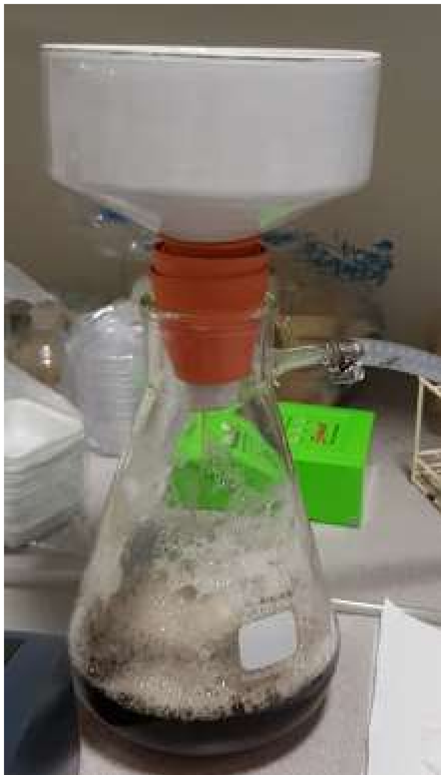


Figure 16: Suction filtration of papain hydrolysate after centrifugation

3.5 Ultrafiltration

Using a Millipore spiral cartridge with a molecular weight cut-off of 10 kDa, the hydrolysate was further filtered separating the peptides of molecular weight higher than 10 kDa from the ones lower than 10 kDa. Both the retentates and permeates of the hydrolysates were kept for incorporation into NTP.

Figure 17: Ultrafiltration of papain hydrolysate

3.6 Moisture content

The hydrolysates, pellets, retentates and permeates obtained were dried in the Contherm Thermotec 2000 oven at 70°C to determine moisture content. Drying time was varied depending on the state of the sample. The pellets from each hydrolysis experiment were dried in the oven for two nights while the hydrolysates, retentates and permeates were dried overnight. The moisture content of each of the samples were calculated using the following equation:

$$moisture\ content\ (\%) = \frac{(initial\ mass - final\ mass)}{initial\ mass}\ X\ 100 \qquad (3)$$

Where, initial mass is the mass of the sample before drying, and the final mass is the mass of the sample after drying.

3.7 Size distribution

The molecular weights of the hydrolysates, retentates and permeates obtained were determined by passing the sample through the GE Healthcare AKTA Explorer gel filtration column. The samples were injected

into the chromatograph using 1.20 μm and later 0.45 μm syringe filter into a GE Healthcare Superdex 200 10/300 and later its successor Superdex 200 Increase column for analysis of the molecular weights. A 50 μl sample loop was used to load the samples into column and 0.02M phosphate buffer with 0.1M NaCl at pH 7 was used as the running buffer. The flow rate was 0.75 ml/min. The elution peaks obtained through the gel filtration were detected through UV absorbance at 280nm.

3.7.1 Buffer

21.73 g of di-sodium hydrogen phosphate, dihydrate ($Na_2HPO_4.2H_2O$) and 10.73 g of sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) were mixed in 1 litre of distilled water to obtain 0.2M phosphate buffer. This buffer was diluted 1 in 10 with distilled water and 58.4g sodium chloride (NaCl) was added to make 0.02M phosphate buffer with 1M NaCl. 0.02M phosphate buffer with 1M NaCl was diluted 1 in 10 to make 0.02M phosphate buffer with 0.1M NaCl which was used as a running buffer for size exclusion chromatography. The pH of this phosphate buffer was 7.

3.8 Concentration

The concentration of each of the samples obtained were calculated using the absorbance at 280nm. The absorbance was determined on Shimadzu UV-VIS Spectrophotometer (UV-1700) using distilled water as blank. A 1:10 and 1:100 dilution of the hydrolysate was done depending on the absorbances obtained at each dilution. The presence of heme in each of the samples were determined by measuring its absorbance at 585nm. Since the concentration of each of the samples required should be free of heme protein, the final concentration obtained was calculated by subtracting the heme concentration from the total protein concentration.

3.8.1 Calculating concentration at 280 nm

Series of dilutions were made using 10 mg/ml BSA whose concentrations were measured at 280nm. Obtaining the linear trend from these

concentrations, the following equation was used to calculate the concentration of peptides in the hydrolysates, retentates and permeates (Refer Appendix C):

$$y = 0.592x + 0.0138 \quad (4)$$

Where, y is the absorbance measured at 280 nm and x is the concentration in mg/ml.

3.8.2 Calculating heme concentration

Heme concentration was calculated using the molar coefficient of oxygenated haemoglobin at 585 nm. Using the molar coefficients of HbO_2 at 584 nm and 586 nm, the molar extinction coefficient at 585nm was calculated. This value was later included in the calculations for obtaining heme concentration.

$$Concentration = \frac{Absorbance\ x\ 64500}{30620} \text{ [63]} \quad (5)$$

Where, 64500 is the molar mass of haemoglobin and 30620 is the molar extinction coefficient of HbO_2 at 585 nm.

3.9 Concentrating samples

The hydrolysates, retentates and permeates obtained from the ultrafiltration were concentrated to obtain 45% solids by continuous mixing at 60°C.

Figure 18: Concentrating enzyme mixture hydrolysed bloodmeal permeate and retentate

3.10 Preparing hydrolysate based NTP

3.10.1 Sample preparation

The sample preparation for NTP were formulated according to the following table (

Table 9). Accounting for the solids and water content in the hydrolysates, the amount of bloodmeal and water added in the mixture was varied to keep the protein and water content constant in all the samples.

Table 9: Formulation of NTP with hydrolysate as plasticiser

Ingredients	Amount (g)		
Bloodmeal	600	575	550
SS		18	
SDS		18	
Urea		60	
Water			
Reference		240	
Low MW alcalase hydrolysate		216	192.1
Enzyme mixture hydrolysate		198.6	157.1
Hydrolysate permeate		212.1	184.2
Hydrolysate retentate		193.2	146.4
Papain hydrolysate		234.9	229.9
Hydrolysate permeate		187.8	135.7
Hydrolysate retentate		218.7	197.3
Hydrolysate			
Reference		0	
Low MW alcalase hydrolysate		49	97.9
Enzyme mixture hydrolysate		66.4	132.9
Hydrolysate permeate		52.9	105.8
Hydrolysate retentate		71.8	143.6
Papain hydrolysate		30.1	60.1
Hydrolysate permeate		77.2	154.3
Hydrolysate retentate		46.3	92.7

In a beaker, sodium sulfite (SS), sodium dodecyl sulphate (SDS) and urea were added in water and mixed together for 15 minutes at 60°C. Once the mixture got cleared, it was added to bloodmeal and the mixture was blended for 5 minutes in a blender. Hydrolysate was added to this mixture and was further blended for 5 minutes along with manual mixing using a spatula in a mixing bowl. The resulting mixtures were stored in zip-lock bags and were refrigerated until used for extrusion. To avoid more moisture getting into the mixtures, they were double zip locked and refrigerated.

Figure 19: NTP blends

3.10.2 Extrusion

Using a die size of 10mm, the hydrolysate plasticised NTP were extruded in a LabTech extruder. The temperature for each zone of extruder was set at 70,100,100,100, 100,100,100,100, 105,110, 120°C. The screw speed of the extruder was kept at 150 rpm and the samples were hand fed to avoid bridging of the feed hopper. During each extrusion, the average time, torque, pressure and the total mass of the extrudate were noted to determine the specific mechanical energy of each extrusion. The extrudate obtained were double bagged in zip lock bags and refrigerated until next use.

Figure 20: Extrusion of NTP using LabTech extruder

3.10.3 Granulation of NTP extrudate

The long extrudate were broken into pieces and were granulated into small granules using a mesh diameter of 4 mm. The total mass of the granulated blends was noted and few grams were removed for their moisture contents at 100°C for three days while the remaining blends were refrigerated in a double zip-locked bags.

3.10.4 Injection moulding of NTP

The granulated NTP blends were moulded into dog bone specimens (ASTM D638-03 using a Boy 35A injection moulder. The mould temperature was set at 60°C while the barrel temperature was set at 100, 110, 115, 120, 120 for the five zones in the barrel. The samples were fed into the barrel using the feeder and the instrument was operated on semi-automatic and manual mode. 10 specimens and impact bars were collected for each granulated formulations and later were subjected to conditioning for seven days at 23°C with 50% humidity. The weights of both the specimens and the bars were checked before and after conditioning for shrinkage.

Figure 21: Injection moulded dog-bone specimens and impact bars

3.11 Material Characterisation

3.11.1 Tensile strength

The specimens were tested for their tensile strength using an Instron 33R-4204 tensile tester by placing each specimen between tensile crossheads. A load of 4.8 kN was used for each crosshead to clamp the specimens. 50 mm extensometer was connected to the specimen after it was mounted between the cells. The data for each specimen was collected at 40 ms interval. Once the specimens were tested for its tensile strength and stress, the broken specimens were dried in the oven to determine their moisture content (100°C for 3 days) while some were grinded into powders for thermal analysis. Liquid nitrogen was poured onto the small part of broken specimen after which they were grinded in a food grinder (Magic bullet) to obtain powders.

Figure 22: Tensile tested specimens (former = extensometer connected to the mounted specimen; latter = broken specimens after tensile testing)

3.11.2 Impact testing

The injection moulded bars were impact tested by placing a notch on each of the bars and measuring their dimensions at the notch area. Using the Charpy test on the Ray Ran Pendulum Impact system, the bars were tested swinging the pendulum with a velocity of 2.9 m/s. The results obtained from this test were noted in kJ/m^2. The dimension of the first bar was inputted into the system and its result was used to convert and obtain actual result of the subsequent bars.

3.11.3 Thermal gravimetric analysis (TGA)

The ash content of each formulated NTP was determined using SDT 2960 Simultaneous DTA-TGA by heating the powdered sample (5 – 20 mg) in the instrument up to 800°C at a rate of 10°C/min and with an air flow of 150 ml/min. The results obtained from this analysis were detremined by converting the raw data into the desired graphs.

3.11.4 Dynamic Mechanical Analysis (DMA)

The broken samples of the impact bars were used to analyse the thermal behaviour of NTP such as glass transition temperature, storage modulus and loss modulus of each of the formulation using Perkin Elmer DMA 8000 Dynamic Mechanical Analyzer. The data for each formulation was acquired over the range of -80 - 150°C with liquid nitrogen to cool the temperature to -80°C and at the frequencies of 0.1, 0.3, 1, 3, 10 and 30 Hz. The heating occurred immediately with the rate of 2°C/min and the results obtained were extrapolated into desired graphs.

3.11.5 X-ray diffraction (XRD)

The structural characteristics and the crystallinity of different formulations of NTP were determined using PANalytical EMPRYEAN. The samples were loaded in auto-sampler and the instrument was set at the following parameters:

Table 10: XRD parameters

Settings	Values
Current	40 mA
Tension	45 kV
X-ray tubing	Copper
Beam optics	
Angle	6° to 60°
Soller slit	0.04 rad
Fixed incident beam mask	10 mm
Programmable divergence slit	
Diffracted beam optics	
Wavelength ($k_{\alpha}1$)	1.54 Å
Large β filter	Nickel
Soller slit	0.04 rad
Anti-scatter slit	7.5 mm
Detector	PIXcel3D detector

Figure 23: XRD instrument

Chapter 4

Results and Discussions

4.1 Initial trials

4.1.1 Papain hydrolysis

The hydrolysis by papain was carried out for 6 hours at both pH 7 and pH 5 since the whole reaction was slowing down in the last hour and a half. This means that after almost 5 hours, the papain has reached its saturation and would no longer hydrolyse any more of the protein.

In these 6 hours, at pH7, there has been slight change in the pH where the pH would drop up to 6 in the initial two hours. As the time increased the change in pH reduced which can be seen in the amount of NaOH required to maintain the pH constant. Similar trend was observed at pH5 hydrolysis where instead of NaOH, HCl was used to maintain the constant pH5.

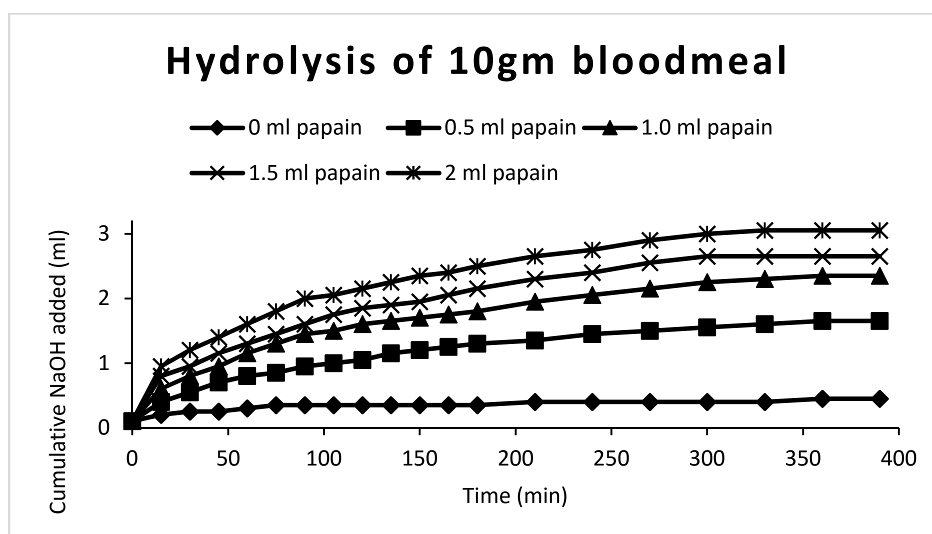


Figure 24: 10g BM pH 7

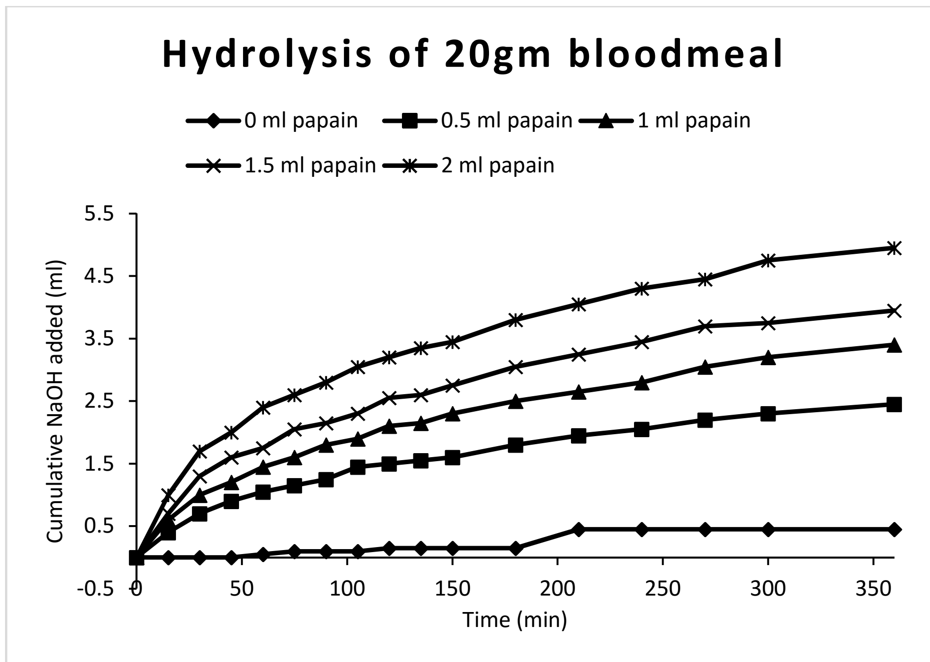


Figure 25: 20g BM pH 7

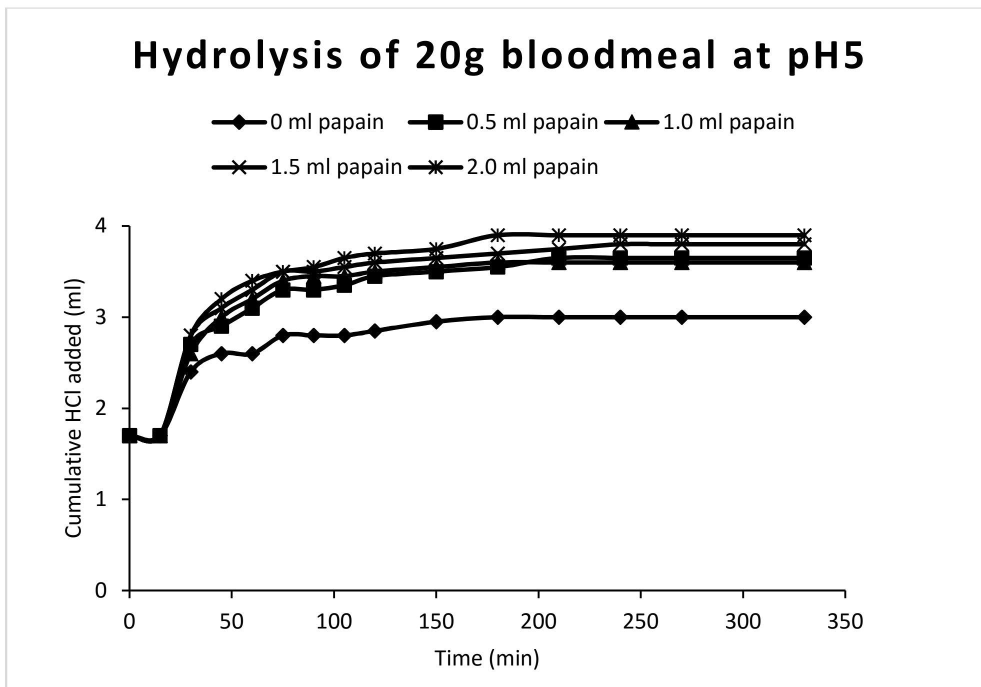


Figure 26: 20g BM pH 5

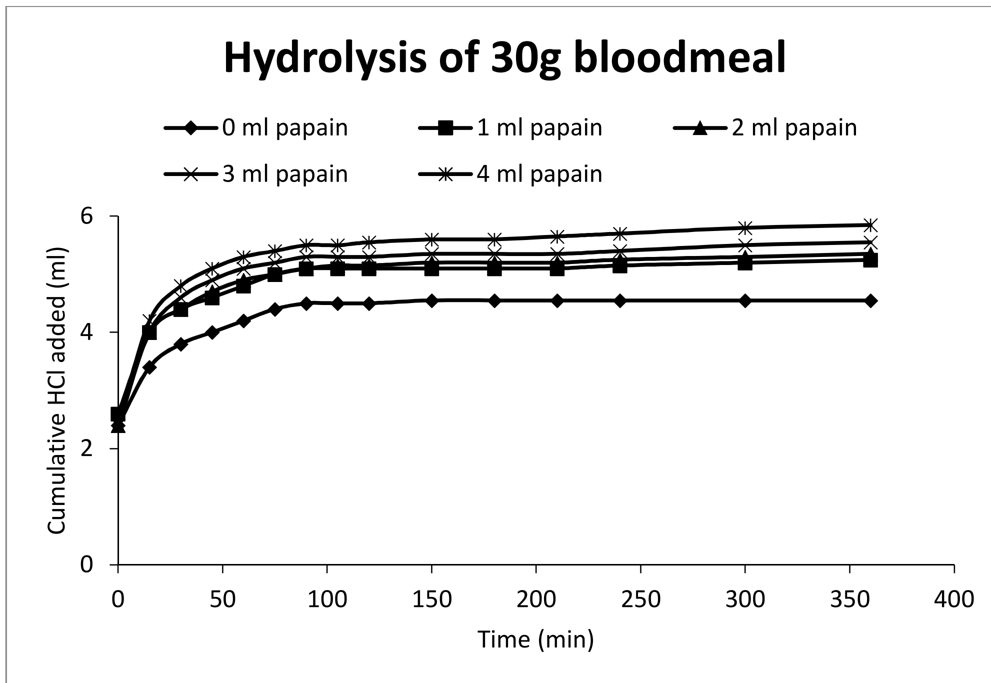


Figure 27: 30g BM pH 5

At pH 7, the rate of reaction (refer **Appendix A**) was almost twice that in 20 g BM than in 10 g. This could be due to the amount of BM present for the enzyme to hydrolyse. The rate also increased with increasing enzyme concentration as can be seen in the following graph.

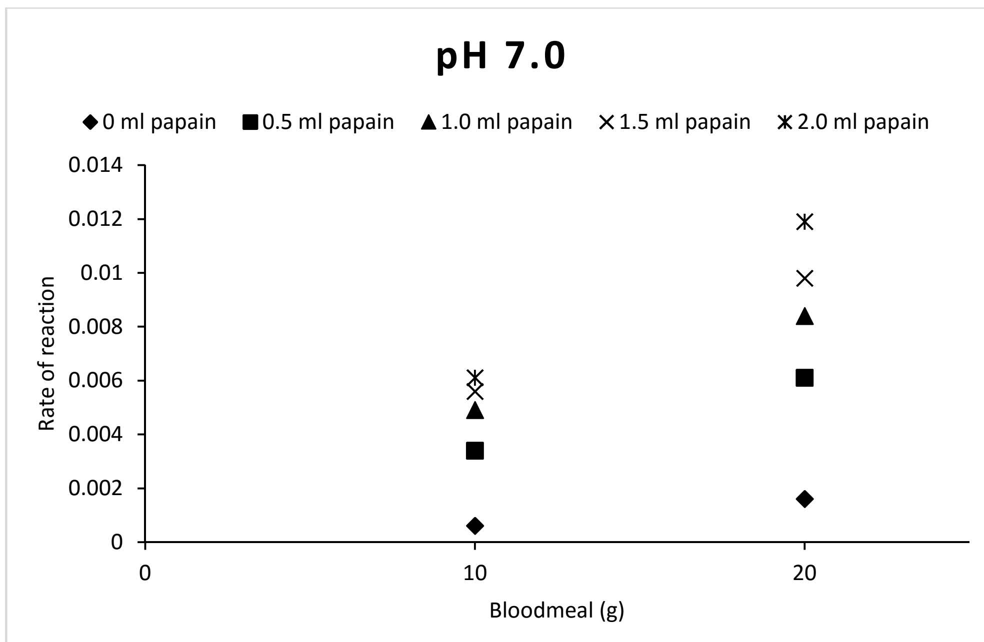


Figure 28: Rate of reaction at pH 7

The degree of hydrolysis (DH) obtained at both 10 g and 20 g BM were in the range of 0.5 to 4% with maximum DH obtained by hydrolysing 10 g with 2 ml papain (3.84%). Similar DH was obtained when 20g BM was hydrolysed at pH5 (DH less than 3%). Degree of hydrolysis corresponds to the number of the peptide bonds cleaved in a protein reaction [64]; low DH means only 3% of the total protein in the BM has been hydrolysed. To raise the DH at pH 5, the enzyme amount added in the reaction was doubled for the same BM concentration, but the results were same. There has been only slight increase in the overall degree of hydrolysis but still less than 3%. Low DH could be attributed to the enzyme composition used for the hydrolysis. In the manufacturer's information sheet obtained, the papain composition was given to be <30% (w/w) papain, glycerol and water. This means less than a gram of papain is hydrolysing 10g and 20g of BM even though its enzyme activity is as high as 6000 protease units per mg (PU/mg). Low degree of hydrolysis also contributed to the low yield of the hydrolysates obtained.

At pH 5, hydrolysing BM provided hydrolysate yield of less than 6%. This contrasted with the information provided in the manufacturer's information sheet where it states that the optimum pH to hydrolyse haemoglobin is pH 5. On the other hand, hydrolysing BM at pH 7 provided yield as high as 25% with highest yield obtained when 10 g of BM was hydrolysed by 2 ml papain. The hydrolysates obtained from the hydrolysis reaction were later subjected to ultrafiltration to separate the peptides of low molecular weights from the high molecular weights, followed by analysis using gel filtration chromatography to determine molecular weights. The permeate solution obtained from this ultrafiltration contained peptides with less than 10 kDa molecular weight while the retentate contained higher than 10 kDa. Though its DH and yield are less, papain hydrolysis provided peptides of intermediate molecular weights. Hydrolysing BM by papain at pH 7 provided one peak of 12 kDa. Relatively smaller peptide sizes of bloodmeal hydrolysates could be attributed to the catalytic activity of papain. As mentioned earlier (**Section 2.3.4.3**), papain has broad specificity of cleaving peptide bonds of basic amino acids and those with hydrophobic side chains

(valine, leucine and isoleucine) while bloodmeal has high lysine, isoleucine and valine (**Table 1**). Due to this, papain is able to cleave most peptide bonds in the substrate.

Hydrolysing at neutral pH and later filtering provided peptides with low salt content. The conductivity of the papain hydrolysates is in the range of 1 to 3 mS with highest conductivity obtained when 30 g BM was hydrolysed with 4 ml papain at pH 5. The amount of salt present in the hydrolysate reduces when they are passed through an ultrafiltration column.

Low DH and low yield had an effect on the overall concentration of the peptides obtained. The concentration of the hydrolysates and later of the ultrafiltrate samples provided concentrations were as low as 2 mg/ml. The maximum concentration for the permeates obtained was 5 mg/ml while for retentate was 29.4 mg/ml. The ultrafiltrate products hydrolysed at pH 5 was far less than as compared to the one hydrolysed at pH 7. The concentrations are somewhat similar for retentates and permeates obtained for pH 5 hydrolysates.

Though hydrolysing BM at low pH provided less yield, it led to discoloration of the substrate providing yellow coloured hydrolysate. This is due to the fact that heme group dissociates at low pH during hydrolytic attack by papain. The above overall result for papain hydrolysed BM is consistent with the result obtained for Gómez-Juárez, Castellanos [65].

4.1.2 Hydrolysis by two enzyme mixture

Hydrolysis was carried out by using enzyme mixture of two pancreatic enzymes, trypsin and chymotrypsin at pH 9 whose enzyme activities are 1250 and 100 units per mg, respectively. The use of enzyme mixture has significantly affected the degree of hydrolysis of BM as compared to papain. Papain hydrolysates has DH not exceeding 3%, while the hydrolysates obtained from this enzyme mixture provided DH as high as 13%. Though the amount of BM that has been hydrolysed was less, it was more compared to BM hydrolysed by papain and considering the enzyme activities of both trypsin and chymotrypsin, it can be determined that trypsin is the major contributor for the hydrolysis of BM.

The hydrolysis trend and the rate of reaction obtained for both 10 g and 20 g BM is similar as can be seen from the figures (**Figure 29**, **Figure 30**, and **Figure 31**). When the hydrolysis was initiated, the pH of the BM mixture went down until pH 7 in the first 30 to 40 minutes after which the changes were slight till the final pH was 8.9. The rate of reaction doubled when the BM concentration was doubled but it has no effect on the increasing enzyme concentration. Changing enzyme amounts to hydrolyse 10 and 20g BM provided almost same rate which means there has been some saturation when enzyme amount was more than 120 mg.

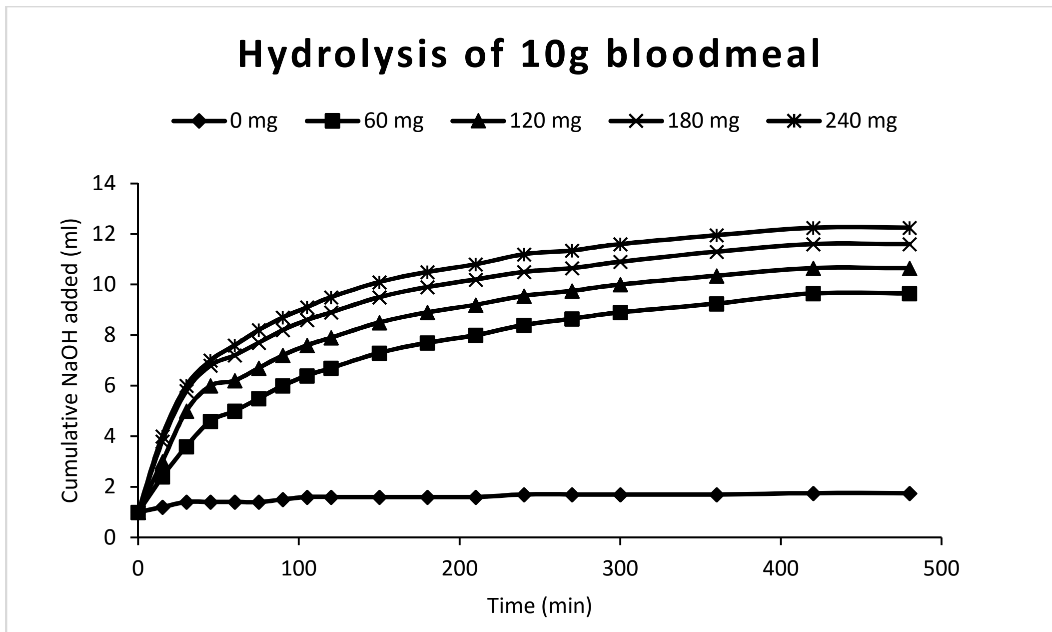


Figure 29: 10g BM pH 9

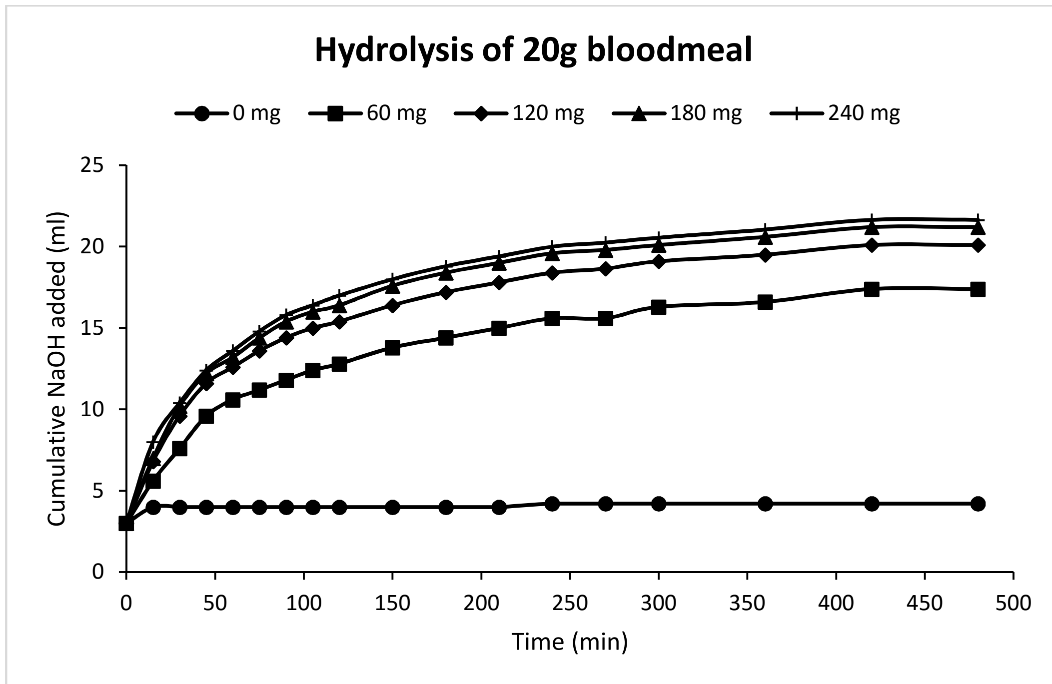


Figure 30: 20g BM pH 9

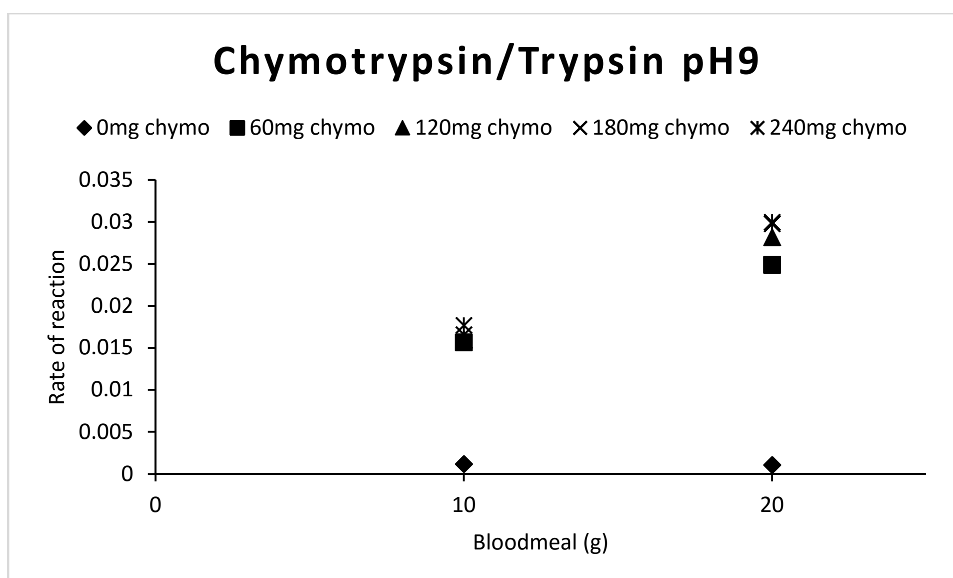


Figure 31: Rate of reaction at pH 9

The use of enzyme mixture as a reaction catalyst has significant effect on the hydrolysis yield. The yields obtained from this hydrolysis reaction were more than 40% and went as high as 70%. The yield obtained from hydrolysing 10 g BM is higher than that obtained from hydrolysing 20 g BM. This is in contrast to the degree of hydrolysis obtained for two BM amounts where the DH for hydrolysing 20g BM (2 to 13%) is higher than the one obtained for hydrolysing 10g (1 to 7%).

Gel filtration analysis of the peptides obtained from this reaction mixture has one peak with high molecular weight of 387 kDa and another with 8 kDa. This contrasted with papain hydrolysates that contained smaller sized peptides. The difference between the peptide sizes could be attributed to the composition of bloodmeal (**Table 1**). Bloodmeal is high in lysine, isoleucine and valine (amino acids with hydrophobic side chains) while chymotrypsin catalyses bonds of aromatic amino acids (**Section 2.3.4.2**) and trypsin, C-terminal of lysine and arginine. Due to less aromatic amino acid content in bloodmeal, the enzyme mixture produced higher molecular sized peptides. These two peptide sizes are separated when the hydrolysates were passed through ultrafiltration. The average molecular weight of the hydrolysate permeates obtained was 6 kDa with the exception of 10 g BM with 240 mg enzyme whose average molecular weight was as low as 3 kDa.

Since the hydrolysis was done at pH 9 and NaOH was added to keep the pH constant, it had led to significant increase in the salt content of the hydrolysate. The conductivity of the hydrolysates obtained from hydrolysing 10 g of BM are in the range of 1 to 3 mS while these values are doubled when the BM concentration was doubled with the highest conductivity of 5.49 mS found when 240mg enzyme was added to 20g BM. After passing the hydrolysate through ultrafiltration column, the conductivity of the retentates reduced significantly by almost half in each of the BM and enzyme concentrations.

Due to significantly high yield obtained by trypsin/chymotrypsin hydrolysis, the concentrations of the hydrolysates are higher than the papain hydrolysates though there is no correlation between the hydrolysate concentrations and the enzyme concentration used to hydrolyse them. The hydrolysate of 10 g BM has the highest concentration of 94.26 mg/ml when it was hydrolysed with 240 mg enzyme. This was followed by 120 mg enzyme with 92.49 mg/ml concentration of the hydrolysate, followed by 180 mg enzyme with 88.96 mg/ml concentration. Ultrafiltering the hydrolysates provided retentate and permeate concentrations of 240 mg hydrolysed bloodmeal to be the highest among its group with 41.45 mg/ml and 8.25 mg/ml, respectively. The concentration obtained by hydrolysing 20 g BM was significantly higher than the one obtained for 10 g BM though its yield is less than the latter. 20 g BM observed highest concentration of 131.28 mg/ml when 180 mg enzyme was added to hydrolyse it followed by 125.78 mg/ml when 240 mg enzyme was added for hydrolysis. Ultrafiltering these products provided the highest retentate concentration of 64.16 mg/ml when 180 mg enzyme was added for hydrolysis and highest permeate concentration of 14.47 mg/ml using the same enzyme concentration. This suggests that the enzyme mixture of trypsin/chymotrypsin reached saturation when 180 mg was added to the bloodmeal concentration which reduces slowly if the enzyme concentration is increased.

4.2 Final hydrolysis

The final hydrolysis reaction by enzyme mixture of chymotrypsin and trypsin, and papain provided degree of hydrolysis and yield similar to the initial trials. Large scale alcalase hydrolysis was performed based on the previous results [62] while papain and trypsin/chymotrypsin hydrolysis was performed with the concentration that provided high yield (10g BM with 200ml water). All the hydrolysis reaction led to the evaporation of water due to heating at high temperatures even though they were covered with aluminium foil. But since the volume of these reactions were large, evaporation of few litres did not affect the yield and overall result. While using high temperatures for hydrolysis reaction, evaporation could not be avoided even though the hydrolysis vessel was covered since some of the steam that contains the hydrolysate would stick to the foil which could not be recovered.

The results of these hydrolyses are summarized in the following table:

Table 11: Large scale hydrolysis

Enzyme hydrolysis	Degree of Hydrolysis (%)	Yield (%)	Concentration of hydrolysate (mg/ml)
Alcalase	14	44	67.23
Trypsin/chymotrypsin	13.9	65.54	100.56
Papain 1	3.72	14.86	381.96
Papain 2	3.50	8.22	260.0

Due to the large volume required of 53 L, papain hydrolysis was carried out in two batches which provided similar DH but different yields and concentrations. This difference could be due to fermentation of the papain 2 which gave one of the biggest challenges during the research. Hydrolysate from enzyme mixture started fermenting during suction filtration of the product which produced a foul smell, making the hydrolysate more viscose, and slowed the filtration. This was overcome by centrifuging the hydrolysate followed by suction filtration which made the whole process faster.

Centrifugation made the hydrolysate less viscous by the centrifugal force separating the unhydrolysed bloodmeal from the hydrolysed one (supernatant). It also reduced the foul smell since the fermented portion settled down with the pellets forming a mustard layer on top of the solids. Since the hydrolysate was thin, it passed through the filter paper effortlessly. The above two steps (centrifugation and suction filtration) are important since the hydrolysate had to be passed through the ultrafiltration column for the separation of the peptides.

The alcalase hydrolysate (AH) had a solids content of 4.1% while the pellets contained 24.2% solids. Similarly, hydrolysate from the enzyme mixture (EH) had a solids content of 4.1% while the pellets has 16.8% solids, while hydrolysate from papain had 1.6% solids and 32.4% solids in the pellets. The low solids content of papain hydrolysate (PH) could be reflected on its yield which is 23% on average.

AH produced the peptide size of intermediate molecular weights (10 kDa to 1 kDa) with one peak around 470 kDa. This peak is prominent in the retentate when the hydrolysate is ultrafiltered providing all the low molecular weights peptides in the permeate. Almost all the hydrolysate passed through the ultrafiltration column producing 194 g (dry weight) of ultrafiltered retentate and 3061 g (dry weight) of permeate.

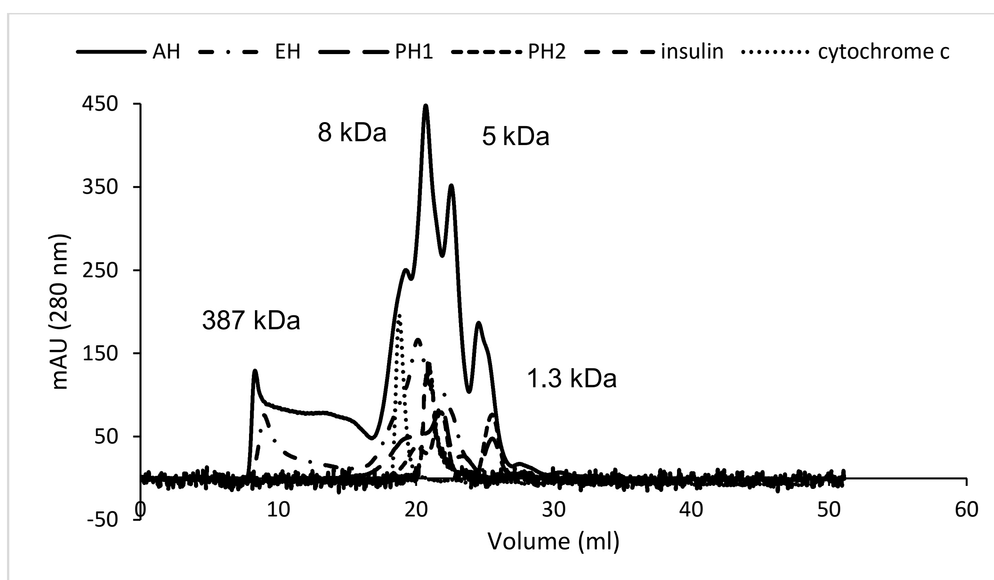


Figure 32: Hydrolysate size distribution

EH has one large peak of 377 kDa with one smaller peak of 8 kDa. When the hydrolysate is ultrafiltered, the 377 kDa peak disappears in retentate. Before injecting the samples in to the column, they were gravity filtered to remove any solid white residue that might have been produced over the period of time in the freezer. The EH permeate filtered quickly but the hydrolysate and the retentate slowed this process producing solid bloodmeal precipitates. It could be speculated that the high mass of 377 kDa had been precipitated out due to protein aggregation. The peak of 8 kDa is separated into smaller peaks of low MW in both retentate (EHR) and permeate (EHP) and this is reflected in their concentrations. Overall, the EH produced average molecular weight of 65.8 kDa, EHR produced 15.7 kDa and EHP produced 6.3 kDa peptides.

Table 12: Concentration of hydrolysates obtained from enzyme mixture

	Concentration (mg/ml)	**Mass (wet weight, g)**
EH	100.56 mg/ml	17259 g
EHR	97.01 mg/ml	4733.7 g
EHP	38.14 mg/ml	6648 g

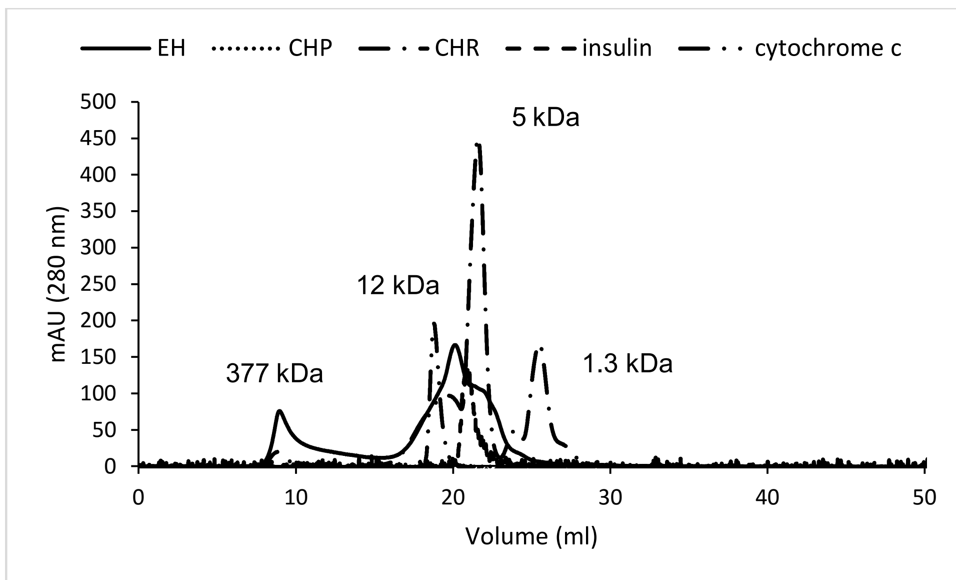


Figure 33: Size distribution of hydrolysates obtained from enzyme mixture

Hydrolysates (PH1 and PH2), retentate (PHR) and permeate (PHP) of papain hydrolysis produced small peptide sizes of less than 8 kDa. PHP has an additional peak around 12 ml (139 kDa) which is unusual since it has been passed through the ultrafiltration column with MWCO of 10 kDa and is a permeate of the hydrolysate (**Figure 34**).

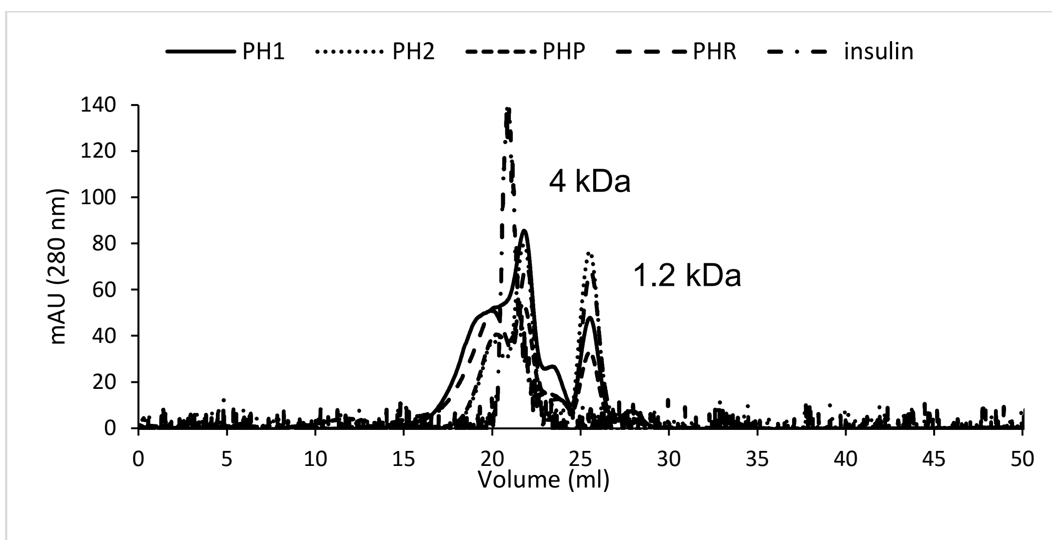


Figure 34: Size distribution of papain hydrolysate

As mentioned earlier (**Section 4.1.1**), the smaller sizes of these peptides are due to the catalytic activity of papain which is reflected in their concentrations (**Table 13**). Comparing the peptide sizes of the alcalase,

enzyme mixture and papain, it could be speculated that the choice of substrate has an effect on the enzyme activity since it's the amino acid content of the substrate that governs the catalytic activity of the enzyme.

Table 13: Concentration of papain hydrolysates

	Concentration (mg/ml)	Mass (wet weight, g)
PH1	381.96	22732.2
PH2	260.03	23154.3
PHR	340.37	6959.7
PHP	195.04	23971.2

Size distribution of the samples were initially performed on Superdex 200 10/300 column with flow rate of 0.5 ml/min and maximum pressure of 1.5 MPa. While performing these distributions, the column resin got compacted due to being used for 10 years (and on the odd occasion being over pressurised). As a result, it had to be replaced with its successor Superdex 200 Increase 10/300. The two columns produced similar distributions but the latter column has a major advantage over the former in terms of its flow rate and maximum pressure (0.75 ml/min and 5 MPa respectively). Due to the higher flow rate of Superdex 200 Increase, the time taken to finish one sample run is less compared to the one taken to finish on Superdex 200.

Ultrafiltering the hydrolysate did not affect its conductivity. The conductivity of the hydrolysates was calculated based on the different concentrations of NaCl (Appendix C). The conductivity of EH was 3.19 mS while that of its retentate was 7.92 mS and permeate 6.80 mS. Similarly, the conductivity of PH1 was 2.23 mS while that of PH2 was 4.86 mS, PHR 3.90 mS and PHP 2.49 mS. High salt content in the hydrolysate and its retentate and permeate could be due to the fermentation of these samples at different stages. This can be observed by comparing the conductivity of the papain hydrolysates (PH1 and PH2 with PH2 being the fermented sample).

4.3 Concentrating the solids content

All the hydrolysates, retentates and permeates were concentrated to obtain 45% solids. After concentrating these samples, the final product obtained was thick. PH and PHP had large total mass obtained (15295 g and 23971 g wet weight, respectively). Due to this large mass, the two hydrolysates were concentrated by heating it in 15 L stainless steel pot. The pot increased the surface area due to which concentration was fast but it also led to the change in colour of the hydrolysate. The hydrolysate colour changed from brown to blackish green which could be because of the oxidation of the stainless steel.

PHP and CHR took longer time to concentrate. Concentrating PH led to complete loss of water producing sticky hydrolysate and slightly less solids content (**Figure 35**). The final solids content obtained after concentrating the hydrolysates is provided below.

Table 14: Final solids % of concentrated hydrolysates

Sample	Concentrated wet mass (g)	Final % solids
AHP	246	47.6%
EH	548.9	35.1%
EHR	845.4	32.5%
EHP	230	44.1%
PH	472.8	77.5%
PHR	390.2	50.3%
PHP	1760.5	30.2%

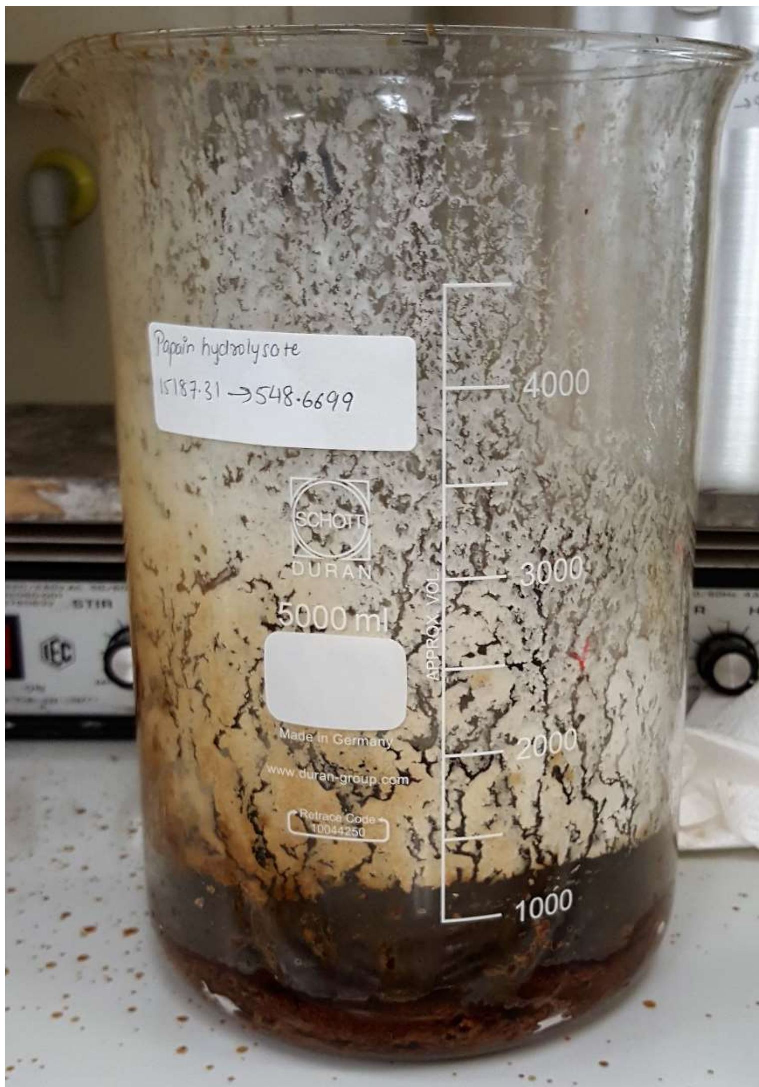


Figure 35: Papain hydrolysate concentrate

4.4 NTP production

Bloodmeal has moisture content of 6.72%. As a result, the addition of more water and concentrated hydrolysate as a plasticiser were varied taking into consideration this water content. All the concentrates were incorporated into NTP blends as plasticiser, except alcalase retentate (AHR). AHR, which after concentrating, produced 30ml of retentate which was not sufficient for incorporation. Approximate total protein in the formulations was maintained at 559.7 g dry basis and water content was maintained at 280.3 g providing formulations present in **Table 9**.

For each hydrolysate concentrate, two batches of formulated bloodmeal blends were made, one with 575g of bloodmeal and other with 550g, the remaining protein coming from addition of the hydrolysate. A standard containing 600g of bloodmeal was made that contained no hydrolysate and was used as a reference for comparing the mechanical, thermal and structural properties. In total, 15 different formulations of crude NTP were prepared for extrusion and injection moulding.

4.4.1 Extrusion

All 15 formulations of NTP were extruded effortlessly. The extrusion of each batch led to few mass losses, partially from discarding the extrudate that gets mixed with the previous batch material and partially from evaporation of water.

Figure 36: Extruded NTP

The standard NTP formulation (denoted as Ref henceforth) was extruded in 13 minutes providing 700 g of extrudate. The mass flow rate of this batch was 0.05 kg/min with specific mechanical energy (SME) of 133,748.6 kJ/kg.

SME is an important parameter to determine extrusion conditions and thus, the characteristics of the extrudate and the final product. The torque, mass flow rate and the screw speed are determined during extrusion with torque affecting the extruded material in terms of its rheological properties, the extent to which the macromolecules could be transformed and the interaction between the additives [66, 67]. Based on the above parameters, SME is calculated using the following equation:

$$SME\ \left(\frac{kJ}{kg}\right) = \frac{torque * screw\ speed}{mass\ flow\ rate}\ \text{[66, 67]} \qquad (6)$$

It should also be noted that the value obtained after calculating SME is high. In real world, this energy is one-tenth of the calculated SME. The high SME is due to the value used for calculating torque. The extruder used in this research calculates motor drive (noted in %) which gives an indication of the torque. Therefore, instead of using torque value in the above equation the motor value was used which produced such large value.

The SME of all the NTP formulations were less than the reference except PHP550 which had slightly high specific mechanical energy (**Figure 37** and **Table 15**). CH575 has the lowest SME of 61,998 kJ/kg compared to others followed by AHP575 (74,507 kJ/kg).

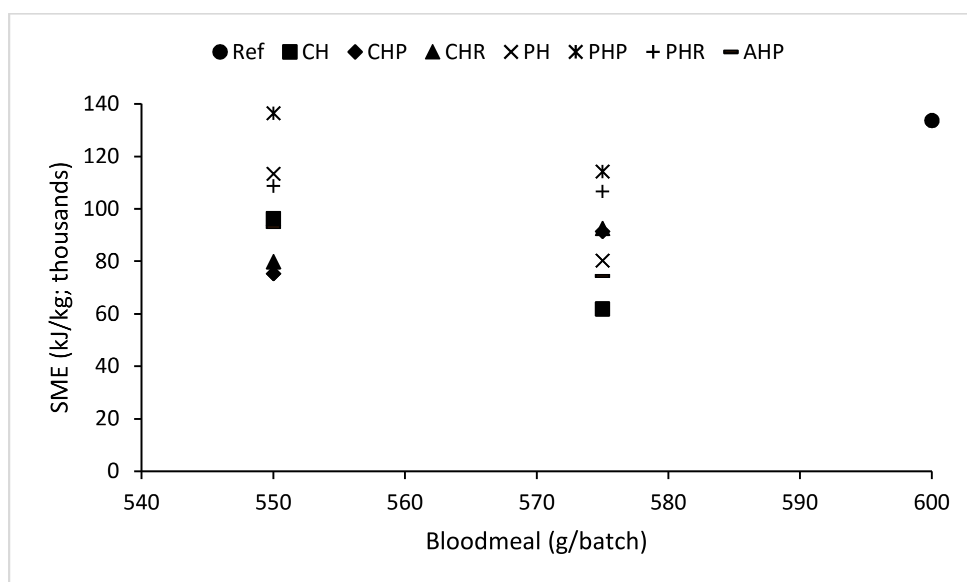


Figure 37: Specific Mechanical Energy of NTP extrudate

Table 15: Specific Mechanical Energy of NTP extrudate

Sample ID	Bloodmeal (g)	Hydrolysate (g)	SME (kJ/kg)
Ref	600	0	133,748.57
CH575	575	66.4	61,998.52
CH550	550	132.9	96,432.13
CHP575	575	52.9	91,535.43
CHP550	550	105.8	75,413.22
CHR575	575	71.8	92,607.39
CHR550	550	143.6	79,881.49
PH575	575	30.1	80,379.11
PH550	550	60.1	113,536.14
PHP575	575	77.2	114,364.91
PHP550	550	154.3	136,517.59
PHR575	575	46.3	106,727.07
PHR550	550	92.7	108,824.95
AHP575	575	49	74,507.75
AHP550	550	97.9	93,191.78

4.4.2 Injection moulding

The extrudates were converted into small granules to be fed into the injection moulding. NTP/hydrolysate of different formulations were injection moulded into perfect specimens and impact bars after which they were conditioned for 7 days at 23°C and 50% humidity. The weight loss of the tensile specimens and impact bars before and after conditioning are noted in the following table (**Table 16** and **Table 17**).

Table 16: Weight of tensile specimens before and after conditioning

Sample name	Before Conditioning	After Conditioning
Reference	9.7323	8.5088
AHP575	10.1122	8.3529
AHP550	10.0775	8.4362
CH575	9.9918	8.3884
CH550	10.4144	8.8241
CHP575	10.1309	8.5320
CHP550	9.9957	7.6938
CHR575	9.9612	8.2949
CHR550	10.1099	8.3552
PH575	10.2456	8.5442
PH550	10.2707	8.5478
PHP575	10.0173	8.3714
PHP550	10.0807	8.3632
PHR575	9.9442	8.2643
PHR550	9.9724	8.2534

After conditioning in the humidity chamber, the weight of the tensile specimen was around 8 g and the impact bars around 3 g. Conditioning lead to loss of water providing the specimens with similar weight.

Table 17: Weight of impact bars before and after conditioning

Sample name	Before Conditioning	After Conditioning
Reference	3.6838	3.1686
AHP575	3.7343	3.0926
AHP550	3.7516	3.1280
CH575	3.7606	3.1839
CH550	3.8304	3.2595
CHP575	3.7327	3.1683
CHP550	3.7349	3.1709
CHR575	3.7274	3.1171
CHR550	3.7099	3.0675
PH575	3.7606	3.1608
PH550	3.7862	3.1772
PHP575	3.7362	3.1359
PHP550	3.7602	3.1610
PHR575	3.6889	3.0832
PHR550	3.6876	3.0598

The injection moulded NTP/Ref had white residues on it which increased as after conditioning (**Figure 38**) while the other batches of plastic had none. The white residues could have formed due to mixing of some purged material with granulated Ref (since this had been the first injection moulded batch) or due to migration of urea from the composition [49]. Since the amount of residue increased with days after conditioning, it could be speculated that it is urea that is migrating steadily. One possible reason for migration of urea from the composition could be due to the lack of plasticiser present (other than water) that binds the additives together.

Figure 38: Tensile specimens of NTP with no hydrolysate as plasticiser

The moisture content of NTP/hydrolysate is similar throughout the different formulations. This can be seen in the following table.

Table 18: Moisture content of NTP/hydrolysates

sample	moisture content (%)
Ref	8.90
CH575	8.33
CH550	8.40
CHR575	8.39
CHR550	9.02
CHP575	8.61
CHP550	8.61
PH575	8.84
PH550	8.81
PHR575	8.00
PHR550	8.57
PHP575	8.22
PHP550	8.63
AHP575	8.57
AHP550	8.51

4.5 Characterisation

4.5.1 Mechanical

The tensile strength of the NTP with no hydrolysate (Ref) is 17.6 ± 2.53 MPa with 0.0112 ± 0.0024 mm/mm strain. Its secant modulus is 2944.69 ± 469.34 MPa while its impact strength is 2.551 ± 0.734 kJ/m^2. From each batch, most of the specimens broke at the edge due to its brittleness.

The tensile strength of NTP in each formulation is dependent on the amount of hydrolysate present as a plasticiser. In each formulation, the tensile strength decreased with increase in hydrolysate content. This was consistent in the previous experiments [62] and also can be seen by comparing the tensile strength of Ref with each formulation of the hydrolysate (**Table 19**). Similar trend is seen in the secant modulus, strain at break and toughness (Refer to **Appendix F** for the whole summary). The standard deviation for tensile strength is in the range of 1 to 4 MPa, for secant modulus is in the range of 300 to 900 MPa, for strain it is 0.002 to 0.5 mm/mm, for toughness 002 to 0.15 J/m^3 and for impact strength it is 0.4

to 1.5 kJ/m^2. This concludes that the amount of hydrolysate as a plasticiser governs the mechanical properties of the NTP.

Table 19: Mechanical properties of NTP

	secant modulus (MPa)	tensile stress (MPa)	Strain (mm/mm)	Toughness (J/m^3)	Impact testing (kJ/m^2)
Ref	2944.69	17.60	0.0112	0.1684	2.551
AHP550	1349.67	7.88	0.1921	0.0624	2.659
AHP575	1746.17	11.57	0.0111	0.0739	3.051
CH550	1081.90	8.62	0.0126	0.0715	1.704
CH575	1508.27	14.38	0.0159	0.1805	2.361
CHP550	1523.60	12.48	0.0162	0.1336	2.41
CHP575	1755.14	14.34	0.0119	0.1240	2.176
CHR550	1290.99	9.81	0.0139	0.0992	1.388
CHR575	1667.07	14.66	0.0138	0.1303	2.834
PH550	1992.31	11.98	0.0166	0.1510	2.199
PH575	2090.19	11.69	0.0105	0.1074	2.078
PHP550	1158.43	12.07	0.0193	0.1630	3.111
PHP575	2456.97	15.23	0.0151	0.1658	2.686
PHR550	1253.11	11.59	0.0185	0.1444	2.86
PHR575	1921.57	15.89	0.0167	0.1948	3.581

The impact strength of few formulations of NTP has been improved when compared it with reference (2.551 ± 0.734 kJ/m^2). The impact strength of CH, CHP and PH is lower than the reference. CHR having 575g of bloodmeal has the lowest impact strength among the group while its other formulation with 550g BM have impact higher than the reference. The slight correlation between the tensile and impact strength of each NTP can also be seen with thermoplastic having lower tensile strength has low impact strength as well.

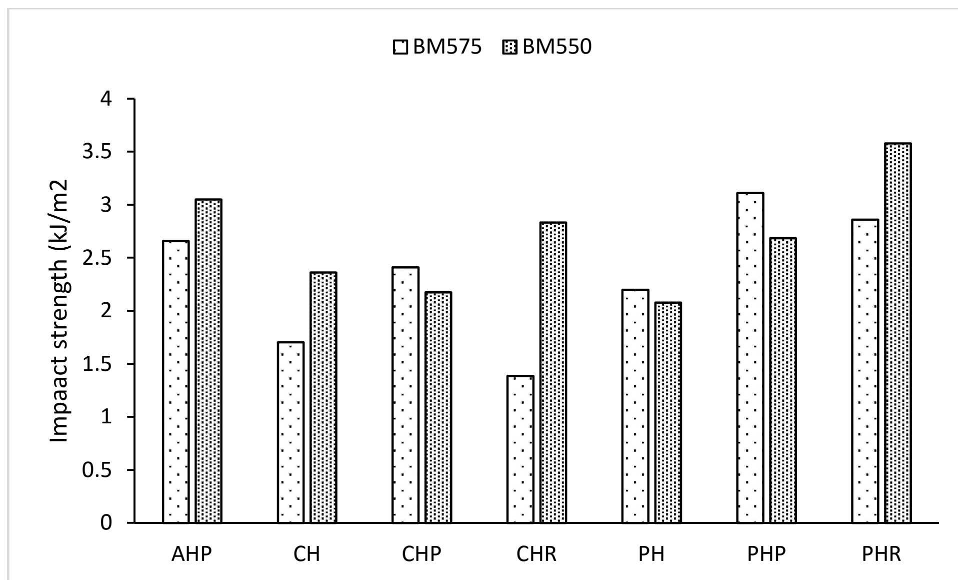


Figure 39: Impact strength of different NTP formulations (Ref had impact strength of 2.551 kJ/m^2).

For both papain hydrolysates and enzyme mixture hydrolysates, the mechanical properties are similar irrespective if that hydrolysate has been passed through the ultrafiltration column or not. This could be due to the fermentation of both the hydrolysates that removed the high molecular weight peptides providing low size peptides, and increased the salt content. As these hydrolysates were fermented, difference in the mechanical properties was not observed. It would be interesting to see whether these properties are affected if the peptide sizes are high or low and whether the non-fermentation of the hydrolysate affect these properties.

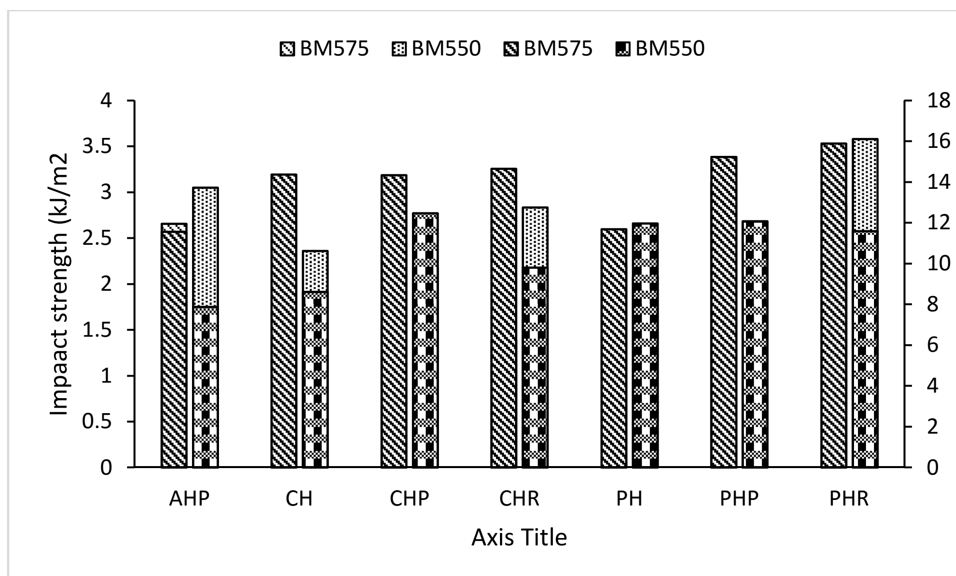


Figure 40: Correlation between tensile and impact strengths

4.5.2 Thermogravimetric analysis

Thermogravimetric analysis was carried out on the different NTP formulations. Thermogravimetric analysis determines the change in the sample weight caused by volatilization, reaction, absorption from gas phase at high temperatures [68]. The TGA thermogram for NTP (**Figure 41**) consists of four parts that relates to the mass loss: **elimination of water** from room temperature to 150°C, **decomposition of plasticiser** (150°C to 230°C), **cleavage of weak covalent bonds** disrupting the peptide bonds between the amino acid (230°C to 380°C) and lastly, **cleavage of strong disulphide and ionic bonds** above 380°C leading to total plastic degradation [66, 69]. NTP with hydrolysates does not contain any chemical plasticisers – the plasticisers added are protein hydrolysates – therefore there is no plasticiser decomposition change around 150°C to 230°C range

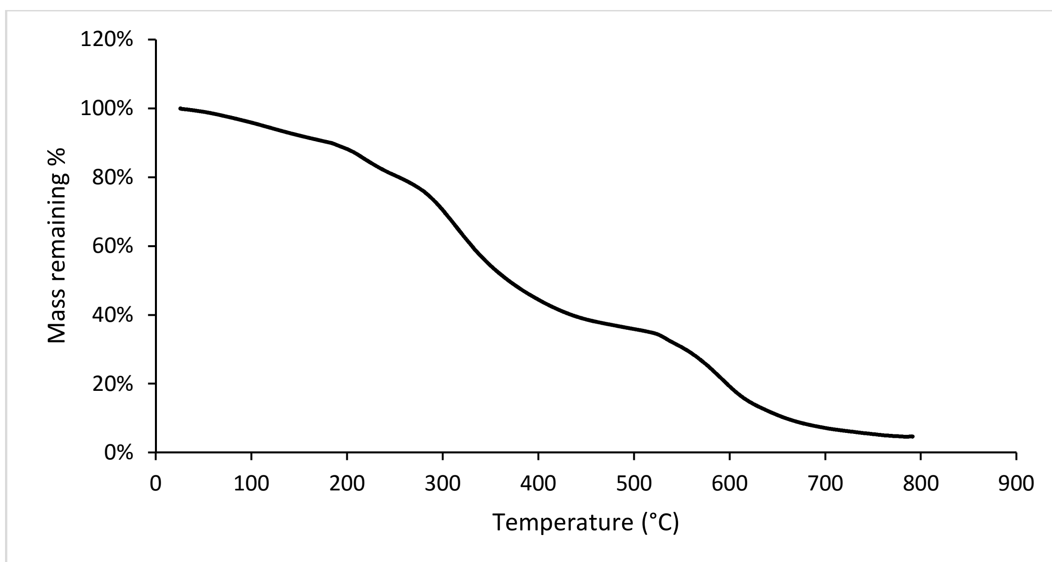


Figure 41: Typical thermogram of NTP

The thermogram of NTP/alcalase hydrolysates (**Figure 42**) is similar to the Ref thermogram except in the range of 500-600°C where the NTP/hydrolysates decompose earlier than the Ref implying that the NTP/alcalase hydrolysates is less thermostable. A similar trend can be seen with NTP/enzyme mixture hydrolysates (**Figure 43**) and NTP/papain hydrolysates (**Figure 44**) which implies the NTP with no hydrolysate as a plasticiser is the most thermostable of all. Adding hydrolysates lowers the average protein molecular weight in NTP resulting in it being less thermostable.

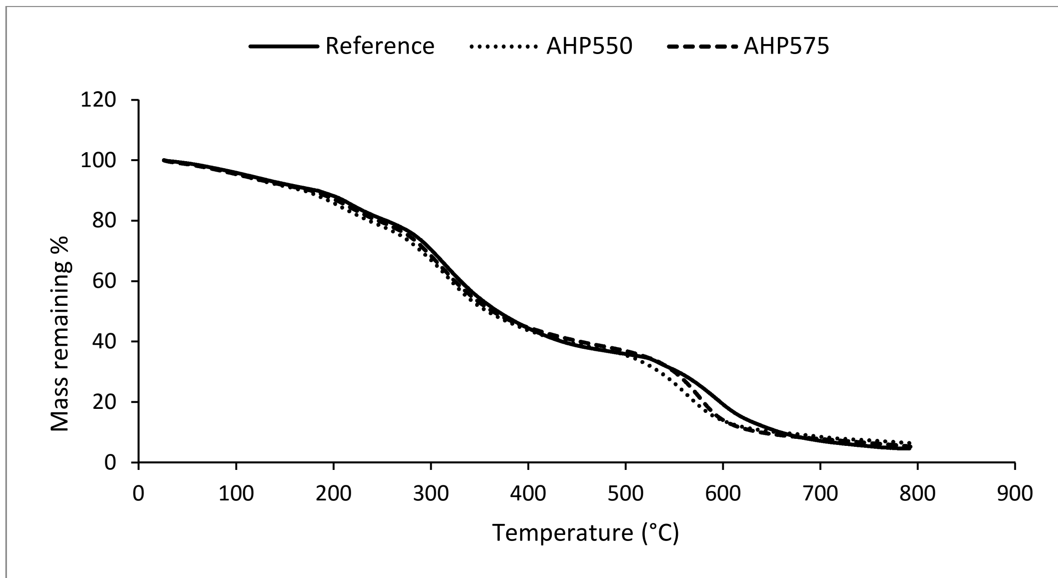


Figure 42: Comparing TGA of NTP/alcalase hydrolysate with reference

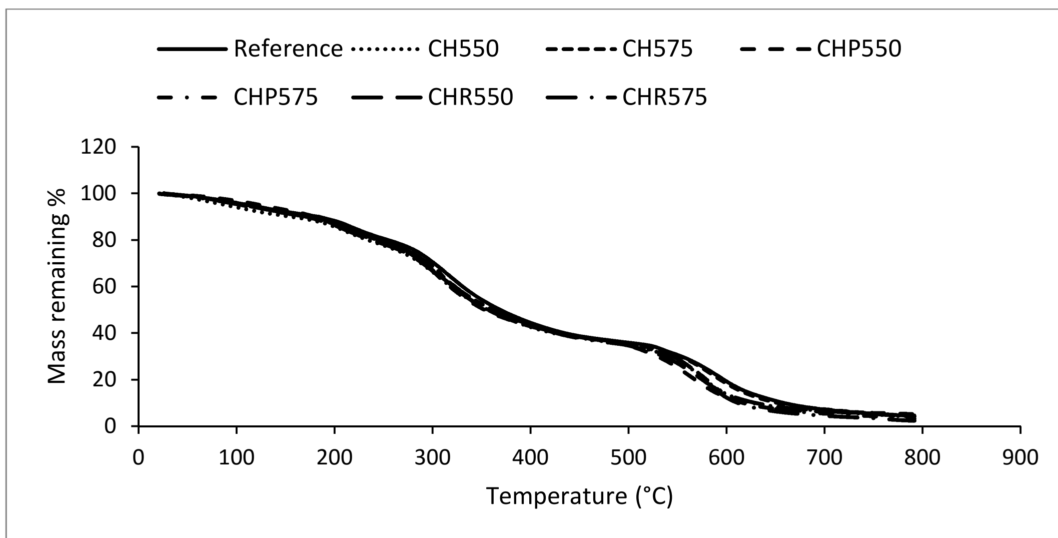


Figure 43: Comparing TGA of NTP/enzyme mixture hydrolysate with reference

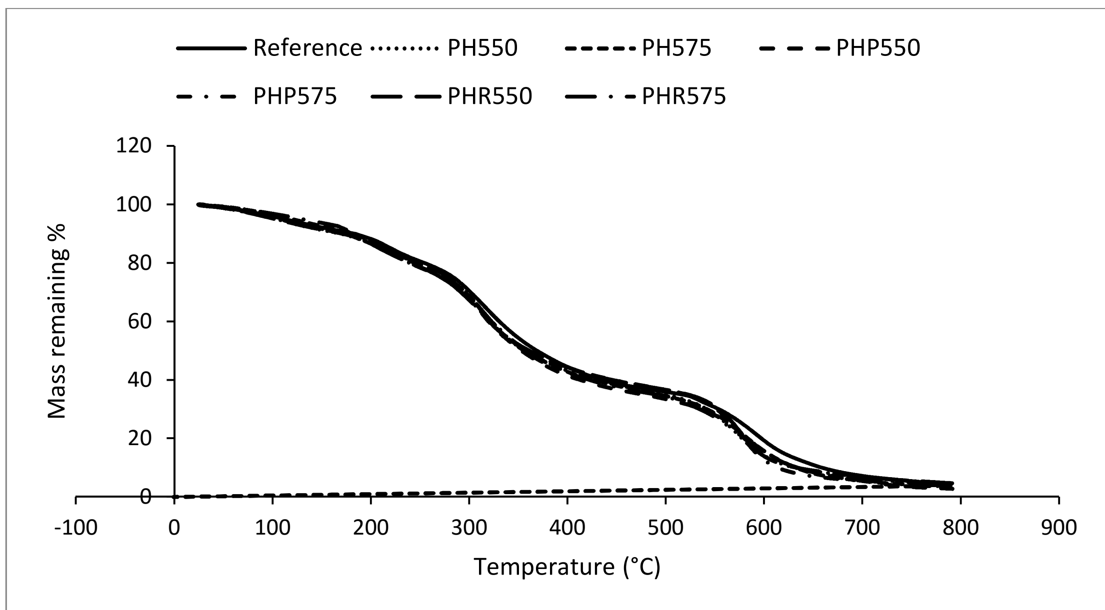


Figure 44: Comparing TGA of NTP/papain hydrolysate with reference

4.5.3 Dynamic Mechanical Analysis

NTP with different formulations has a glass transition temperature in the range of 70 - 80°C with Ref having a T_g of 77.8°C. This temperature contrasts with T_g of pure NTP noted earlier [70]. The difference in these T_g could be attributed to the formulation of this NTP where the protein content of the plastic was kept constant and was prominent.

Table 20: Glass transition temperature of NTP/hydrolysates

Sample	Glass transition temperature (Tg) (°C)
Ref	77.8°C
AHP550	73.8°C
AHP575	77.9°C
EH550	75.9°C
EH575	78.1°C
EHR550	72.2°C
EHR575	77.1°C
EHP550	76.3°C
EHP575	78.8°C
PH550	74.9°C
PH575	76.6°C
PHR550	77°C
PHR575	80.8°C
PHP550	73.7°C
PHP575	78.9°C

NTP with AHP as plasticiser has T_g lower than the Ref with 72°C (**Figure 47**) while it is similar for enzyme mixture hydrolysates and papain hydrolysates (**Figure 45** and **Figure 46**). Comparing **Table 20** with **Figure 45**, **Figure 46** and **Figure 47**, it can be concluded that T_g of NTP is affected by the amount of hydrolysate present in the formulation. The glass transition temperature of NTP decreases as the amount of hydrolysate is increased. Similar trend can be seen in their loss modulus and storage modulus (Refer to **Appendix D** and **E** for graphs of loss modulus and storage modulus).

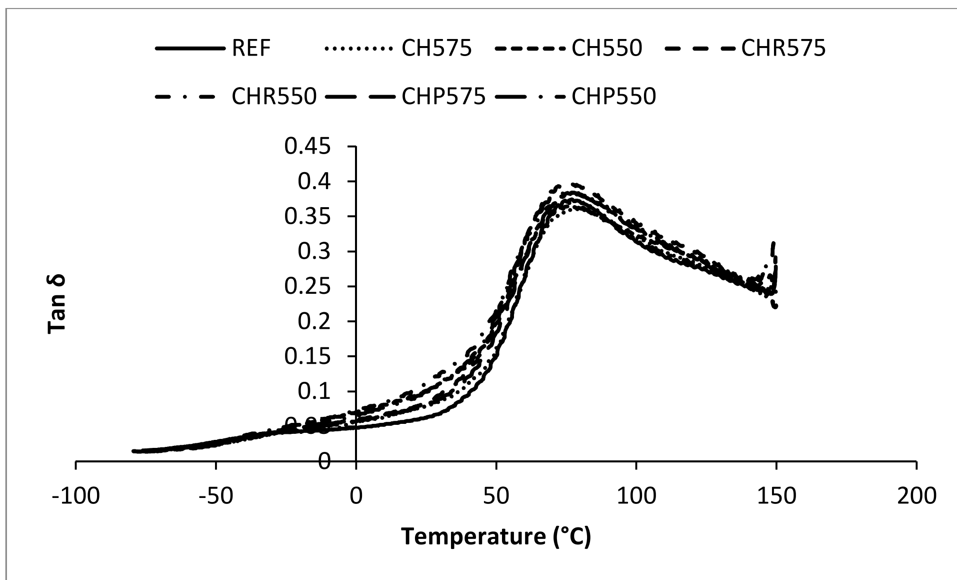


Figure 45: Glass transition temperature of NTP having enzyme mixture hydrolysate as plasticiser

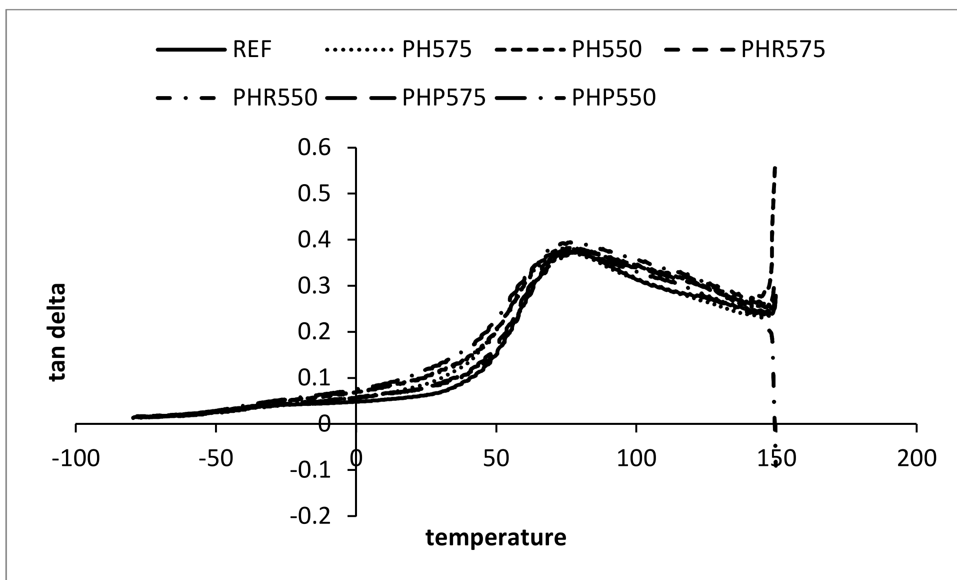


Figure 46: Glass transition temperature of NTP with papain hydrolysate as plasticiser

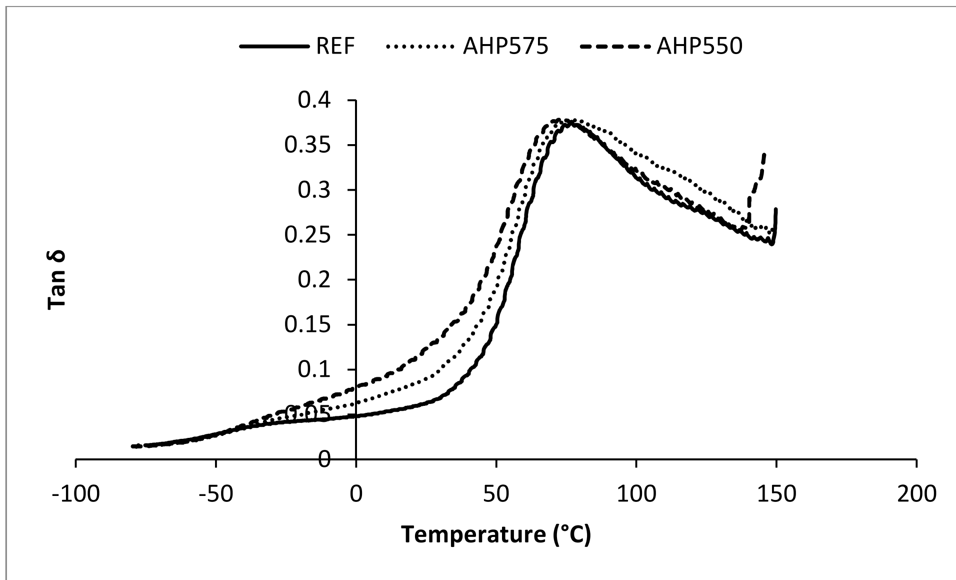


Figure 47: Glass transition temperature of NTP alcalase hydrolysate permeate as plasticiser

4.5.4 Structural

The intensity obtained from XRD was plotted against angle (2θ) which was smoothed with 11 point. Baseline was corrected between 4° and 60° while the peak area was calculated under this baseline region. Cubic spline was used to determine the amorphous region. The spline was fitted between 4° and 60° so that the spline just touched the minima. Amorphous area was then calculated, and based on this value crystalline area was determined by subtracting the amorphous area from the total area. % crystallinity was calculated by [71]

$$X_c\% = \left(\frac{A_c}{A_a + A_c}\right) \times 100\% \tag{7}$$

Where A_c, area of crystalline phase; A_a, area of amorphous phase; X_c, percentage of crystallinity.

NTP with no hydrolysate has a % crystallinity of 23%. Comparing this crystallinity with different hydrolysates (**Figure 48**), it is observed that papain hydrolysates had higher variations that other hydrolysates. NTP with 550g

BM and papain retentate as a plasticiser as a lowest % crystallinity among the three as well as among the group while the original hydrolysate and permeate has % crystallinity higher than the Ref (28% and 26%, respectively). This trend of papain hydrolysate is reversed in NTP when 575 g BM was added with retentate having high % crystallinity among its group (27%) followed by the permeate with 25% and original hydrolysate with 18%. Comparing within each batch of papain hydrolysate (comparison with respect to BM amount), it can be seen that original hydrolysate (PH) and retentate (PHR) have massive difference in their crystallinity while the permeate crystallinity is almost similar. The difference between the crystallinity with each group (PH, PHP and PHR) could be attributed to its hydrolysate content. Increasing hydrolysate content in papain hydrolysates increases % crystallinity while it decreases when hydrolysates are increased in PHR. There is no difference to the % crystallinity when the hydrolysate content is increased in PHP.

NTP with enzyme mixture hydrolysates as a plasticiser had no such trend observed. The % crystallinity is similar for different formulation of enzyme mixture and BM amount. Within each batch of enzyme mixture hydrolysate, with respect to BM mass, permeate (EHP) and retentate (EHR) has slight difference in the % crystallinity while the enzyme mixture hydrolysate has no difference at all. This difference between the crystallinity within each group (EH, EHP and EHR) could be due to its hydrolysate content. Increasing hydrolysate content in EH has no effect on the crystallinity of NTP, % crystallinity decreases in EHP with increasing hydrolysate content and increasing hydrolysate in EHR increases % crystallinity.

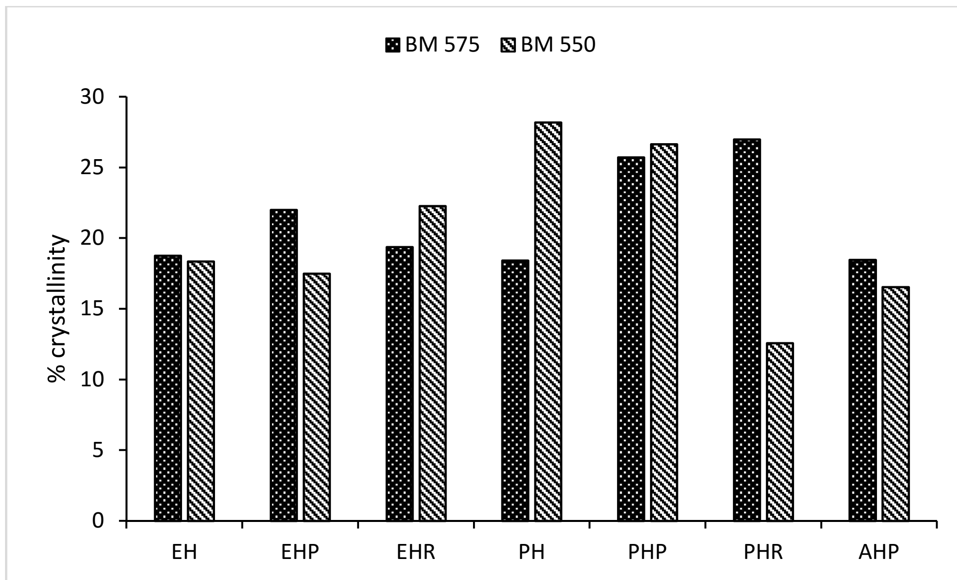


Figure 48: % crystallinity of NTP

% crystallinity of NTP with AHP as plasticiser is similar irrespective to the hydrolysate content.

Within the % crystallinity of three permeates, it can be seen that increasing hydrolysate content in AHP and EHP decreases % crystallinity while it has no effect on the increasing hydrolysate content of PHP. On the other hand, hydrolysate content for the retentate greatly affected its crystallinity. Increased hydrolysate content increased crystallinity of papain retentate and decreased in enzyme mixture retentate.

Chapter 5

Conclusion

The aim of this research was to produce a low molecular weight plasticiser from bloodmeal to replace tri ethylene glycol. This was achieved using alcalase, chymotrypsin/trypsin, and papain enzymes to hydrolyse bloodmeal. Peptides were obtained with molecular weights of 5-12 kDa, all three enzymes gave low degrees of hydrolysis of around 3% (papain) to 13%-14% for chymotrypsin/trypsin and alcalase. Chymotrypsin/trypsin gave the highest yields of 65%, while papain gave a yield of 23% and alcalase of 45%. Rate of hydrolysis was dependent on the amount of enzyme added and bloodmeal concentration, and was also dependent on pH. A low salt content was achieved by carrying out hydrolysis at neutral Ph ultrafiltering the hydrolysate.

Large scale hydrolysis was carried out to produce hydrolysate for inclusion in NTP as a substitute for triethylene glycol. Hydrolysate was also ultrafiltered to produce a permeate with a molecular weight of less than 10 kDa and a retentate. Extrusion of mixtures with hydrolysate produced extrudates with lower specific mechanical energy than standard NTP, and the extrudate had a good appearance visually. Tensile strength, impact strength, secant modulus, energy at break, and glass transition temperature, and thermal stability generally decreased with increasing the amount of hydrolysate added. Standard NTP had tensile strength of 17.6 MPa, impact strength of 2.55 kJ/m^2, secant modulus of 2944 MPa and glass transition temperature of 77°C, while adding hydrolysate reduced its tensile strength, impact strength, secant modulus, strain and energy at break. Impact strength of the NTP/hydrolysate was similar to the standard NTP. Therefore, the hydrolysate was effective as a plasticiser.

Recommendations for future work include:

Investigation of thermal stable proteins such thermolysin, but which is very expensive, because it can produce low molecular weight peptides.

Difficulties were encountered processing the large volumes of hydrolysate with some hydrolysate starting to ferment as it was cooling down. IT is recommended that method of rapidly cooling the hydrolysate is used in future work, or adding a preservative to prevent microbial activity. It also took a long time to centrifuge and filter the hydrolysate to remove unhydrolysed bloodmeal, so a continuous centrifuge or one that can take large volumes of hydrolysate is recommended.

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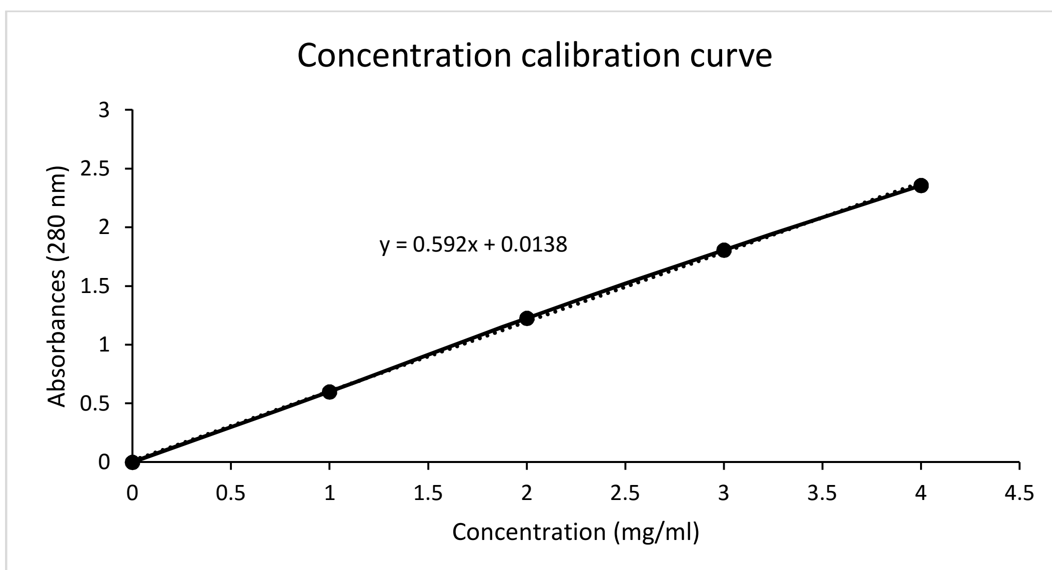
Appendix

Appendix A

Calibration curve used to calculate hydrolysate concentration using known BSA concentrations.

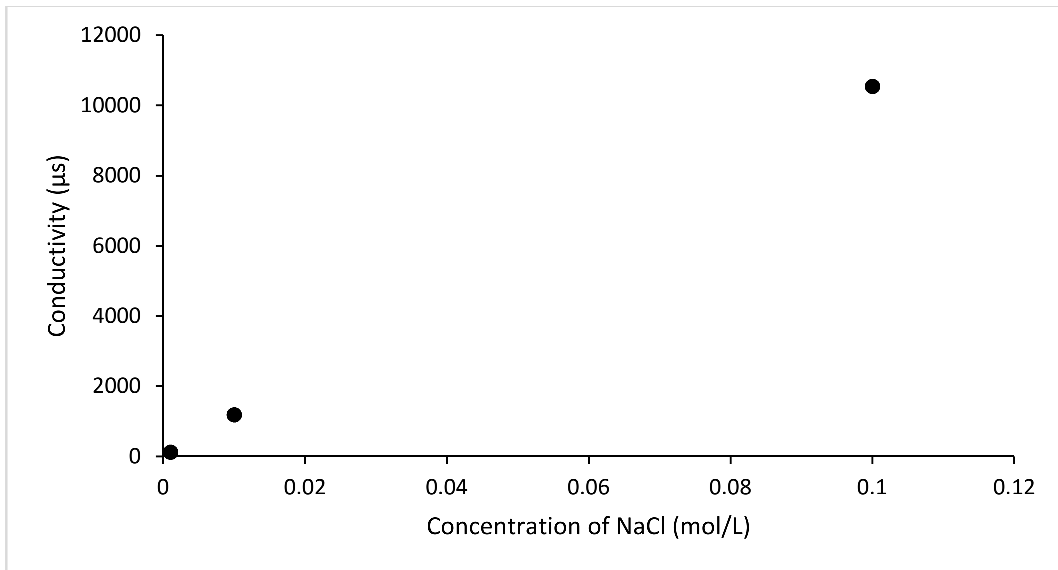


Appendix B

Hydrolysis rate of reaction was calculated by plotting linear trend on each hydrolysis curve and obtaining equations (in the form y = mx + c) for each curve. The m value for each curve is the rate of reaction.

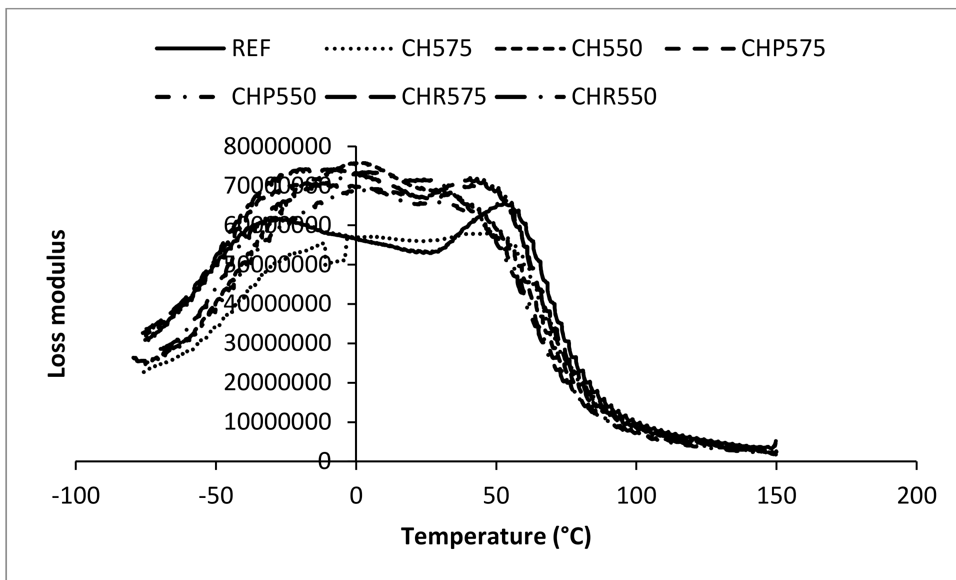
Appendix C

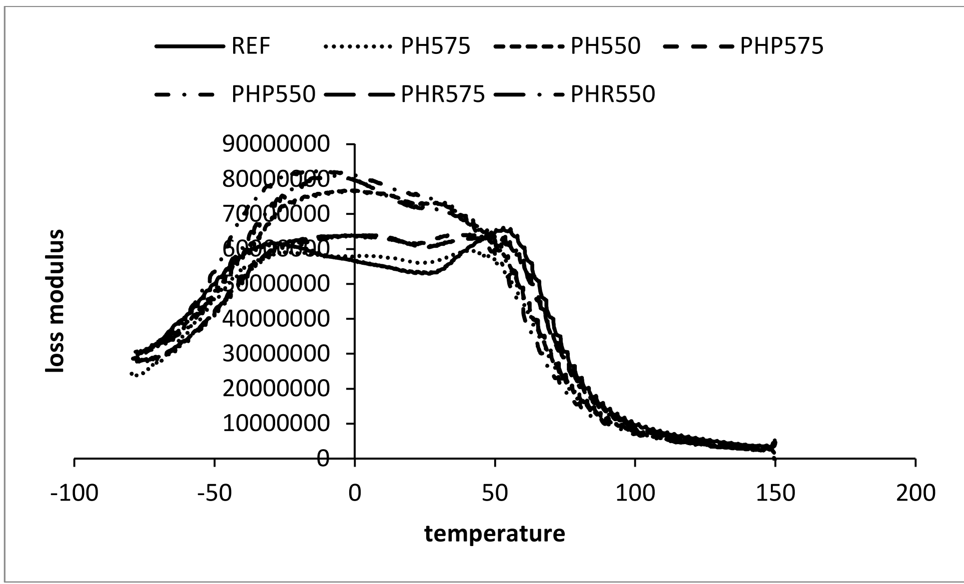
Conductivity calibration curve

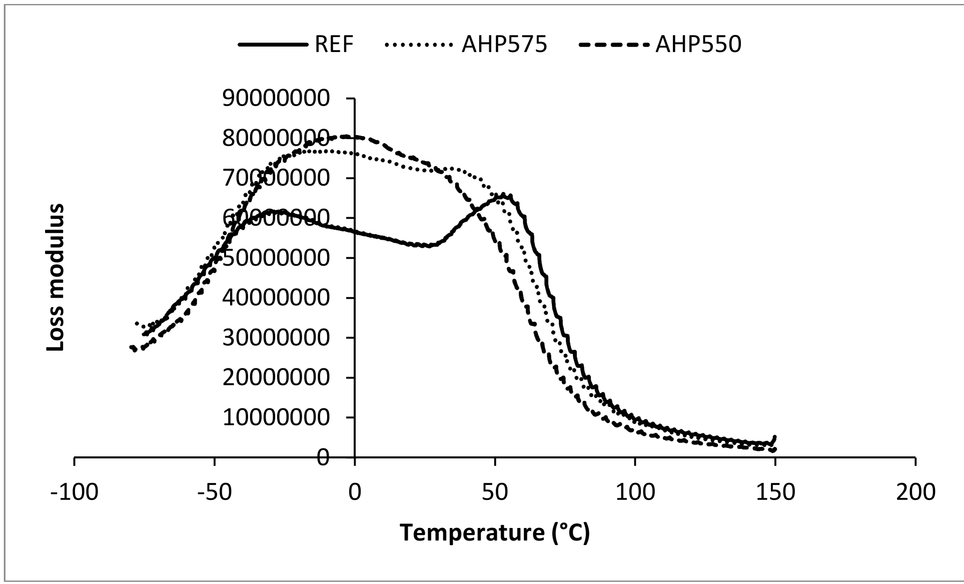


Appendix D

Loss modulus of NTP/hydrolysates

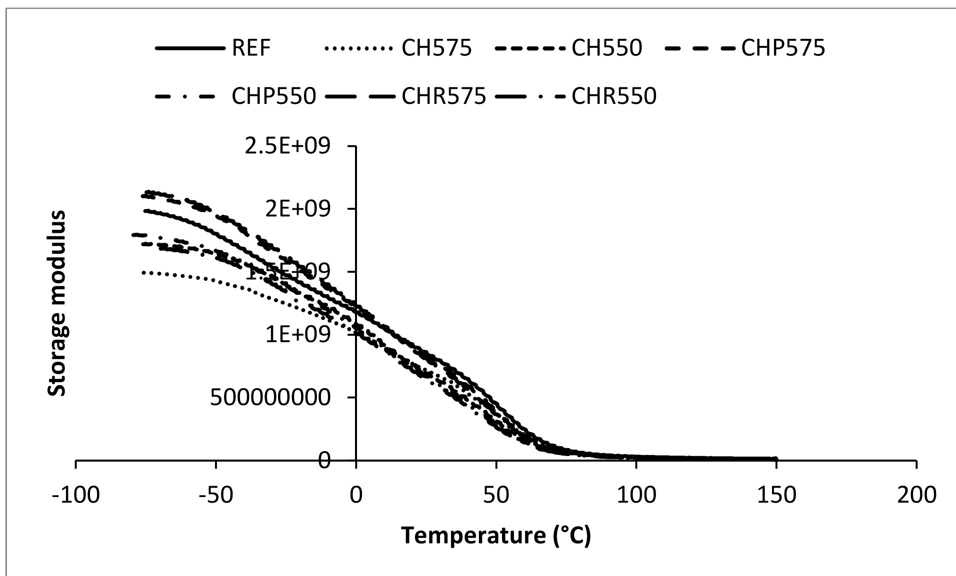


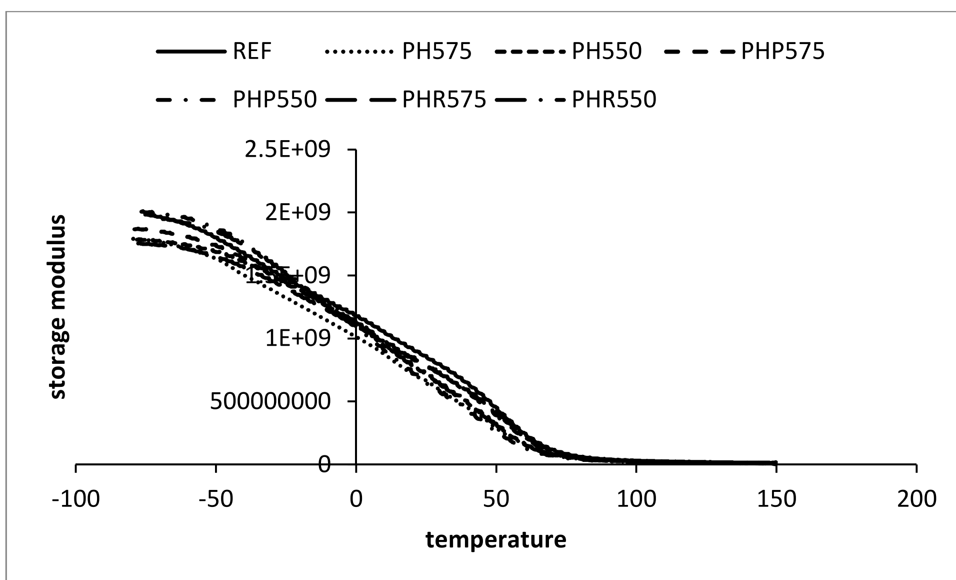

REF
PH575
PH550
PHP575
PHP550
PHR575
PHR550
90000000
80000000
70000000
60000000
50000000
40000000
30000000
20000000
10000000
0
-100
-50
0
50
100
150
200
loss modulus
temperature

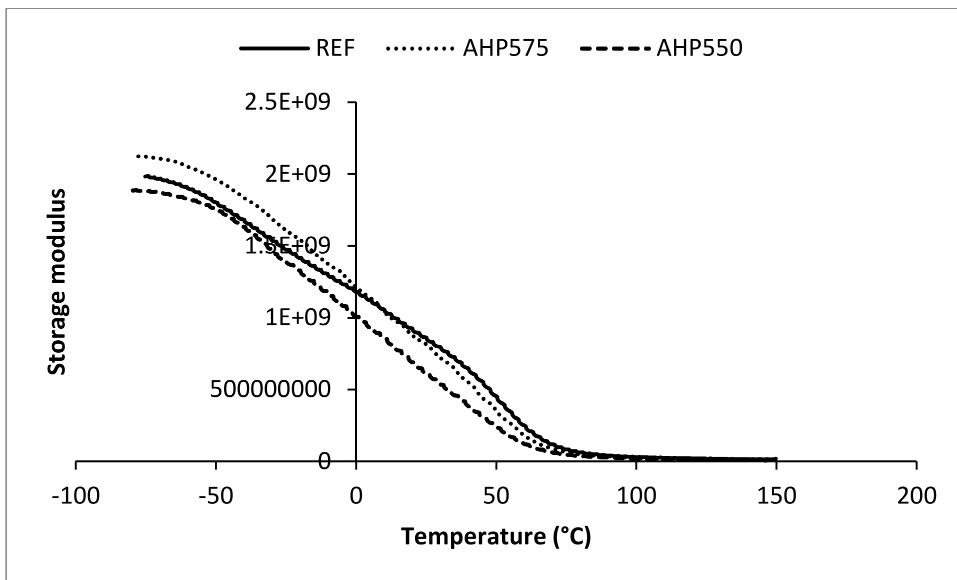

REF
AHP575
AHP550
90000000
80000000
70000000
60000000
50000000
40000000
30000000
20000000
10000000
0
-100
-50
0
50
100
150
200
Loss modulus
Temperature (°C)

Appendix E

Storage modulus of NTP/hydrolysates






REF
AHP575
AHP550
Storage modulus
2.5E+09
2E+09
1.5E+09
1E+09
500000000
0
-100
-50
0
50
100
150
200
Temperature (°C)

Appendix F

Summary table for mechanical properties of NTP with hydrolysate as plasticiser

	secant modulus (MPa)	std dev (MPa)	tensile stress (MPa)	std dev (MPa)	Strain (mm/mm)	std dev (mm/mm)	energy at break (J/m^3)	std dev (J/m^3)	Impact testing (kJ/m^2)	std dev (kJ/m^2)
Ref	2944.69	469.34	17.60	2.53	0.0112	0.0024	0.1684	0.0889	2.551	0.734
AHP550	1349.67	321.25	7.88	1.13	0.1921	0.5686	0.0624	0.0215	2.659	0.897
AHP575	1746.17	387.94	11.57	2.21	0.0111	0.0031	0.0739	0.0347	3.051	0.559
CH550	1081.90	192.19	8.62	1.80	0.0126	0.0036	0.0715	0.0371	1.704	0.397
CH575	1508.27	317.70	14.38	3.83	0.0159	0.0070	0.1805	0.1472	2.361	0.831
CHP550	1523.60	720.04	12.48	2.60	0.0162	0.0052	0.1336	0.0768	2.41	0.522
CHP575	1755.14	626.37	14.34	4.56	0.0119	0.0042	0.1240	0.0992	2.176	0.72
CHR550	1290.99	437.41	9.81	3.26	0.0139	0.0067	0.0992	0.0867	1.388	0.477
CHR575	1667.07	456.91	14.66	3.56	0.0138	0.0037	0.1303	0.0645	2.834	0.837
PH550	1992.31	921.09	11.98	2.01	0.0166	0.0049	0.1510	0.0784	2.199	1.15
PH575	2090.19	357.37	11.69	4.17	0.0105	0.0053	0.1074	0.1035	2.078	0.755
PHP550	1158.43	398.29	12.07	2.65	0.0193	0.0074	0.1630	0.1050	3.111	1.541
PHP575	2456.97	909.66	15.23	2.83	0.0151	0.0049	0.1658	0.0863	2.686	0.626
PHR550	1253.11	700.68	11.59	2.27	0.0185	0.0056	0.1444	0.0783	2.86	0.611
PHR575	1921.57	885.43	15.89	3.34	0.0167	0.0054	0.1948	0.1045	3.581	0.826