University of Alberta

Valorization of waste protein biomass for bio-based plastics, composites and adhesives development

by

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Abstract

The increasing demand and cost escalation of raw materials for industrial chemicals, materials and energy impel the development of sustainable strategies of resource utilization. Such resource demand spurred investigation for the utilization of agricultural wastes and by-products. The emergence of bovine spongiform encephalopathy (BSE) reduced most of the traditional uses of rendered animal meals such as blood meal, meat and bone meal as animal feed. The purpose of this research was to valorize rendering industry wastes for preparation and synthesis of bio-based products.

Specified risk materials (SRM) were hydrolyzed according to two protocols approved by the Canadian Food Inspection Agency and Food and Drug Administration and the recovery of proteinacious fragments for non-food/feed value-added applications were evaluated. Cleavage of recovered proteinacious materials and other characteristics were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion high performance liquid chromatography (SEC-HPLC), and free amino acid determination methods.

The recovered protein hydrolyzates were modified through chemical crosslinking to develop a thermosetting protein based plastics. The plastics made by the crosslinking of protein hydrolyzates with epoxy resins exhibited promising thermal and mechanical performance. Based on these results, biocomposites were also developed by using the protein thermosets as matrices and reinforcing with filling fibers of hemp, woven roving and chopped strand mat glass fibers. Results

showed that the biocomposites developed in this research also exhibited promising flexural strength, tensile strength and tensile modulus; despite relatively poor moisture resistance. In another platform, an adhesive for engineered wood composites such as oriented strand board, was developed from the hydrolyzed material. The performance of the engineered wood with the use of the protein based adhesive was evaluated against a commercial adhesive and a standard.

In summary, this study has showed that using the knowledge of polymer chemistry and material science, the otherwise hazardous waste SRM can be valorized into a feedstock for a range of useful applications including bio-based plastics, adhesives, biocomposites etc. This approach avoids the cost of landfilling, perceived competition of biomass feedstock with food production while at the same time producing an alternative income source for the rendering and livestock industries.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AH	Alkaline hydrolysis
ASTM	American Society for Testing and Materials
BSE	Bovine spongiform encephalopathy
CFIA	Canadian Food Inspection Agency
CSA	Canadian Standard Association
CSM	Chopped stand mat
DMA	Dynamic mechanical analysis
DSC	Differential scanning calorimeter
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GC	Gas Chromatography
HE	Hemp
HPLC	High performance liquid chromatography
LSD	Least significant difference
MDI	Diphenylmethane diisocynate
Mn	Number average molecular weight
MUF	Methyl urea formaldehyde
Mw	Weight average molecular weight
OSB	Oriented strand board
PF	Phenol formaldehyde
PrP^{c}	Cellular Prion Protein
PrP^{sc}	Scrapie infected Prion Protein

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC-HPLC	Size exclusion high performance liquid chromatography
SEM	Scanning electron microscopy
SRM	Specified Risk Materials
Tg	Glass transition temperature
TGA	Thermogravimetric analysis
TH	Thermal hydrolysis
UF	Urea formaldehyde
WR	Woven roving

Chapter 1

Introduction and objectives

Canada has a large agricultural sector. Animal agriculture comprises a substantial portion of this overall agricultural sector, with wastes being generated along the supply chain of slaughtering, handling, catering, rendering etc. In addition, the emergence of bovine spongiform encephalopathy (BSE) reduced most of the traditional uses of rendered animal meals such as blood meal, meat and bone meal as animal feed. Some tissues known as specified risk materials (SRM) that have been sold as meat and bone meal are completely banned from food/feed and fertilizer application unless they go through an approved treatment in many countries including Canada, the US and EU [1-3].

Thus, the investigation of government approved hydrolytic methods as a gateway to safely convert potentially infectious material containing SRM obtained from rendering operations provides opportunities for the development of novel valuable applications and products. Such an approach addresses the environmental concerns and economic loss associated with several million tonnes of these materials already deployed in landfills across North America. Moreover, it provides a much-needed renewable feedstock for high value applications. The objective of this research was to expand our understanding of proteinacious waste utilization, using novel biorefining approaches and to develop a broader protein modification platform for utilizing proteins as building blocks of materials using the concepts of polymer and material science. The hydrolysis patterns of SRMs treated under a range of conditions was investigated using High performance

liquid chromatography (HPLC), Gas chromatography (GC), Fourier transform infrared spectroscopy (FTIR), and Thin layer chromatography (TLC). The modification patterns and macroscopic properties of the developed materials were also investigated using Differential scanning calorimetry (DSC), Thermogravimetric analysis (TGA), mechanical testing and other tools.

Specific objectives of the thesis were:

- Hydrolysis of SRM according to government approved protocols and recovery of valuable feedstock for bio-based industrial applications
- Development of technology platform for the modification of the recovered proteinacious material into polymeric materials
- Development, property evaluation and improvement of bio-based materials made out of the recovered peptides and proteins

As such, the development of various industrial, bio-based products including bio-based thermosetting plastics, biocomposites, and bio-adhesives have been exclusively studied and reported in the subsequent chapters of this research thesis.

In Chapter 2, the use of slaughterhouse waste and rendering industry products as renewable biomass for the preparation and synthesis of products and the respective valorization methods is critically reviewed. It is shown that the products that can be prepared from the transformation of such materials are similar in terms of properties and performance to fossil fuel derived materials. The review focuses on valorization progresses achieved on conversion of protein, fat and ash rich biomass obtained from animal processing co-products into high

value products, such as bio-based plastics, flocculants, surfactants, adhesives, bio-fuels, solvents and drop-in chemicals, minerals and fertilizers.

In Chapter 3, thermal and alkaline hydrolysis of SRM according to Canadian Food Inspection Agency and Food (CFIA) and Food and Drug Administration (FDA) of the US approved protocols were conducted. The study also investigated the recovery and characterization of proteinaceous material by varying the hydrolyzing water concentration for thermal hydrolysis and alkaline solution concentration for the alkaline hydrolysis [4]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Size exclusion high performance liquid chromatography (SEC-HPLC), and free amino acid determination methods were adopted to evaluate the extent of protein cleavage and the molecular weight distribution of the protein hydrolyzates, and to identify the reactive functionalities available. The cleavage of proteins and lipids, generation and degradation of free and total amino acids, and generation of organic acids was also studied as a function of hydrolysis temperature (180, 200, 220, 240 and 260 °C) at subcritical conditions for 40 min as reported in Chapter 4. These works demonstrated the technical feasibility of recovering safe protein hydrolyzates and other ingredients from hazardous protein biomass as a potential industrial resource. The recovered protein hydrolyzates (discussed in Chapter 3 and 4) were used as feedstock for the development of plastics, biocomposites and adhesives reported in subsequent chapters.

Chapter 5 investigated modification of the hydrolyzed proteins through chemical crosslinking to develop thermosetting plastics [5]. In this chapter, the crosslinking

of protein functional groups with epoxy resins were studied using Fourier Transform Infrared Spectroscopy (FTIR). Furthermore, the performance of the resulting thermoset plastics including mechanical strength, solvent resistance at acidic, neutral and basic pH, and thermal properties were investigated and reported. The dependence of the proteinacious plastics performance on the concentration of the hydrolyzed protein in the epoxy resin that influence the viscosity of the pre-mix that in the end change the crosslink density of the thermoset was discussed.

The development of biocomposites based on the protein thermosetting plastics developed by incorporating hemp fiber, and two types of glass fibers (woven roving and chopped strand mat) is discussed in Chapter 6 [6]. The other objective of the chapter was to investigate the mechanical, thermal and moisture absorption performances of the resulting composites. The effects of matrix and filling fiber variation and long term moisture exposure on the performance of the developed plastics were also investigated. Scanning electron microscopy (SEM) studies on the fracture surface before and after 1 month water soaking exhibited that the relatively unremarkable moisture resistance was attributed to the poor interaction of the matrix with the fiber that may require interfacing the matrix and reinforcing fibers.

In Chapter 7, the development of an adhesive technology platform for oriented strand boards (OSB) using hydrolyzed SRM protein extract as feedstock was investigated. The crosslinking of proteins with highly reactive isocynate functional group containing diphenylmethane diisocyanate (MDI) to form a

networked and rigid matrix for OSB adhesives, and evaluation of OSB performance made as such against a control were among the major objectives of this chapter. This study contributes to the global demand of substituting formaldehyde component in common wood adhesives including urea formaldehyde and phenol formaldehyde as a result of its recent categorization as carcinogenic chemical [7]. The last chapter, Chapter 8, provides a concluding discussion that presents the overall valorization of an otherwise hazardous proteinaceous waste as a feedstock for the development of bio-based materials including plastics, composites and adhesives.

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Chapter 2

Literature review: Valorization of slaughterhouse and rendering industry wastes and co-products for industrial chemicals, materials and energy: review[1]

2.1. Introduction

The modern industrial system is built on efficient and large-scale utilization of non-renewable hydrocarbon feedstock. In order to address pressing economic and environmental concerns, significant research efforts are underway to develop technology platforms that enable chemical building blocks compatible with the present hydrocarbon infrastructure using feedstock from renewable and low-value or waste streams. The conversion and utilization of non-edible agricultural feedstock, agricultural by-product and waste streams is a centerpiece in this strategy owing its abundance and relatively widespread geographical accessibility [1]. Animal agriculture constitutes a substantial portion of the overall agricultural sector, with a significant quantity of non-utilized and underutilized by-products that has direct impact on the profitability of the sector not only in the form of loss of revenue but also due to added cost of disposal.

Animal livestock operators report that 40 - 60% of the weight of livestock (including poultry) is not consumed by humans [2]. These non-edible portions of slaughtered animals are collected and processed by the rendering industry to recover hide and skin, fats and oils and protein rich meals. In 2013, the North American rendering industry recycled approximately 27.8 billion kilograms of

[1] A version of this chapter has been submitted for peer-reviewed publication: Tizazu Mekonnen, Paolo Mussone, David Bressler. *Crit. Rev. Biotech.*

perishable material generated by the beef, swine and poultry processing facilities, food processing, supermarkets and restaurants [3]. While a significant portion of the fat fraction is absorbed by the oleochemical industry for the production of cosmetics, feed additives, industrial commodity chemicals, pharmaceuticals and more recently for biofuel production [3, 4], the protein stream finds only limited and low-value market applications. Rendered protein meals such as blood meal, meat meal, meat and bone meal and feather meal are used as ingredients to pet food, aquaculture, poultry and livestock feed industry (Figure 2.1).

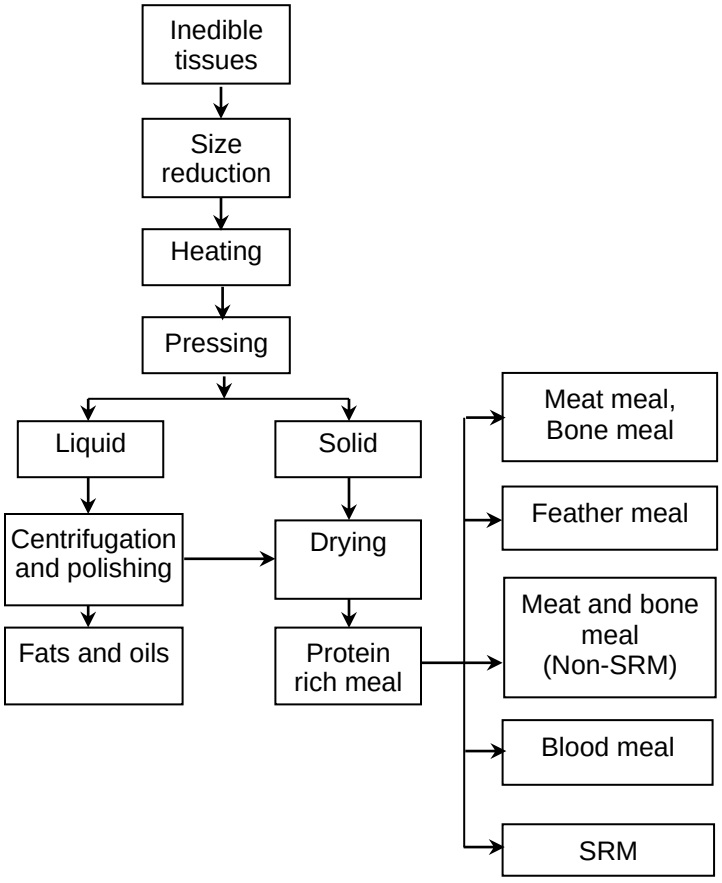


Figure 2.1. Basic process flow of rendering to recover fats and oils and protein rich meal products.

The rendering industry is crucial as it is closely intertwined with the livestock sector, the environment and public health. It is important to emphasize that without rendering process, serious disposal crisis from slaughterhouse, farms, food processors, and restaurants would occur that could challenge environmental and public health [2, 5]. Currently dry rendering is the most common rendering process that uses physical, thermal and chemical processes to convert waste animal products, by-products and mortalities into usable proteins and fats (Figure 2.1). While the current rendering process is shown to destroy foodborne pathogenic microorganism laden raw materials, including *Clostridium perfingens*, *Listeria monocytogens*, *Campylobacter jejuni* and *Salmonella* species [6], it does not inactivate prion proteins that are known to cause transmissible spongiform encephalopathies [2].

The emergence of BSE in the 1980s in England and later in the European Union, Japan, North America and other countries (shown in Figure 2.2) changed the whole paradigm of rendering product utilization. While the use of animal meal (such as blood meal and meat and bone meal) has since been limited to only non-ruminant animals in Canada and the US, the EU has banned it from all animals including fish apart from pets and fur animals as of 2013 [7]. Certain cattle tissues, known as Specified Risk Materials (SRM), are completely banned from use as animal feed, pet food or fertilizer applications in many countries that placed SRM control. These tissues are also effectively categorized as hazardous waste because they are the highest risk tissues to contain BSE causing misfolded proteins, known as prions.

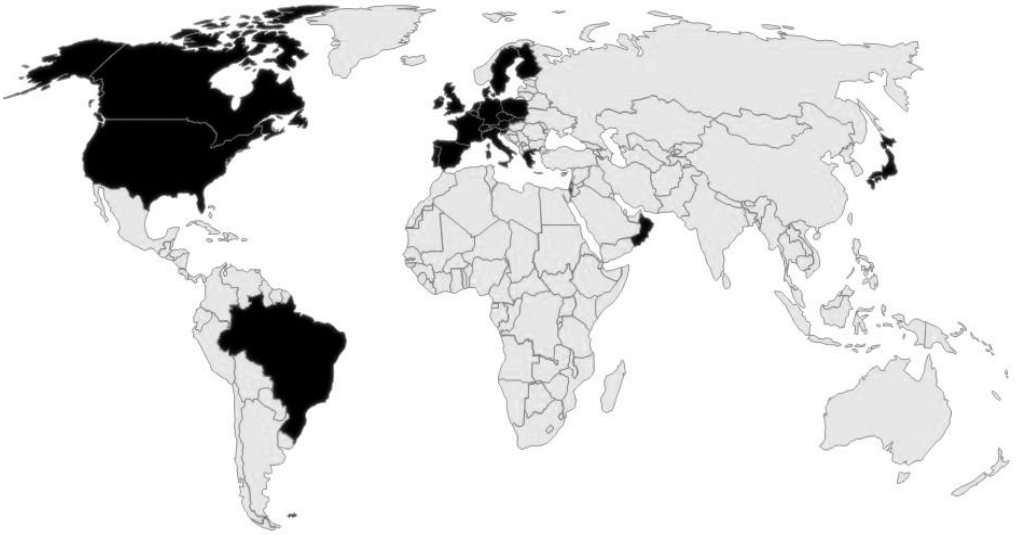


Figure 2.2. Geographical distribution of countries that reported BSE confirmed cases since 1989, based on the data reported by World Health Organization for animal health [8].

As a result of the implementation of these provisions, significant quantity of slaughterhouse wastes and rendering industry lost their economic value. This review addresses current research progress achieved with regard to valorization of these animal co-products for bio-based industrial applications.

2.2. BSE emergence and its economic impact

BSE belongs to a group of fatal neurological diseases of mammals known as transmissible spongiform encephalopathies (TSEs), commonly referred to as "mad cow disease". TSEs include the Creutzfeldt Jakob Disease (CJD) in humans, scrapie in sheep, and chronic wasting disease (CWD) in deer and elk. BSE has been reported in several European countries, Canada, Israel, Japan, the US, Brazil [8] etc. (shown in Figure 2.2).The infectious agent causing TSE is a misfolded, β-sheet rich protein known as prion PrP^{Sc}, particularly in the nervous

and lymphatic systems [9]. Prion proteins are extremely resistant to natural degradation and conventional decontamination techniques such as heat, chemical disinfectants, boiling, irradiation and cooking that are effective with microorganisms [10-13]. This obviates that regular rendering process could not destroy prions that exist in infected animals.

2.2.1. BSE emergence and its economic impact: the case of Canada

In 1997, Canada adopted a feed ban that prohibits proteins derived from most mammals from being fed to ruminant animals (such as cattle, sheep, goats, deer, elk bison etc.) acting on the recommendations of the World Health Organization. Following the discovery of the first BSE positive cow in 2003, Canada defined high risk tissues known as specified risk material (SRM), and excluded them from the human food chain, and from being fed to ruminants but not to other species [14]. In 2007, the Canadian Food Inspection Agency (CFIA) implemented an enhanced feed ban that eliminates SRMs not only from ruminant and human food chain, but also from all animal feed, pet foods and fertilizer applications [15]. SRMs, which include the skull, brain, trigeminal ganglia, eyes, spinal cord, and dorsal root ganglia from cattle over 30 months of age and the distal ileum and tonsils from cattle of all ages are illustrated in Figure 2.3.

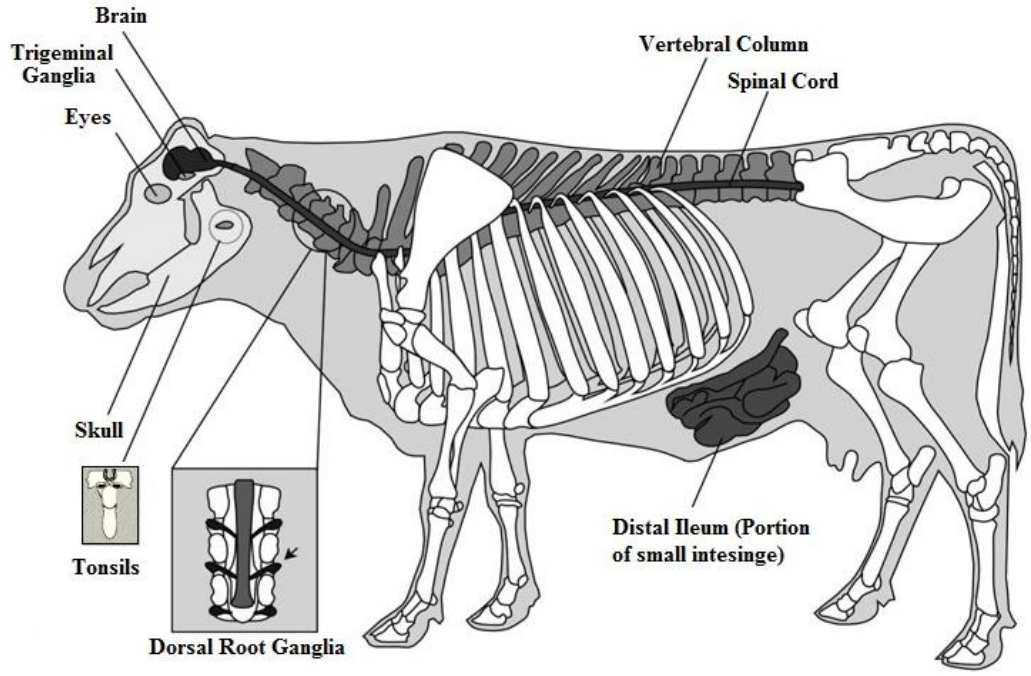


Figure 2.3. Specified risk material tissues of cattle in Canada and the US [16].

After the 2003 BSE outbreak in Canada, the US government banned imports of Canadian beef to minimize the risks to the US beef industry. Moreover, the US placed Canada under its BSE restriction guidelines and stopped accepting any ruminants or ruminant products from Canada [17]. Pre-2003, more than 18 % of all live cattle export, and 32 % of all beef slaughtered within Canada, was exported to the United States [18]. For example, Canada exported more than 47 % of its beef production to the world market in 2002, however that entire export opportunity was closed in 2003 [19]. The disruption of these exports caused severe repercussions to the country's livestock sector. Pre-2003, prices for cattle in Canada and the US were closely aligned. The crisis however resulted in price collapse in Canada and price rise to a record high in the US [17, 18, 20]. Canada beef producers gained some relief as the US partially lifted its ban in

August 2003, by allowing imports of boneless beef from Canadian cattle slaughtered less than 30 months of age.

In response to the discovery of BSE in 2003, Canadian renderers restructured their facilities into species specific by segregating handling of ruminant and non-ruminant in separate plants [21]. In order to comply with the enhanced feed ban of the 2007, a second restructuring involving the construction of entirely separate infrastructures, including dedicated SRM lines, was implemented. Furthermore, the dedicated line processed SRM has to be separately stored, and trucked to a government-permitted landfill [15, 21].

The most common method of handling SRM in Canada remains rendering to recover lipids while the remaining fractions are landfilled. Currently, over three hundred thousand tonnes of such rendered SRM, previously sold as meat and bone meal, are disposed of to the landfill annually or incinerated, posing economic challenges to the rendering industry with repercussions to the whole livestock industry. These challenges include costs attributed to segregation of SRM from non-SRM animal tissues, segregation of processing lines to handle SRM and non-SRM tissues, and costs associated with SRM storage, transporting and disposal fees. Disposal tipping and transportation fees range from $75 to $200 (depending on jurisdiction) per tonnes on average [12]. The cost of producing tallow for feed has also increased as a result of the regulatory requirement that bans the feeding of ruminant fats containing more than 0.15 percent of insoluble impurities, since most tallow now has to be polished to achieve the standard set by the regulation [15]. The non-SRM meat and bone meal, and blood meal are

still allowed to be fed to non-ruminant animals such as hogs, poultry etc. (Table 1). However, only Philippines and Indonesia are the existing export market destinations for Canadian meat and bone meal beside domestic consumption.

2.2.2. BSE emergence and its economic impact: the case of the US

Similar to Canada, the US also banned feeding mammalian tissue to ruminants in 1997. In response to the discovery of the first BSE infected cow in Washington state of the US, beef importers, notably Japan, Korea and Mexico began importing from alternative supplies that curtailed beef and cattle exports from the US that resulted in drop-off of domestic prices for cattle and beef [19]. These resulted in new regulations to enhance protections against the spread of BSE and to reassure consumers that beef was safe to consume [22]. These regulations include listing of SRM tissues and banning these tissues from human and animal consumption. Canada [16] and the US [23] enlisted similar high risk tissues (SRM) as illustrated in Figure 2.3.

The current status on the utilization of animal meals as feed, food and fertilizer in Canada and the US is shown in Table 2.1.While by-products such as meat and bone meal and blood meal are still an important segment of income for the meatpackers, renderers, and the overall livestock industry, significant value was lost by the disposal of SRM that used to be sold as meat and bone meal. Furthermore, the reduced export demand of the rendered products, price decline as a result of its restriction and cost incurred for SRM disposal were added burden to the industry.

Table 2.1. Feed ban controls on rendered animal products in Canada and the US [15, 23].

Rendered meal	Ruminants	Non-ruminants	Pets	Fertilizer
Blood meal (ruminants)	NA	A	A	A
MBM (ruminants)	NA	A	A	A
Blood meal (non-ruminants)	NA	A	A	A
MBM (non-ruminants)	NA	A	A	A
Fish meal	NA	A	A	A
SRM	NA	NA	NA	NA

A- Allowed; NA – Not allowed

2.2.3. BSE emergence and its economic impact: the case of European countries

The recognized association between BSE and feeding of ruminant derived meat and bone meal (MBM) to cattle led to the introduction of the 1988 UK ban on feeding ruminant-derived proteins to ruminants [24]. The UK introduced also a complete ban of other high risk bovine tissues, such as the brain and spinal cord from human food, and from entering rendering facilities that still produce MBM for consumption by non-ruminants [25]. The 1992 downturn of BSE incidence in the UK [8] is an implication of the effect of the feed ban in 1988. The reason for the delay could be because of the possibility of BSE exposure at calfhood and the

average age at which cattle display clinical signs of BSE, that is around five years [25].

Additionally, the UK in 1995, and the European Union in 2001, banned the use of all processed mammalian protein in fertilizers and feeds for farmed animals [26]. The European Union's ban includes the prohibition of feeding these mammalian proteins to non-ruminants such as chicken and hogs. Similar hazardous SRM tissues that are completely removed from the food chain of animals and fertilizer applications in North America (Figure 2.3) are also completely removed across the European Union. Moreover, the use of mechanically recovered meat from all ruminant bones are also banned in 2001 [27]. From 1996 to 2005, the UK implemented a ban that eliminated the use of a cattle older than thirty months for human consumption [27]. This regulation turned European rendering industry into an almost waste disposal industry.

Though the BSE outbreak involved relatively small numbers of livestock outside the European landmass, policy and regulatory implementation has still resulted in large changes in protein feedstuff markets. The changes on the use of rendering products and by-products occurred even in countries with no confirmed cases of BSE, as every country imposed regulations to prevent the spread of the disease [20]. This caused a far-reaching impact to consumers, rising environmental costs associated with disposal of hazardous materials, loss of value for the rendering industry, including a decline in value of meat and bone meal; and costs of supply disruptions and substitutions within the feed market sector

[20]. These created a strong demand for technological solutions to valorize the animal co-product biomass into non-food/feed value added applications.

2.3. Valorization of animal processing co-products and wastes

Animal co-products are composed of three principal ingredients: proteins, lipids and ashes in varying quantities. The perishability and inherent variability of the ingredients in each stream represents a major challenge for direct processing of such materials into valuable products. Rendering industries produce low moisture content products (less perishable) that are protein and ash rich co-product and waste biomass (such as blood meal, meat and bone meal, feather meal and SRM) and lipid rich biomass including fat and grease. With regard to rendered SRM waste there are four government approved methods of disposal in Canada [16] and the US [23]: alkaline hydrolysis, thermal hydrolysis, incineration, and landfilling at designated sites. However, rendering of SRM to recover fats and landfilling the remaining fractions is by far the most common SRM disposal method in North America [12] and combustion and incineration in EU countries [28]. Valorization of these animal co-products and wastes into high value products using biorefinery approach is shown in Figure 2.4, and the conversion to various bio-based products is reviewed in the following sub-sections.

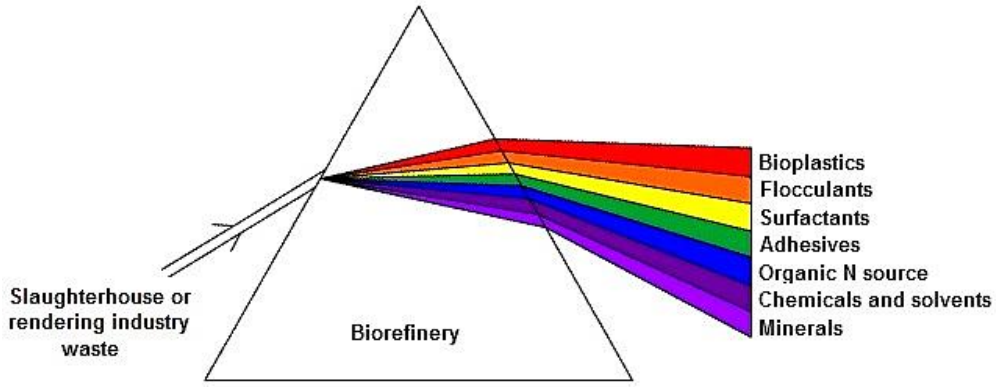


Figure 2.4. Valorization model of slaughterhouse and rendering industry waste.

2.3.1. Conversion of protein biomass into bio-based plastics

Plastics are amorphous and semi-crystalline organic polymers covering a wide range of polymerization products suitable for the manufacture of diversified products. Worldwide annual plastics production is expected to surpass 300 million tons by 2015 [29] representing trillions of dollars in terms of global economic returns [30]. Plastics are highly valued materials that constitute the second largest petroleum application only next to utilization in production of energy-related commodities [31]. The material science community has been striving for decades to generate bio-based plastics to displace or complement synthetic plastics.

The first generation of bio-based polymers developed from renewable feedstocks is represented by poly (lactic acid) (PLA), polyhydroxyalkanoates (PHAs), starch plastics, cellulose esters and protein based plastics. Other bio-based plastics, such as bio enriched polyurethane manufactured using modified vegetable oils, polyethylene monomers derived from the dehydration of bio-ethanol that have at least have partial renewable source constitute a set of

emerging technologies that are expected to make a significant market impact in the near future [32]. The focus of the second generation of bio-based polymers is on developing materials from the efficient utilization of inedible or waste streams or low-value by-products from rendering and slaughterhouses, food processing and agricultural industries [33].

In general, the utilization of proteins as a bio-based material feedstock hinges of the rational exploitation of the functional groups associated with the end group of protein chains and the amino acid residues for chemical modification. Moreover, their capability to form a continuous and cohesive matrix received attention for the production of biodegradable plastics films and sheets [32, 34, 35]. A notable example of successful utilization of low-value animal protein is represented by bloodmeal. Bloodmeal is non-hazardous with regard to BSE contamination, in addition to the high protein concentration it contains (about 90%) [36] that avoid the common protein concentrating efforts in other rendering products such as meat and bone meal, that allow its direct modification into thermoplastics. Bloodmeal thermoplastic processing has been extensively studied by researchers at Waikato University in New Zealand and commercialization of this technology is underway [33, 36-42]. Protein based films and coatings in general are known to be stiff and brittle due to the extensive intermolecular interactions between protein chains through hydrogen bonding, electrostatic forces, hydrophobic bonding and disulfide cross-linking similar to other plant proteins [43]. Hence there is a strong necessity of modification to achieve a useful material.

Plastic materials based on bloodmeal protein can be adequately formulated with specific modifiers to produce seeding trays, planting pots and mulching films that are cost competitive with petroleum based plastics for similar applications [38, 44-46]. Plasticizers are incorporated to improve processability, reduce brittleness and modify the properties of the final structure [32, 47]. Denaturants are added to disrupt secondary structures such as beta sheets and increase chain mobility during processing [37, 48, 49].

Other animal protein streams, including meat and bone meal and chicken feathers meals have been successfully converted into thermoplastic and thermosetting materials. Some studies reported direct processing of animal meals [38, 42, 46, 49] into protein plastics, others researches reported [50-52] the use of extracted proteins for the development of protein-based plastics. As shown in Table 2.2, direct processing of bloodmeal with 20% plasticizers resulted in a plastic with high ductility. On the other hand, direct processing of meat and bone meal into plastics with 30% plasticizer exhibited lower elongation. The reason for this observation can be attributed to the physical and chemical characteristics, including the amino acid composition, of the feedstock. Bloodmeal is composed of about 90 % proteins [36], whereas meat and bone meal is composed of about 50 % protein and 35 % ash [46]. Ash in meat and bone meal acts as inorganic reinforcing filler making the meat and bone meal based plastic a composite with high modulus and low ductility behavior.

The marked elongation difference observed between 30% propylene glycol and 30% ethylene glycol plasticized chicken feather protein extracts (Table

2.2), was attributed to the plasticization efficiency [45]. Dynamic mechanical analysis (DMA) and Fourier transform infrared spectroscopy (FTIR) studies [44] exhibited that ethylene glycol, being a smaller molecule, have higher ability to reduce polymer-polymer associations and interact with the protein polypeptide chains through hydrogen bonds compared to propylene glycol.

Thermosetting protein based plastics developed from crosslinking of hydrolyzed SRM protein extracts with epoxy resin exhibited a different set of mechanical performance compared to the thermoplastic proteins. Such materials are characterized by high tensile moduli, intermediate tensile strengths and relatively low elongations at break (Table 2.2) [52] similarly to synthetic thermosetting plastics [53]. Potential applications of these SRM based plastic materials include adhesives, matrix of composites and biocomposites, concrete and flooring [51, 52, 54].

Table 2.2. Mechanical properties of plastics based on animal co-product and waste as partial feedstock

Resource	TS (MPa)	Modulus (MPa)	ε (%)	Comment
Bloodmeal [38]	5.7	251	53	Plasticized with 20% Tri (ethylene glycol)
Meat and bone meal [46]	3.96	341	2.1	Plasticized with 30% glycerol
Chicken feathers protein extract [45]	22.3	811.2	7.6	Plasticized with 30% propylene glycol
Chicken feathers protein extract [45]	17.8	354	43.8	Plasticized with 30% ethylene glycol
Feather quill protein extract [44]	20.5	529.5	11.2	Plasticized with 30% propylene glycol
SRM hydrolyzate protein extract [52]	58.56	1463	4.34	Used as epoxy crosslinker (30%)
Native chicken feather **[55]**	55.7	4400	14.2	Grafted with methyl acrylate, and plasticized with 30% plasticizer
Native chicken feather **[55]**	206.3	28,800	1.1	Grafted with methyl acrylate, no plasticizer

TS – Tensile strength at break; ε- elongation at break

A major limitation to the industrial deployment of protein-based plastics, with the notable exception of keratin, is their sensitivity towards humidity [56]. For example, Zheng *et al*. [56] reported that soy protein sheets submerged in water for 20 h absorbed moisture up to 180 % of their initial weight. The presence of hydrophilic protein functional groups, including –NH_2, –NH, –OH, –SH is the molecular driver for this phenomenon. A possible solution to this problem could be the formation of covalent bond bridges between proteins hydrophilic moieties to form a thermoset using a crosslinking agent. For example, the crosslinking of hydrolyzed SRM protein extract with glutaraldehyde resulted in only 5-10% weight loss of the resulting bio-based plastic under 24 h water soaking [50].

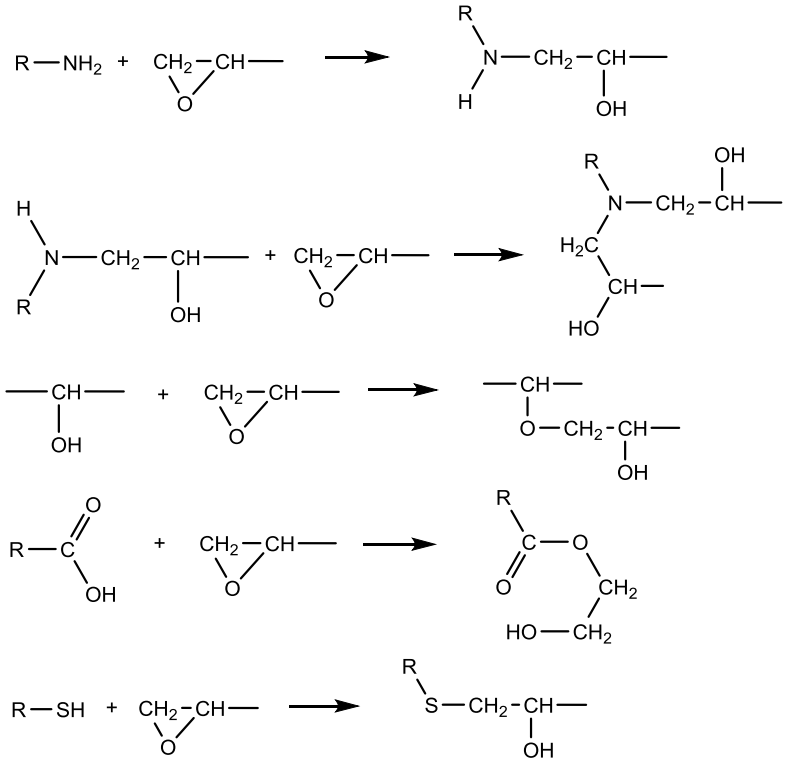


Figure 2.5. Crosslinking reactions between functional groups of proteins (-NH2, -NH, -OH, -COOH and –SH) and epoxide functional group of epoxy resins [52].

SRM protein hydrolyzate extracts are very soluble in water [12]. However, SRM hydrolysates crosslinked with epoxy resins, compounds with epoxide functional groups capable of forming covalent bridge between amine (primary and secondary), hydroxyl, carboxyl, and sulfhydryl groups as shown in Figure 2.5 [51, 52, 57, 58] are virtually insoluble in water. Figure 2.6 shows that the moisture resistance of the SRM based plastics is of the same order of magnitude to other commercial epoxy polymers cured with diamines [59]. The observed variations in water resistance of these plastics were due to variation in crosslinking efficiency as influenced by the molecular dynamics of the hydrolyzed protein in epoxy resin.

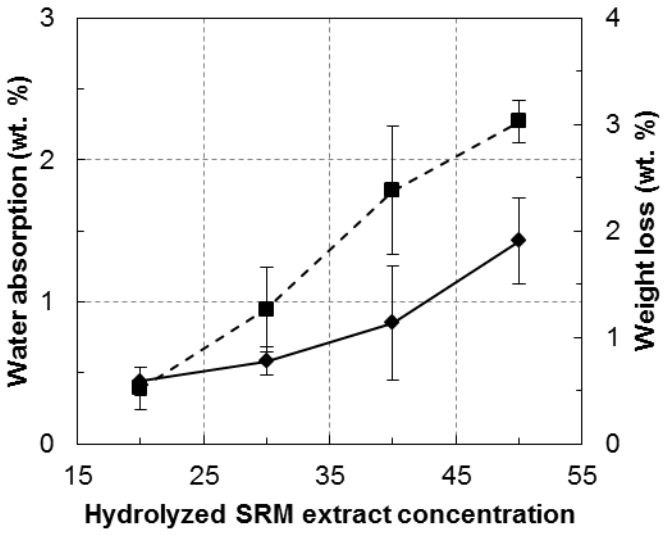


Figure 2.6. Water absorption (-◆-) and weight loss (-■-) performance of epoxy cured SRM extract based plastics at various concentrations based on data from Mekonnen *et al*. [52].

Another strategy pursued to develop thermoplastic materials using animal proteins is the graft polymerization of proteins with synthetic polymers. This

approach keeps the thermoplasticity of the original proteinaceous material that provides ease of processability using standard extrusion, compression or injection molding approaches and possibility of recyclability of the final products. For example, Jin *et al.* [55, 60] recently demonstrated the graft polymerization of native chicken feathers with methyl acrylate for thermoplastic applications. The study showed that under optimal polymerization conditions, up to 97 % monomer conversions, 35 % grafting with an efficiency of 78% was achieved. The films developed by compression molding had high values of tensile strength (206.3 MPa) and modulus (28.8 GPa) (Table 2.2). The poor thermplasticity of native chicken feathers have also been improved through the graft polymerization. Further improvement in ductility was also observed when the developed material was plasticized with glycerol. Therefore, grafting holds the potential to combine the advantage of proteins and the grafted synthetic polymers to generate hybrid materials.

2.3.2. Conversion of protein biomass into industrial flocculants

Another emerging industrial application of animal waste proteins is as feedstock for the production of coagulants and flocculants for wastewater treatment. Flocculation is the process of addition of chemicals to promote the separation of colloidal solid-liquid suspensions through particle aggregation [61]. Coagulants destabilize colloidal suspensions that were formed as a result of electrostatic repulsion between negative charge carrying particles. Flocculation process is one of the most widely used techniques for water and industrial wastewater treatment, sludge dewatering, oil recovery and de-emulsification. The

use of flocculants could also be further extended to applications in soil erosion control and soil conditioning in agriculture and construction [62], oil recovery and de-emulsification [63], water and wastewater treatment [64], paper manufacture [65], concentration during chemical operations such as flotation [63, 65], dewatering and thickening in mineral processing [66] etc. Common synthetic polymers used as flocculants include polyacrylamide, polyacrylic acid, poly (styrene sulphonic acid), poly (diallyl dimethyl ammonium chloride) [64, 67, 68].

While synthetic flocculant polymers can be effective at very low dosage, the resulting flocs are typically unstable under shear, limiting their range of applications. Additional challenges are posed by unsatisfactory chemical stability of some of these synthetic polymers and by the demonstrated toxicity of their monomers to aquatic life forms. These factors have driven the development of non-toxic biomass-based flocculant systems such as starch, guar gum, chitosan cellulose, alginate, glycogen, dextran etc.[68-70]. Microbial extracellular biopolymeric materials have also been investigated as renewable flocculants [71].

A key driver in the utilization of proteins as feedstock for flocculants is their high solubility in aqueous medium and their polyampholite nature combined with the functional groups of the composing amino acids and. The flocculating activity of proteins, similar to other synthetic polymer flocculation, could take place through a variety of mechanisms, including electrostatic patch flocculation and bridging [67, 72, 73]. The potential of proteins as renewable flocculants through various modification techniques is a rapidly growing area of research. Bioflocculants from soy protein [74, 75], egg albumin [76, 77], milk casein [78,

79] modified by methylation has been reported as efficient flocculant for wastewater treatment. While these systems have been demonstrated in the laboratory, concerns over the utilization of high-value edible proteins in industrial applications are likely to persist and discourage the implementation of these systems on a large scale.

The use of meat and bone meal extract and gelatin [80, 81], chicken blood extract [82], bovine blood extract [83], hydrolyzed meat and bone meal extract [84, 85] flocculation potential has been investigated and reported in the literature. Similar to the other renewable feedstocks, modification of protein is essential to enhance its flocculation capability. Most of the reported modification techniques involve charge modification either by varying the pH of the aqueous solution or by capping some functional groups of the protein. The other protein modification technique is grafting of the protein with other synthetic polymers.

The protein charge modification by either varying the pH of the solution or capping the functional groups is to provide the protein net positive charges that would result in binding of the mostly negative charged colloidal particles (such as clay, diatomite etc.) through electrostatic interactions [86]. Since protein molecules achieve a net positive charge below their isoelectric point, acidification of the solution below their isoelectric point provide the desired flocculation activity. The use of divalent cations that could serve as a bridge between the protein and colloidal particles are also reported to improve the flocculation efficacy of animal proteins [82, 84]. Methylation of proteins, usually with alcohols, aims to cap the carboxyl functional group (main negative charge

contributor) that would give rise to a cationic protein [87] (Figure 2.7). As such, methylation could be considered as an alternative to acidification of protein.

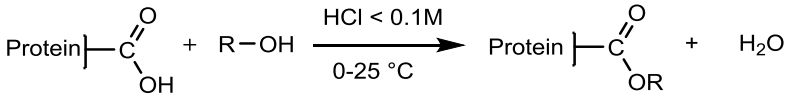


Figure 2.7. Methylation of proteins with alcohols catalyzed by mineral acids.

For instance, the successful development of methylated soy protein based flocculant and its performance was investigated with suspensions of kaolin clay and loam from deposits of volcanic ash by Liu *et al*. [75]. The results were compared with another well studied bio-based flocculant chitosan in terms of clarification efficiency and settling rate at various pH ranges in the same study as shown in Figure 2.8. Results of the study showed that methylated soy protein showed much higher flocculation performance than chitosan at pH 3–7, in terms of both the clarification efficiency and the settling rate. The clarification efficiency of methylated soy was almost the same as that of chitosan at pH 7–10, however the settling rate of floc formed by chitosan increased sharply at pH 7–8 and surpassed that formed by methylated soy protein [75]. While the major limitation of developing methylated protein based flocculant is the abatement of solubility, it is however possible to balance the level of methylation and keep the solubility at the desired level.

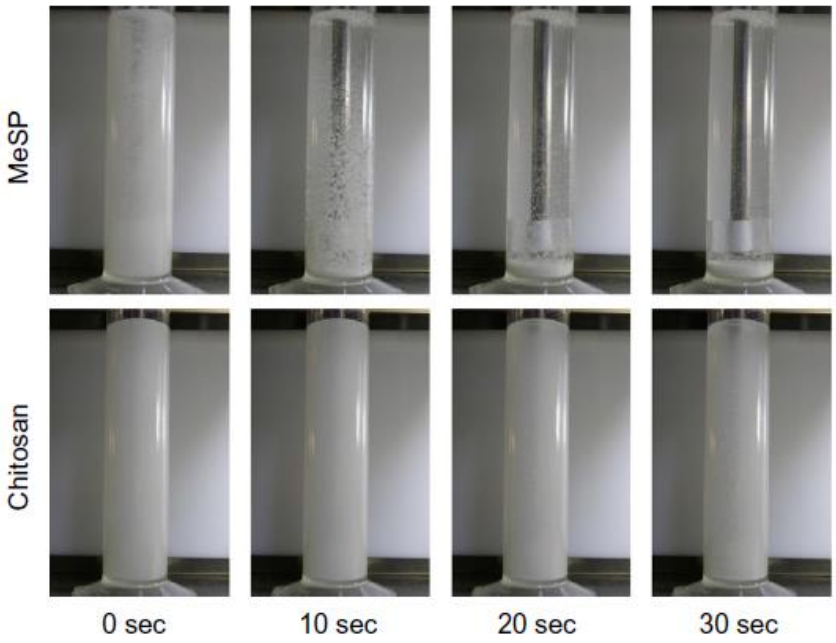


Figure 2.8. Photographs of jar test of kaolin (1wt. % suspension) flocculated with methylated soy protein (MeSP) and chitosan systems. The concentration and pH of the suspensions were 3g/L and 7, respectively. (Adapted from Liu *et al.* [75] Elsevier © 2012).

Grafting of proteins with synthetic polymers is an emerging research area with important application in diverse field of applications including drug delivery, biotechnology and nanotechnology [88-90]. However, studies of grafting of proteins for flocculant applications are nearly absent in the literature. Sinha *et al.* [91] have recently investigated and reported grafting of casein with PAM for flocculant application. According to the groups investigation, the grafted co-polymer of casein and PAM exhibited a higher hydrodynamic volume/higher radius of gyration of the macromolecule [91] that led to higher flocculation efficacy [92]. Higher grafting was indicated to be positively correlated to higher flocculation efficacy due to the more cationization achieved by the $–NH_2$ of the

PAM [91]. This innovative approach could be tailored to graft hydrolyzed animal proteins as such or onto other polymers.

2.3.3. Conversion of protein biomass into wood adhesives

The remarkable progress in the development of engineered wood products over the past three decades allowed the utilization of low-value plant wood species as well as forest and mill residues. Engineered wood products such as particle board, medium density fiberboard (MDF), parallel and laminated strand lumber, oriented strand board (OSB), plywood, are high-tech, high performance, dimensional stability and freedom from defects, that are totally integrated with other construction materials [93]. An important element in all modern industrial production technologies of engineered wood composites including oriented strand board, particle board, glued laminated timber (glulam) and plywood is the use of petrochemical adhesives such as urea-formaldehyde (UF), methyl urea formaldehyde (MUF), phenol-formaldehyde (PF), and isocyanates (MDI). These adhesives however are derived from non-renewable sources and some of their constituting components have deleterious effect on human health and the environment. For example, isocyanates can react rapidly with many compounds present in human bodies, posing safety issues during manufacturing and handling [94, 95]. They are also known asthma inducers and sensitizers [95]. Formaldehyde, a major component in the manufacture of UF, PF or MUF, on the other hand has been classified as a human carcinogen by the International Agency for Research on Cancer [96]. Substantial research efforts have been placed into

substituting these ingredients with safe and renewable materials derived from biomass feedstock.

Proteins have been used for centuries to prepare adhesives until petroleum-based adhesives started to dominate the market in the early twentieth century [97]. Bone glues have been traditionally used in carpentry and blood used as an additive in plywood glue-mixes [98]. Modern chemical modifications have built on these early knowledge and allowed the development of engineered wood adhesives based on bovine and porcine blood meal [99], blood protein extract [100], whey protein [101], casein [100], spent hen (a by-product of the poultry industry) protein extract [102], meat and bone meal protein extract [103], chicken feather protein extract [104]. Historically animal protein based glues were the adhesives of choice for paper manufacture and converting, bookbinding, text sizing, abrasives, gummed tape matches, and a variety of other applications [105].

Amino acids such as cysteine, lysine, and tryptophan present in animal proteins contain polar functional groups that interact with components of wood such as cellulose, hemicellulose and lignin. However, these functional groups need to be exposed from an otherwise prohibitive internal hydrogen bonding among themselves [103]. Controlled breakage of these protein internal bonding through the use of denaturants and chemical modifications are necessary to produce an effective adhesive with suitable bond strength and moisture resistance. Another common modification of proteins for adhesive application is chemical crosslinking [103, 106]. The crosslinking give rise to a rigid protein based adhesive with higher bond strength and water resistance. Some of the common

protein crosslinking agents that can be applicable for adhesive development in the literature include aldehydes (glutaraldehyde, glyoxal) [103], [50, 107], epoxy resins [51], maleic anhydride [108] etc. Figure 2.9 below shows the reaction of secondary amino group of protein with the dialdehyde functional group of glyoxal forming a networked and more rigid structure.

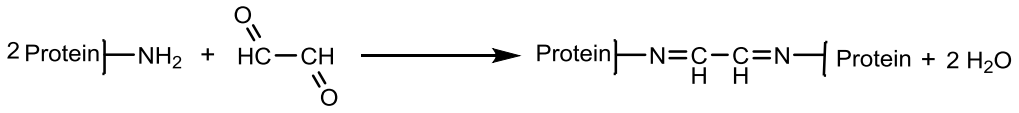


Figure 2.9. Crosslinking reactions between protein and glyoxal.

Unfolding of native globular proteins and as a result exposing hidden polar functional groups also enhance the interaction of proteins with the constituents of the wood. For instance, sodium dodecyl sulphate (SDS), an amphipathic molecule containing a polar group with a long hydrophobic carbon tail, is one known chemical that can cause protein unfolding. The denaturation and unfolding takes place by the binding of hydrocarbon tail of SDS with protein exposing normally buried regions [109]. Such unfolding could be brought about by one or a combination of the following: (i) electrostatic repulsion between the charges of SDS bound proteins (ii) penetration of the hydrocarbon tail of SDS into the polar regions of the protein; (iii) binding induced changes in the protein–hydrogen ion equilibrium, resulting in an increase in electrostatic repulsion between charged species [109-111]. Unfolding and treatment of proteins by urea [102, 108, 112], cationic detergents such as hexadecyltrimethyl ammonium bromide, and dodecyltrimethyl ammonium bromide [113], alkali and protease

enzymes [114, 115] were also shown to improve adhesive performance of several animal and plant proteins.

2.3.4. Conversion of protein biomass into other applications

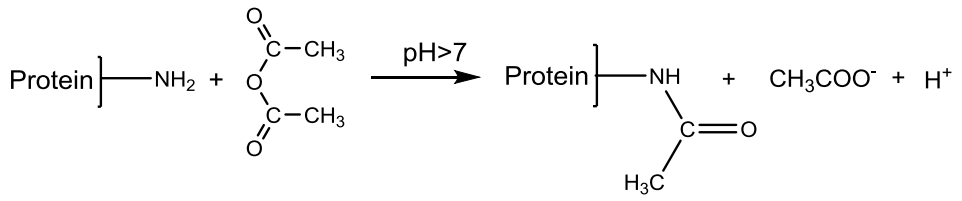
Surfactants and firefighting foams

Surfactants are important industrial chemicals widely used in the manufacture of household cleaning, industrial process, personal care and cosmetics, agricultural and food products and other industrial applications [116, 117]. The applications of surfactants in microbiology and biotechnology and several other industries including petroleum oil recovery, bioremediation, emulsification and de-emulsification applications, pharmaceuticals and therapeutics, downstream processing etc. has been excellently reviewed by Singh *et al*. [118]. Surfactants are characterized by a universal molecular structure consisting of hydrophilic and hydrophobic moieties with main properties of tendency to adsorb at interfaces, reduction of surface or interfacial tension, foaming properties, and emulsification. Proteins are amphiphilic molecules that exhibit surface activity. Given their biodegradability and low toxicity, the use of agricultural protein, including modified amino acids and hydrolyzed proteins as surfactants is well documented in the literature. Applications include foaming agents in food products [119], personal care products [120] and firefighting foams [121-124].

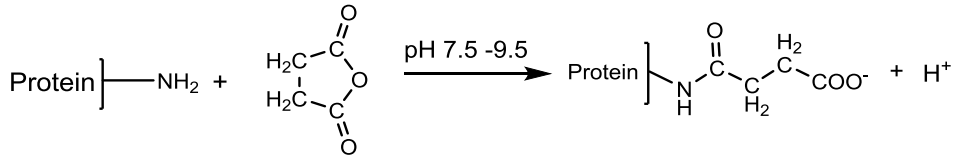
While native and denatured proteins can be used unmodified for these applications, several modification strategies have been pursued with the goal to improve their surfactant properties. For example, chemical modifications such as

phosphorylation through dry heating in the presence of pyrophosphate [125], acylation [126], acetylation and succinylation [127-129] has been shown to improve the foaming capacity and stability. Acetylation and succinylation modifications of proteins for surfactant preparation is shown in Figure 2.10 (a & b). Lin and Chen [128, 129], investigated the covalent attachment of hydrophobic functional groups in improving the surface activity of soy protein and gelatin hydrolyzates. Results of the investigation showed increasing the hydrophobic character of the proteins and hydrolyzates resulted in a greater reduction in surface tension and an improvement in surface activity [128, 129].

A study on the use of bovine blood and rendered blood meal protein for the development of firefighting foams exhibited that mild hydrolysis improved foaming ability [121]. The reason for the foam ability improvement as a result of the hydrolysis is due to the change in the hydrophilic and hydrophobic moieties. The same study showed that attaching hydrophobic groups such as fatty acids (C_2-C_{18}) to mildly hydrolyzed blood proteins greatly improved the foam stability of protein based surfactants. The variation in amino acid profile with the feedstock protein and the processing conditions cause variation in functional group and the balance of hydrophobic and hydrophilic functional groups. Thus, modifications in hydrophobicity or hydrophilicity of proteins depending on the protein feedstock could balance the functional groups to achieve the best surface-active properties.



(a)



(b)

Figure 2.10. Chemical modification of protein (a) Acetylation with acetic anhydride and (b) Succinylation with succinic anhydride [121, 129-131].

Hydrolysis of proteins and chemical acylation of the resulting polypeptides with acid chlorides in order to produce anionic lipopeptide surfactants is also reported [126]. Similar acylation modifications using amino acid feedstocks was also reported [132]. The resulting lipopetides surfactants have comparable foaming capacity and foam stability to commercial surfactants [126, 132]. The surface active properties of the formulations, such as critical micelle concentration, surface tension, foaming capacity and stability were shown to be dependent on the alkyl chain length [126]. For instance for the peptide based surfactant, shorter is the lipophilic chain, higher is the foaming capacity [126]. For the amino acid based surfactants on the other hand, the most desirable surface properties were observed for the longer chains [132]. In summary, the use of

waste or low value proteins with suitable modification is another potential area of utilization.

Microbial nitrogen source, organic fertilizer and biogas

The use of protein hydrolyzates in other applications after functional utilization has been extensively reported. For example, peptones produced through enzymatic and alkaline hydrolysis of rendered animal protein including blood meal, meat and bone meal and feather meal to support the growth of microorganisms has been evaluated by Garcia *et al*. [133]. Small peptides and amino acids have also been used as foliar fertilizers [134]. Espinoza *et al.* [135] reported potential of protein hydrolyzates that have high amino acid content in enhancing plant growth, climate tolerance, chlorophyll concentration, and chelation transport of micronutrients [135].

Waste protein biomass may also be integrated in the generation of biogas through anaerobic digestion for the production of electricity and thermal energy. Towards this end, the co-digestion of specified risk material together with cattle manure for biogas production has been studied by Giloyed *et al.* [136]. The study showed that the inclusion of SRM as substrate at the studied composition (10% and 25% wt. /wt.) increased the production of methane during anaerobic digestion of manure and may offer a means of deriving economic value from the disposal of SRM. At mesophilic temperature (37 °C), anaerobic digestion of manure with 10 % SRM substitution increased methane yield production by 83%. A 25 % SRM substitution at the same conditions on the contrary has increased the methane yields by 161 % compared to the control, where 100 % manure and no SRM were

used. At thermophilic temperature (55 °C), a 45% and 87% increase in methane production was observed for a 10 % and 25 % SRM inclusion, respectively [136]. The study further showed a logarithmic reduction possibility of bovine cells with retention times above 6 days, suggesting that degradation of BSE causing prions via anaerobic digestion took place. Since this method did not include a direct or *in vivo* study of prion infectivity test by anaerobic composting, together with the fact that SRM composting is not permitted in North America as safe SRM disposal techniques, this approach cannot be applied on a large scale. Nevertheless, the high methane production potential reported in this study showed that the use of SRM treatment (thermal or alkaline hydrolysis), or the use of other safe rendering industry products such as meat and bone meal, blood meal and feather meal could have interesting potential for biogas production. The use of SRM for similar application followed by downstream processing of the digestate prior to disposal is an acceptable option, provided that the economics of such process is viable.

An example of an industrial scale venture that converts animal waste protein to energy is represented by Biorefinex Inc., a company based in Lacombe, Alberta, Canada. Biorefinex's technology [137] is based on a thermal hydrolysis reaction protocol to process animal byproducts, mainly SRM, other organic residue and waste materials. This process destroys BSE causing prions and other pathogens [138] and yields dry pelletized organic fertilizer, liquid organic fertilizer, inedible tallow, biogas etc. (Figure 2.11). The novelty of the company's process is the possibility of integrating all processes together and the flexibility of the approach where a number of various products can be processed.

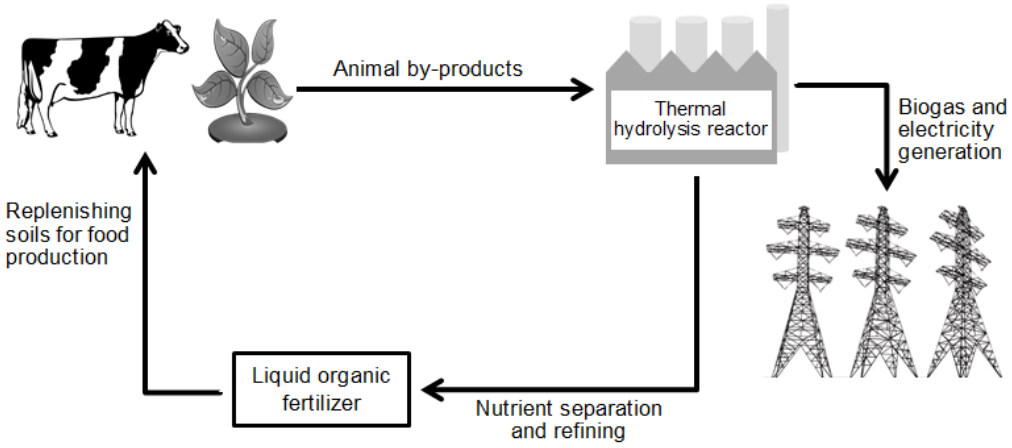


Figure 2.11. Biorefinery concept of Biorefinex for the conversion of SRM and other organic wastes into various industrial products (www.biorefinex.com, with permission © BioRefinex, Inc. Canada).

2.4. Conclusion and outlook

The rapid industrialization of modern society drives increasing demand of chemical building blocks. In order to address growing environmental concerns related to resource depletion and sustainability, low-value or waste streams must be efficiently upgraded to high value useful product. This is a particularly pressing issue for the slaughterhouse and rendering industry animal waste protein in advanced economies.

The emergence of the bovine spongiform encephalopathy changed most of the traditional uses of the wastes generated from these industries. While the values of some rendering products (for example blood meal, meat and bone meal etc.) became extremely limited, SRM tissues were completely banned from use as either feed/food or fertilizer application and effectively handled as hazardous waste. In Canada and the US alone, more than 13 million tonnes of rendered SRM

(www.biorefinex.com) are disposed of in landfills or incinerated annually, posing economic challenges to the rendering industry with repercussions to the whole livestock industry. These challenges include costs attributed to segregation of SRM from non-SRM animal tissues, segregation of processing lines to handle SRM and non-SRM tissues, and costs associated with SRM storage, transporting and disposal fees.

This review work presented the technological state-of-the art and emerging trends in the conversion of rendering and slaughterhouse wastes with a specific focus on the production of chemicals and materials. A modern biorefinery approach aiming to valorizing all ingredients and co-products of the animal processing industry waste including protein, fat, and glycerol was underpins the discussion presented here. The protein fraction can be valorized into components that are useful for applications including bio-based plastics, adhesives, flocculants, surfactants, nitrogen rich fertilizers and microbial feedstock. The fat fraction on the contrary can be valorized into products and co-products that are utilizable in the biodiesel and oliochemical industries. The ash fraction can also be valorized into minerals and fertilizers. While there are still challenges of heterogeneity of the compositions of these materials, there is huge potential of transforming these and other organic waste materials into safe, environmentally positive, and valuable products at competitive cost.

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Chapter 3.

Recovery and Characterization of Proteinacious Material Recovered from Thermal and Alkaline Hydrolyzed Specified Risk Materials[2]

3.1. Introduction

The rendering industry processes inedible tissues, offal, blood and bones from slaughtered farm animals to recover hides, purified fats and proteins [1]. The outbreaks of bovine spongiform encephalopathy (BSE) in North America and Europe had a profound impact on the use of the protein fraction, which previously was marketed as a feed ingredient for cattle, poultry, pets and aquaculture. In 2001, the European Union banned the use of all processed mammalian protein in feeds for farmed animals [2]. Similarly, the United States Food and Drug Administration (FDA) eliminated SRM from all animal feed [3]. In 2007, the Canadian Food Inspection Agency (CFIA) implemented an enhanced feed ban that eliminates SRMs from all animal food, pet foods and fertilizer applications. SRMs, which include the skull, brain, trigeminal ganglia, eyes, spinal cord, and dorsal root ganglia from cattle over 30 months of age and the distal ileum and tonsils from cattle of all ages (illustrated in Figure 3.1), are believed to be the highest risk material to contain prions in undiagnosed animals [4].

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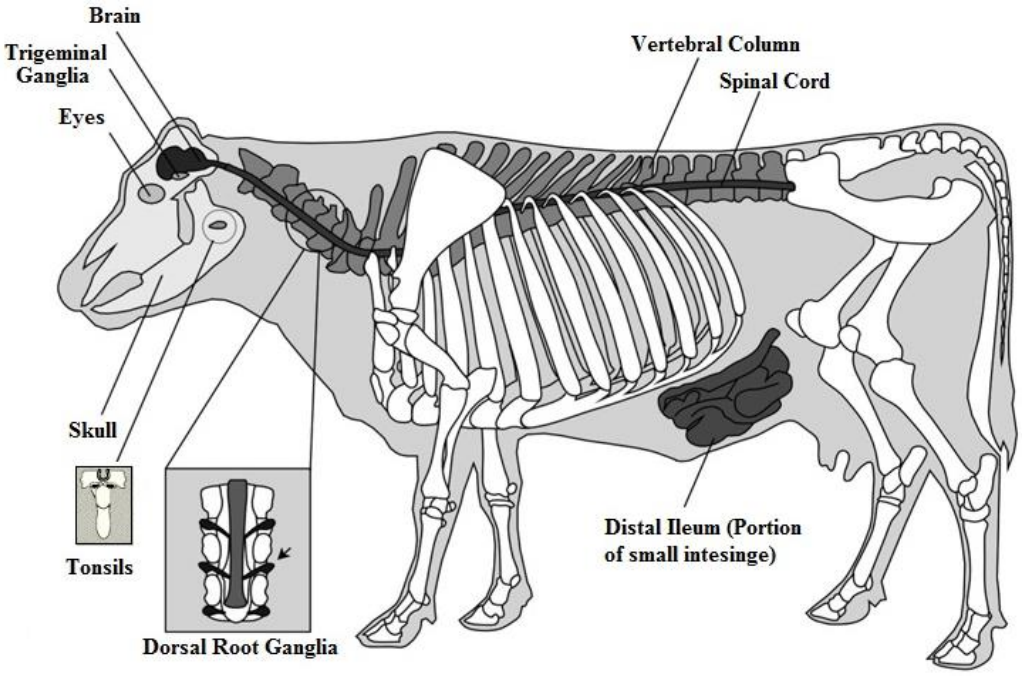


Figure 3.1. SRM in cattle **[4].**

The form of infectious agent thought to cause BSE and several transmissible spongiform encephalopathies (TSE) is a misfolded protein known as prion. In diseases such as BSE, the host-encoded proteins exist as PrP^{C} (cellular) in the non-infected host and as the misfolded PrP^{Sc} (scrapie) in the infected-host which is thought to be the infectious agent. PrP^{Sc} proteins are extremely resistant to natural degradation and conventional decontamination techniques such as heat, chemical disinfectants, boiling, irradiation and cooking [5, 6] and this has been a major scientific and practical challenge resulting from the BSE epidemic [7]. In Canada, the Canadian Food Inspection Agency and in the US Food and Drug administration approved four methods for SRM disposal: incineration, land burial at designated facilities, alkaline hydrolysis and a thermal hydrolysis protocol [3, 8].

In Canada, SRM is rendered to recover lipids while the remaining fractions are landfilled. Currently, over three hundred thousand tonnes (www.biorefinex.com) of such rendered SRM are disposed of to the landfill annually, posing economic challenges to the rendering industry with repercussions to the whole livestock industry. These challenges include costs attributed to segregation of SRM from non-SRM animal tissues, segregation of processing lines to handle SRM and non-SRM tissues, and costs associated with SRM storage, transporting and disposal fees. Disposal tipping fees range from $75 to $200 (depending on jurisdiction) per tonnes and transportation costs are $250 per tonnes on average (www.biorefinex.com). These circumstances constitute a strong drive to develop and market non-food/feed industrial applications from this waste stream.

Proteins have been used as renewable feedstock to develop value-added products such as bioplastics [9-13], adhesives [14], flocculants [15, 16], as well as foaming agents and other surfactants [17]. Protein concentrates, peptides and amino acids can also be used as fertilizers [18] and as ingredients in microbial growth media [19]. Non-SRM animal proteins in particular have been the subject of growing scientific attention. Laboratory experiments have demonstrated their potential use as partial plasticizer replacements [20], as fuel, blended with peat pellets in fluidized combustors [21, 22], and as agents of agricultural crop protection in potato cultivars [23]. On the other hand, to our knowledge, there is only one existing commercial attempt to process SRM using CFIA hydrolytic protocols to produce plant nutrients and biogas [24] in Canada. Despite progress, the full industrial implementation of novel applications of animal-derived protein,

including SRM, remains hampered by the limited economic value of the derived products and by incomplete understanding of fundamental protein hydrolyzate molecular structure and properties.

The aim of this study is to address these knowledge gaps by identifying, for the first time, parameters such as molecular size, free amino acid release during hydrolysis, extraction and solubility of proteinacious materials recovered from hydrolyzed SRM according to CFIA and FDA approved methods. The central hypothesis of this work is that both hydrolytic methods can be used as a gateway to safely convert potentially infectious materials from rendering operations into valuable feedstock for economically viable industrial applications. This research begins to address the environmental concerns and economic loss associated with several million tonnes of such materials already deployed in landfills across North America.

3.2. Material and Methods

3.2.1. Materials

Samples of rendered SRM in this study were provided exclusively by Sanimax Industries, Inc. (Montreal, QC, Canada). These samples were handled in a biosafety level II laboratory, according to CFIA protocols for safe handling, hydrolysis and disinfection of SRM [8]. The total protein, fat and ash content of the rendered SRM were 44.05%, 8 % and 27.2 %, respectively on dry weight basis. The following chemicals were obtained from Sigma-Aldrich (St. Louise, MO, USA): NaOH (99.6 % purity); NaCl (99 %); Na_2HPO_4 (99.6 %); KH_2PO_4 (99.8%); $MgCl_2$ (99%), acetonitrile (99.9%); methanol (99.9%); acetic acid

(HPLC grade, 99.7%); sodium acetate trihydrate (99 %); and hexane (HPLC grade, 99.9%). The following chemicals were sourced from Fisher Scientific (Fair Lawn, NJ, USA): HCl (37%); SDS (99+ %); blue dextran (2 MDa, analytical grade); ferritin (440 kDa, 10 μg/mL); β-amylase (200 kDa, analytical grade); alcohol dehydrogenase (150 kDa, analytical grade); albumin (66 kDa, ≥99%); cytochrome C (13.6 kDa, analytical grade); aprotinin (6.5 kDa, ≥98%); Vitamin B-12 (1.36 kDa, 99%);glycine (75 Da, 99+%); and Coomassie Brilliant Blue G (90+ %). Acrylamide (40% acrylamide/Bis solution, electrophoresis purity reagent) was obtained from Bio-Rad (Hercules, CA, USA) and filter paper (Whatman 4, diameter 11cm, pore size 20-25 μm) was purchased from Whatman (Cambridge, United Kingdom) and used as received.

3.2.2. Hydrolysis of rendering material

Rendered SRM samples were primarily composed of coarse brownish colored granulated particles, and included small but visible bone particles (Figure 3.2a). Alkaline hydrolysis and thermal hydrolysis reactions were performed according to CFIA and FDA approved techniques. Alkaline hydrolysis of SRM was conducted at a temperature of 150°C and a pressure of at least 400 kPa in a hydroxide solution calculated on a mass per mass basis equal or greater than 9% of the SRM input material, which corresponds to 15 % sodium hydroxide (NaOH) for a period of 180 minutes per cycle, in an enclosed pressure vessel [8].

The alkaline hydrolyses were conducted by systematically varying the quantity of the alkaline solution to assess the effect of solution concentration on the level of hydrolysis. This was accomplished by adding 60 mL, 100 mL, 200 mL and 500

mL of alkaline solution containing 15 % (w/v) of NaOH to 100 g of dry SRM into the pressure vessel to yield 37.50 %, 50.00%, 66.67% and 83.33%, respectively of alkaline solution to the total weight of material in the pressure vessel. Thermal hydrolysis reactions were conducted at 180°C and 1200 kPa for a period of 40 minutes per cycle in an enclosed pressure vessel that is suitable for the purpose required [3, 8]. It must be noted that CFIA does not mandate the minimum water load for the thermal hydrolysis. In our laboratory experimental setup 50 mL of water per 100g was enough to ensure complete soaking of the SRM load. The effect of solution concentration on the level of hydrolysis was estimated here by using 50 mL, 100 mL, 200 mL and 500 mL of distilled water added to 100 g of dry SRM into the pressure vessel for thermal hydrolysis giving 33.33 %, 50.00%, 66.67% and 83.33%, respectively of water to the total weight of material (mass/total mass basis) in the pressure vessel. Hydrolysis reactions were carried out in triplicate for the evaluation of molecular size reduction of protein fragments and release of free amino acids. All reactions were conducted in a dedicated 2 L stainless steel pressure vessel (Parr Instrument, Moline, IL, USA) with a maximum pressure rating of 13,769.5 kPa (2000 psi).

SRM samples handling and reactor loading was performed in a biosafety cabinet located in a biosafety level II lab at the University of Alberta. The biosafety cabinet and the outside of the reactor vessels were decontaminated with 5% Environ LpH for 30 min [25] followed by 70% ethanol after each SRM handling.

a **b** **c**

Figure 3.2. (a) Rendered SRM as received (b) Thermal hydrolyzate of SRM (c) Alkaline hydrolyzate of SRM (Diameter of each container is 9 cm).

3.2.3. Physical and chemical characterization of hydrolyzed rendering material

Physical and chemical characteristics such as molecular weight distribution, free amino acid profile and solubility were measured pre- and post-hydrolysis.

3.2.3.1. Electrophoresis and size exclusion chromatography

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and size exclusion high performance liquid chromatography (SEC-HPLC) were used to evaluate the degree of protein cleavage and measure molecular size distribution of unhydrolyzed SRM and hydrolysis products.

SDS PAGE: Proteinacious material was extracted by solubilizing 0.5 g of SRM and 1 g of the hydrolyzed SRM in 10mL of 2.5 % (w/v) SDS and Milli-Q water, respectively and vortexing followed by centrifugation at 15,700 × g each for 5 min. The prepared protein extracts were fractionated using a mini-protean II electrophoresis unit (Bio-Rad Laboratories, Richmond, Calif., USA) tricine-SDS PAGE according to the method developed by [26]. The stacking gel and the

resolving gel contained 4% and 10% polyacrylamide, respectively. Protein standards with molecular weight ranges of 10 – 250 kDa and 1.4 – 26.6 kDa (Bio-Rad Laboratories, Inc., Hercules, Ca, USA) were utilized and Coomassie Brilliant Blue G-250 was used as a tracking dye. After completion, the gels were stained with a solution containing 0.1% Coomassie Brilliant Blue, 40% methanol, and 7% acetic acid, and then de-stained using a solution of 40% methanol and 7% acetic acid.

SEC-HPLC: Proteinacious materials were extracted and diluted with the mobile phase to yield approximately 30% protein, centrifuged at 15,400 × g for 5 min, pH neutralized with 0.1 N NaOH, and filtered through a 0.22 µm filter (Millipore, Bedford, MA, USA) before loading into a Varian Prostar 210 HPLC equipped with an autosampler (Varian Inc., Walnut Creek, CA) and a variable wavelength UV detector. The injection volume was 100 µL. Two size exclusion columns in series (Superdex 200 10/300 GL and Superdex Peptide 10/300 GL, GE Healthcare Biosciences AB, Uppsala, Sweden) were used. The optimum molecular weight separation range of Superdex 200 column is 10 kDa to 600 kDa while the superdex peptide column's is 100 to 7000 Da. The chromatographic system was calibrated with the following external standards dissolved in buffer solution: blue dextran (2000 kDa), ferritin (440 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), cytochrome C (13.6 kDa), aprotinin (6.5 kDa), Vitamin B-12 (1.36 kDa) and glycine (75 Da). The mobile phase was 0.15 M Na_2HPO_4 (adjusted to pH 9 with 1 N NaOH) in HPLC grade water (Milli-Q System; Millipore, Bedford, Massachusetts) containing 5 % acetonitrile at a

flow rate of 0.5 mL/min at room temperature. The eluted compounds were detected by UV absorbance at 210 nm.

3.2.3.2. Free amino acid quantification

Amino acid quantification of recovered protein hydrolyzates were accomplished with an HPLC equipped with a fluorichrom detector (excitation 340 nm emission 450 nm). Recovered hydrolyzate samples were derivatized using the O-phthaldialdehyde as per the method reported by Jones and Gilligan [27]. Sample separations were achieved using a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco) equipped with a Supelco guard column (4.6 x 50 mm, Supelco, Oakville, ON, Canada) packed with Supelco LC-18 reverse phase packing (20 - 40 µm).The derivatized samples were eluted with a gradient composed of 0.1M sodium acetatetrihydrate buffer (eluent A) and methanol (eluent B).for a total analysis time of 45 min. An amino acid solution containing 2.5 µmol/mL of each amino acid purchased form SIGMA-Aldrich (catalogue # AA-S-18) was used as an external standard. Furthermore, two internal standard solutions consisting of 25 µmol/mL β-amino-n-butyric acid (BABA) and ethanolamine (25 µmol/mL), were included in each prepared sample according to the protocol developed by [28].

3.2.4. Protein recovery

The recovery of proteinaceous material after SRM hydrolysis was carried out using the following techniques: salt buffer extraction, acid extraction, membrane

filtration and water extraction. The percentage of protein recovery was quantified to evaluate the efficiency of each extraction method.

Salt extraction: Each of the hydrolyzed samples was extracted using the method developed by Park and co-workers [14]. A salt solution (pH 6.5) consisting of 100 g of hydrolyzed sample was extracted with 450 mL of salt solution consisting of 18.0 g NaCl, 0.23 g $MgCl_2$, 4.10 g KH_2PO_4 and 4.30 g Na_2HPO_4 under agitation at 200 rpm for 30 min in a shaker (Innova lab shaker, New Brunswick, Canada). The supernatant was separated from the residue by centrifugation (7000 × g for 30 min) followed by hexane extraction to remove fats and lipid residues. The raffinate was then collected and vacuum filtered (Whatman no 4) and freeze-dried.

Acid extraction: To separate the protein fraction from the hydrolyzate, thermally hydrolyzed proteinaceous materials were first solubilized in 0.1 N HCl at pH 2.5 and then agitated at 200 rpm for 30 min on a shaker. The supernatant was separated from the residue by centrifugation at 7000 × g for 30 min and filtered (Whatman no 4). The supernatant was then extracted with hexane to remove fats and oils. The raffinate was collected and precipitated with 0.1N NaOH at its isoelectric precipitation point (pH 4.5). The precipitated protein was washed with water, neutralized with 0.1N NaOH and freeze-dried for total nitrogen quantification. Acidic protein extraction of the alkaline hydrolyzate was not attempted as the volumes of acid and base needed to acidify and then neutralize would result in an undesirable salt level in the final dried protein.

Water extraction: 40 g of thermal and alkaline hydrolyzed protein were solubilized in 180 mL of Milli-Q water and agitated at 200 rpm for 30 min followed by centrifugation at 7000 × *g* for 30 min and vacuum filtration (Whatman filter paper no 4) to remove insoluble tissues and bone particles. The filtered supernatant was then extracted with 540 mL of hexane to remove lipids. The raffinate was then freeze dried under reduced pressure and the total nitrogen was quantified using Dummas method and multiplied by a Jones factor of 6.45 [29] to calculate total protein.

Membrane filtration: Solution neutralization is required after alkaline hydrolysis reactions to assure safe handling. In this case, this step is carried out with HCl producing salts such as NaCl which can be removed using membrane filtration. Furthermore, salt extraction of proteinacious material (as described above) resulted in accumulation of unnecessary salt in the final solids. Pellicon XL 5 kDa (Millipore, MA, US) ultrafiltration membranes were used to recover salt-reduced retentate from hydrolyzed SRM post salt extraction. 900 mL of the salt extracted hydrolyzates were pumped (Peristalic pump, 10-600 rpm, Barnant Co. Barrington, IL, USA) through the 5 kDa membrane at 30 mL/min until 600 mL of permeate and 300 mL of retentate was collected. The retained proteins were then freeze-dried and the total nitrogen was measured using Dumas method and multiplied by a factor of 6.45 to calculate the purity of the total protein recovered [29]. The yield was also calculated from the mass balance of the mean of the proteinaceous material recovered from the SRM feedstock.

3.2.4. Protein solubility

Protein solubility was determined using a method validated for food proteins such as whey protein concentrate, soy protein, egg white or sodium caseinate [30]. 500 mg of freeze-dried protein extract was stirred in 500 mL solution of 0.1 M NaCl for 1 h to form a smooth dispersion. Aliquot of the dispersion was then transferred to beakers and the pH was adjusted from 2.0 to 9.0 using 0.l N HCl and 0.1 N NaOH solutions. The pH was intermittently monitored and maintained for another 1h under stirring. After centrifugation at 20,000 × *g* for 30 min, the dissolved protein concentration was determined according to the biuret assay [31]. The percent solubility was calculated as the mass of protein dissolved, divided by the mass of protein in the original sample. Triplicate analyses were performed on each sample.

3.2.5. Statistical analysis

All experiments were replicated at least three times and results were expressed as mean value ± standard deviation. The statistical analyses of the data were conducted using the statistical software package Minitab version 15. To identify significant differences among mean values, single factor analysis of variance (ANOVA) was applied to the data populations involved, according to the LSD criteria with a 95% confidence level ($P < 0.05$).

3.3. Results and discussion

3.3.1. Protein hydrolyzate molecular size

One of the primary goals of this work was to evaluate the degree of protein cleavage occurring during hydrolysis conducted using the protocols described above. The characterization of pre- and post-hydrolysis protein molecular size provides an insight of the level of hydrolysis by these approved techniques of hydrolysis and enables to get critical physical information to design value-added materials applications. The SRM hydrolyzate had a strong pungent smell and a color ranging from dark red in the case of thermal hydrolysis to dark brown for the alkaline hydrolysis (Figure 3.2b and c). The hydrolysis (both thermal and alkaline) broke down clumps of the tissues into soluble fraction and a residue rich in bone fragments. Figure 3.3 shows a typical SDS PAGE experiment conducted in 50% water. SRM proteins are characterized by a relatively broad size distribution, with a predominance of large molecular sizes (>100 kDa) as demonstrated by the clusters centered at or near the upper limit of the marker scale. Proteins from unhydrolyzed SRM are also characterized by two bands appearing at approximately 18 and 14 kDa, possibly representing hemoglobin subunits previously documented in meat and bone meal proteins analysis [15].

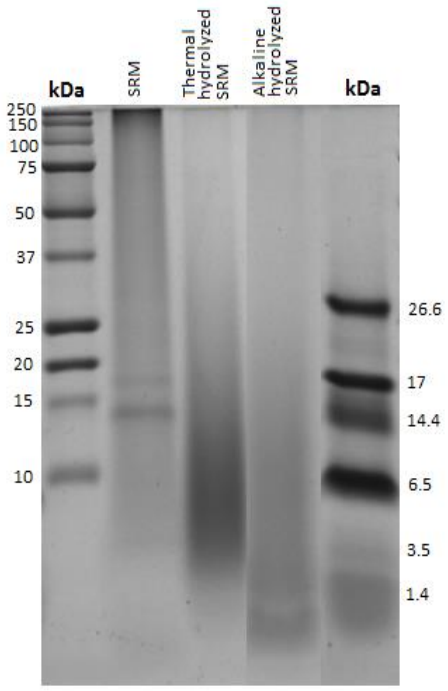


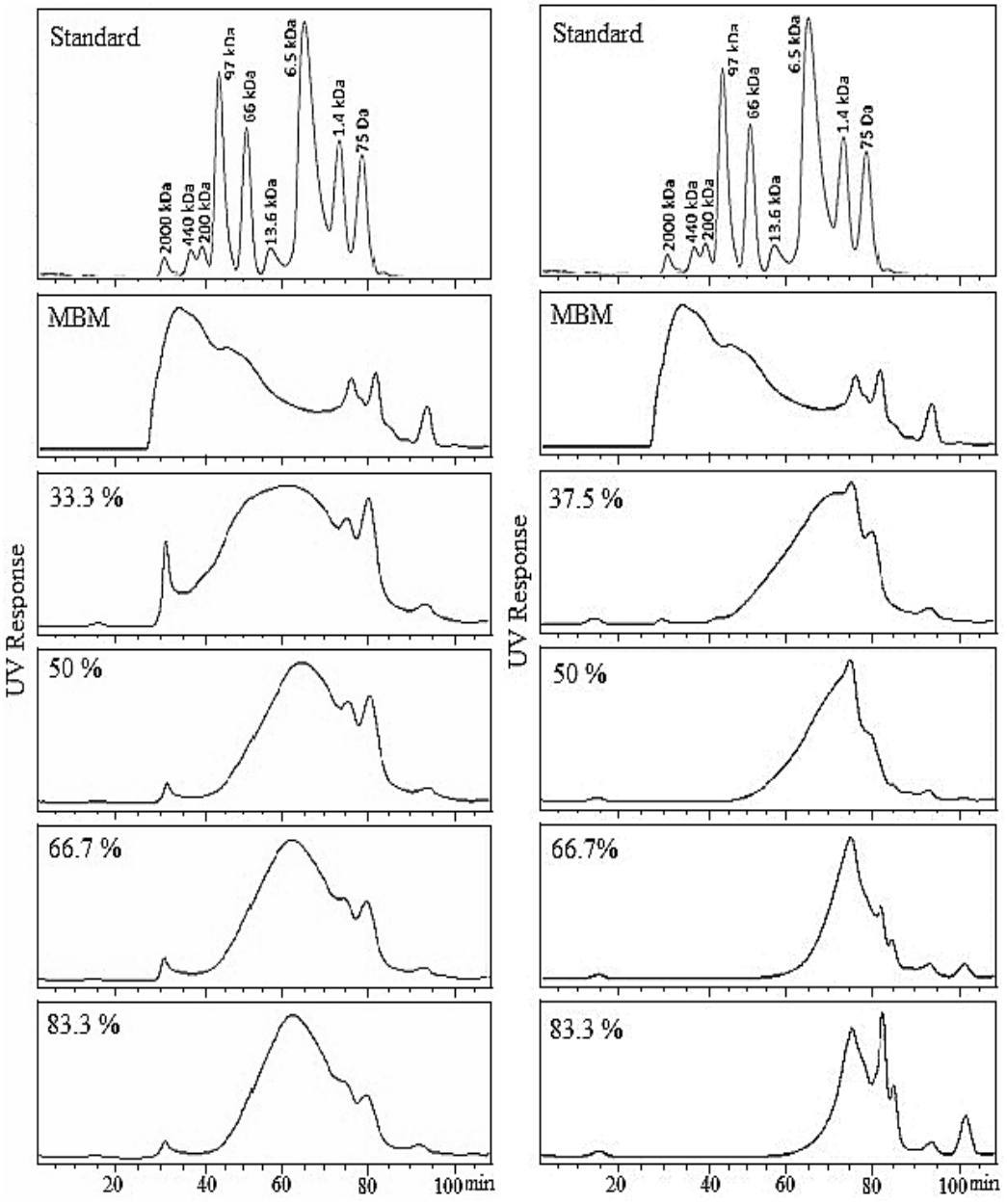
Figure 3.3. SDS PAGE of SRM and its counter thermal and alkaline hydrolyzate at 50 % solvent concentration

General conclusions regarding the molecular weight of SRM protein fractions should be made cautiously. The scientific literature about non-SRM animal protein characterization is sparse and clearly points to a wide variability of molecular characteristics not only as a function of geographical rendering plant location, but often even within the same plant location as a function of time and processing conditions at site [29, 32]. It is entirely plausible that such variability could occur also in the case of SRM. Protein cleavage is evident in both thermal and caustic hydrolysis samples as most of the hydrolyzed proteins were concentrated below the 25 kDa marker. Whereas the proteins from thermal hydrolyzed SRM smeared primarily between 15 and 5 kDa, the proteins from caustic hydrolysis smeared to less than 1 kDa. This result can be explained by

considering that the alkaline protocol (pressure, total cycle time) is more severe than the thermal protocol thus a higher degree of cleavage is plausible. In addition, the presence of alkali is known to exert a catalytic effect on the hydrolysis reaction [33, 34], increasing the likelihood of greater protein cleavage.

Post-hydrolysis proteinaceous material was further characterized by SEC-HPLC to assess the effect of water dilution on the molecular size distribution of the hydrolyzed protein material. Figure 3.4 shows (from top to bottom) the elution profile chromatograms of external standard, meat and bone meal (MBM) protein extract and chromatograms of SRM hydrolyzate extracts under various water and alkaline solution concentrations. In these experiments, unhydrolyzed MBM protein extract obtained from non-SRM tissues was adopted as a benchmark *in lieu* of the unhydrolyzed SRM to obviate safety concerns arising from using unhydrolyzed SRM on HPLC. The molecular weight of unhydrolyzed MBM protein extract was broadly distributed over a wide range of molecular weights, with three unidentified peaks below 1 kDa. More importantly, this result clearly indicates, for the first time, that a significant portion of the native MBM (and presumably SRM owing the similarities in amino acid profiles) proteins is characterized by molecular weight exceeding the 250 kDa, highlighting the limitations of standard SDS-PAGE analysis. This finding explains the very limited solubility of both SRM and MBM proteins in aqueous solutions previously reported in the literature [32] and confirmed also in this study. Figures 3.4a and 3.4b show the distribution as a function of water dilution. In both thermal and caustic hydrolytic protocols, increasing relative amounts of water

translated into a greater degree of protein cleavage and yielded proteinacious material with a relatively narrower distribution molecular weights compared with the material pre-hydrolysis. This is a positive characteristic for value-added applications that rely on homogenous protein feedstock that can be easily dissolved and recovered from aqueous solvents. It should be noted that at each given water dilution level, the alkaline protocol produced smaller and more homogenous proteinaceous product. A possible explanation is that, at the alkaline hydrolysis condition (180 ºC) specified by the CFIA or FDA protocol, most of the water will be in vapor form resulting in a much more concentrated alkali solution that catalyze the breakdown of proteins [35] to short chain peptides and amino acids. At 83.3% total alkali solution containing 15% w/v NaOH; most of the proteins were broken down to short peptides below 10 kDa, and with a relatively narrow molecular weight distribution range owing to the more water and NaOH per a given weight of SRM. Such intense hydrolysis could be valuable for applications such as nitrogen source for plants that require short chain peptides and amino acids.



a b

Figure 3.4. SEC-HPLC of a) thermal hydrolyzed SRM in 33.33 %, 50.00%, 66.67% and 83.33% water to the total mass b) alkaline hydrolyzed SRM in 37.50 %, 50.00%, 66.67% and 83.33% alkaline solution (15% w/v NaOH) to the total mass.

In summary, SDS-PAGE and SEC-HPLC experiments demonstrated that both thermal and alkaline hydrolysis protocols were effective gateways to attaining proteinaceous material with a smaller and more uniform molecular weight distribution compared to the SRM feedstock. Moreover, alkaline hydrolysis cleaved the feedstock protein more severely than the thermal hydrolysis.

3.3.2. Free amino acid profiling

Free amino acid release was studied as an additional measure of the level of protein breakdown. Higher amount of total free amino acids released implies higher degree of protein hydrolysis, resulting in reduction of chain length. The other purpose of this study was to fill the knowledge gap of the reactivity of the recovered proteinacious material because their potential use as feedstock for further chemical processing and modification highly depends on knowledge of the moieties left on the protein side chains [36]. The more free amino acids were released; less peptide-bound amino acids would remain resulting in less reactive moieties on the hydrolyzed protein chain.

Table 3.1. Free amino acid profile of thermal hydrolyzed SRM in 33.33 %, 50.00%, 66.67% and 83.33% water to the total mass.

Solution composition	Average free amino acid (mg/g)			
	33.3 %	50 %	66.67 %	83.3 %
Aspartic acid	0.36 ±0.12[a]	0.66 ±0.08[a]	1.06 ±0.08[b]	1.56 ±0.04[c]
Glutamic acid	0.08 ±0.01[a]	0.10 ±0.02[ab]	0.12 ±0.03[b]	0.21 ±0.04[c]
Serine	0.14 ±0.05[a]	0.31 ±0.08[ab]	0.44 ±0.04[b]	0.63 ±0.02[b]
Histidine	0.04 ±0.03[a]	0.10 ±0.02[ab]	0.11 ±0.02a[b]	0.16 ±0.05[b]
Glycine	1.04 ±0.04[a]	2.11 ±0.27[b]	1.77 ±0.03[b]	2.19 ±0.04[b]
Threonine	0.33 ±0.11[a]	0.62 ±0.15[b]	0.48 ±0.10[ab]	0.39 ±0.01[ab]
Alanine	1.25 ±0.19[a]	1.76 ±0.05[ab]	2.18 ±0.15[b]	1.88 ±0.01 [ab]
Tyrosine	0.28 ±0.16[a]	0.51 ±0.02[b]	0.74 ±0.11b[c]	0.75 ±0.08[c]
Tryptophan	0.02 ±0.04[a]	0.12 ±0.03[ab]	0.16 ±0.21[b]	-
Methionine	0.12 ±0.03[a]	0.19 ±0.02[ab]	0.22 ±0.10[b]	0.22 ±0.05[b]
Valine	0.17 ±0.02[a]	0.29 ±0.08[b]	0.31 ±0.16[b]	0.26 ±0.01[b]
Phenylalanine	0.10 ±0.03[a]	0.21 ±0.02[b]	0.15 ±0.01[ab]	0.18 ±0.02[ab]
Isoleucine	0.18 ±0.06[a]	0.28 ±0.03[b]	0.23 ±0.10[ab]	0.17 ±0.03[ab]
Leucine	0.27 ±0.09[a]	0.48 ±0.08[a]	0.34 ±0.16[a]	0.47 ±0.01[a]
Ornithine	-	-	-	0.04 ±0.00[a]
Lysine	1.31 ±0.29[a]	1.96 ±0.12[c]	0.70 ±0.03[ab]	0.33 ±0.06[b]
Total	5.69 ± 1.43[a]	9.76 ±1.08[b]	9.03 ±1.57[b]	9.44 ± 1.08[b]

Value – mean ± standard deviation (n=3), means with the same superscript letters within a column are not significantly different at $P < 0.05$ level.

Table 3. 2. Free amino acid profile of alkaline hydrolyzed SRM in 37.50 %, 50.00%, 66.67% and 83.33% alkaline solution (15% NaOH) to the total mass.

Solution composition	Average free amino acids (mg/g)			
	37.5 %	50 %	66.7 %	83.3 %
Aspartic acid	2.38 ±0.09^{a}	3.50 ±0.66^{a}	15.80 ±1.03^{b}	11.63 ±1.44^{b}
Glutamic acid	0.38 ±0.04^{a}	1.39 ±0.37^{b}	27.31 ±2.55^{c}	25.78 ±2.80^{c}
Serine	0.65 ±0.11^{a}	1.13 ±0.46^{a}	0.19 ±0.04^{a}	0.21 ±0.06^{a}
Histidine	-	-	4.62 ±0.28^{a}	0.76 ±0.12^{b}
Glycine	8.27 ±0.42^{a}	10.80 ±2.12^{ab}	13.71 ±0.29^{b}	24.93 ±2.49^{c}
Threonine	0.22 ±0.25	-	-	-
Alanine	0.38 ±0.07^{a}	0.17 ±0.20^{a}	0.16 ±0.02^{a}	0.27 ±0.03^{a}
Tyrosine	10.79 ±1.63^{a}	9.85 ±0.86^{a}	32.11 ±1.12^{b}	26.37 ±2.50^{b}
Tryptophan	1.15 ±0.80^{a}	1.04 ±0.96^{a}	3.35 ±0.24^{a}	2.12 ±0.07^{a}
Methionine	0.53 ±0.03^{a}	0.66 ±0.19^{a}	1.90 ±0.06^{b}	1.95 ±0.12^{b}
Valine	0.70 ±0.17^{a}	0.81 ±0.06^{a}	2.31 ±0.15^{b}	5.57 ±0.20^{c}
Phenylalanine	0.68 ±0.08^{a}	1.09 ±0.32^{a}	5.70 ± 0.26^{b}	5.79 ±0.27^{b}
Isoleucine	0.40 ±0.14^{a}	0.48 ±0.21^{a}	1.17 ±0.11^{a}	3.57 ±0.14^{b}
Leucine	1.13 ±0.25^{a}	1.89 ±0.31^{a}	10.78 ± 0.85^{b}	10.84 ±0.84^{b}
Ornithine	0.60 ±0.34^{a}	2.06 ±0.59^{b}	4.85 ±0.28^{c}	7.57 ±0.46^{d}
Lysine	0.75 ±0.30^{a}	1.96 ±0.17^{b}	5.65 ± 0.48^{b}	8.61 ± 0.59^{d}
Total	29.03 ± 1.86^{a}	36.83 ± 1.51^{b}	100.91 ± 9.78^{c}	135.97 ± 4.84^{d}

Value – mean ± standard deviation (n=3), means with the same superscript letters within a row are not significantly different at $P < 0.05$ level.

Table 3.1 and Table 3.2 report the free amino acid profile of thermally and caustically hydrolyzed SRM respectively as a function of water and caustic

solution dilution. For both protocols, increased dilution of the protein in water resulted in a greater total free amino acids release. This finding is in line with previous studies that documented how water can act as both a general base catalyst and a nucleophile leading to more hydrolysis of amides and thereby a greater release of free amino acids [35]. However, the alkaline protocol produced a greater amount of free amino acids than the thermal hydrolysis, owing to the severe cleavage of proteins through such hydrolysis. High concentrations of glycine and alanine are generally associated with animal protein meals alkaline hydrolysis [32]. This is because alkaline reaction conditions favor the decomposition of other amino acids such as serine and threonine into glycine and alanine. In addition, high concentration of these two amino acids in both hydrolyzates reflects the preferential hydrolysis of the collagen fraction present in SRM, a phenomenon that has been documented in the alkaline hydrolysis of MBM [32]. This hypothesis is further supported by the remarkable increase in the release of aspartic acid, glutamic acid and leucine. All amino acids in both protocols showed significant variations as function of water dilution with the exception of leucine in thermally hydrolyzed SRM and alanine and tryptophan in solutions from alkaline hydrolyzed samples. While we do not have a definitive explanation for this phenomenon, we speculate that, similarly to other alkaline hydrolyzed protein meals, this could be due to partial degradation of such amino acids in alkali solutions. Our findings also highlight the similarities between SRM and MBM as our results aligned with previous studies conducted recently by Rutherford [37] and by Garcia and collaborators [32].

3.3.3. Protein extraction

The economically viable, rational design of protein-based value-added applications depends also on the availability of relatively concentrated and water soluble feedstock. For example, adhesives based on soy proteins have been developed from soy concentrate rather than from soybeans directly. In this work, three methods for protein extraction post hydrolysis reactions have been tested as summarized in Table 3.3: 1) salt extraction; 2) salt extraction followed by ultrafiltration; 3) acid extraction; 4) water extraction.

Table 3. 3. Protein concentration and yield of proteinacious products recovered from hydrolyzate by different methods.

Recovery methods	Thermal hydrolyzate		Alkaline hydrolyzate	
	Protein concentration (%)	Yield (%)	Protein concentration (%)	Yield (%)
Salt extraction	70.59 ± 2.56^{a}	38.6	54.34 ± 3.48^{a}	25.0
Salt extraction and ultrafiltration	83.04 ± 1.95^{b}	33.0	71.68 ± 1.74^{b}	22.0
Acid extraction	77.24 ± 1.46^{b}	35.1		
Water extraction	91.04 ± 1.73^{c}	42.1	67.41 ± 0.76^{c}	27.6

Value – mean ± standard deviation (n=3), means with the same superscript letters within a column are not significantly different at $P < 0.05$ level.

The freeze-dried, salt extracted, solids from thermally hydrolyzed SRM were 70.56 % proteinacious material. This result is in line with data published by Park *et al*., [14] who reported a 77.8% protein concentration starting from a MBM sample that contained 48.1% protein. A possible explanation for higher animal

protein solubility in salt may be the increase in solubility of salt soluble microfibril proteins [14]. Insoluble proteins such as actinomycin protein are known to dissociate into soluble actin and myosin at high salt concentration [38]. In addition, the presence of $MgCl_2$ ions allows the protein polar groups to interact with the electronegative Mg ion [39]. On the other hand, salt extraction from the alkaline hydrolyzate resulted in only 54.34% protein concentration. As discussed previously, the alkaline hydrolysis protocol produced a greater amount of free amino acids. It is possible that the amino acid fraction lacking polar moieties might not have been solubilized in the strong ionic salt solution used here, resulting in a lower protein concentration. This protocol encompasses both salt extraction and neutralization of the alkaline hydrolysis produce and therefore salts were collected in the final dried protein.

In order to eliminate salt from the protein concentrate, a potentially detrimental component for downstream applications, ultrafiltration was incorporated to the extraction step prior to drying. The ultrafiltration membrane used in this study had a molecular weight cut off 5 kDa, allowing short peptides, amino acids and salts into the permeate. As expected, ultrafiltration resulted in a significant proteinacious material concentration increase in both hydrolyzates (Table 3.3). However, the yield obtained from salt extraction followed by membrane filtration of both the thermal and alkaline hydrolyzates was lower, because of the loss of nitrogen containing components such as small peptides and amino acids together with the salt into the permeate.

The acid extraction (pH 2.5) disrupts the hydrolyzed proteinacious material via electrostatic repulsion resulting in better solubilization. Higher solubility of extracted proteins was observed at high and low pH (Figure 3.5). The solids collected through acid extraction followed by isoelectric precipitation and freeze-drying were 77.24 % proteinacious material. That means the purity of the acid extracted and dried solids was better than those obtained through salt extraction. However, the yield was lower than that of salt extraction. This observation could be due to the fact that SRM contains various types of proteins with marked difference in solubility that may have resulted in loss of proteinacious material during the isoelectric precipitation.

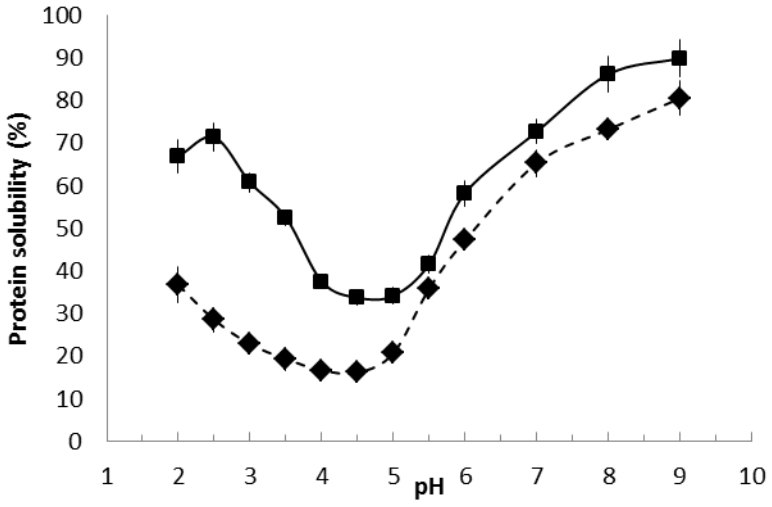


Figure 3.5. Solubility and isolectric point determination curves of thermal hydrolyzed SRM (-■-) and alkaline hydrolyzed SRM (-◆-). The hydrolyzates studied here were from 50% water and 50% alkaline solution for TH SRM and AH SRM, respectively.

The possibility of directly utilizing water to extract proteinaceous material from hydrolyzed SRM after neutralization was also investigated. In this case, water extraction of the thermal hydrolyzate resulted in a protein concentration of 91.04% and in 67.41% protein concentration from caustically hydrolyzed SRM. Different grades of water such as tap water, distilled water and Milli-Q water were also tested, but no significant difference in the final protein concentration was found. While high-concentration solutions can be extracted from thermally hydrolyzed SRM using water, the preparation of similarly concentrated proteinaceous solutions from alkaline hydrolyzed SRM was found to be very challenging, possibly owing to the high concentration of salts during the neutralization of the alkali following the hydrolysis.

3.3.4. Protein solubility

A serious limitation to industrial applications of animal proteins is posed by their low solubility in water. For example, Garcia *et al*. [29] reported a median average solubility of 5.35% for unhydrolyzed MBM and proposed the use of hydrolytic methods to improve this characteristic.

The solubility and isoelectric precipitation point of the water extracted protein hydrolyzates were studied between 2 to 9 pH ranges and are presented in Figure 3.5. The solubility of thermal hydrolyzed SRM was higher than alkaline hydrolyzed SRM in all the pH ranges studied as shown in Figure 3.5. This phenomenon might be due to the difference in the release of free amino acids. The Biuret method was used here to quantify the solubilized proteinacious material. This method, based on binding of cupric ions with functional groups of the protein's peptide bonds forming a colored complex, might have limitation to account for all proteinacious material. As reported in Table 3.1 and Table 3.2, it was depicted that more total free amino acids were released with the severe alkaline hydrolysis than the thermal hydrolysis. It was also observed that some amino acids such as glycine and alanine were the dominant released amino acids from alkaline hydrolysis (Table 3.2) and these amino acids are non-polar that might be contributing to the comparative lower solubility of alkaline hydrolyzed SRM in all pH ranges studied here. An isoelectric precipitation point was observed at pH 4.5 for both thermal and alkaline hydrolyzed SRM. The solubility increased as the pH increased or decreased from the isoelectric pH within the studied range. This is because changing the pH of proteins acquires a net negative

or net positive charge where hydration of the charged residues and electrostatic repulsion results in an increase of solubility. The solubility at neutral pH was found to be 72.6 % and 65.3% for thermal and alkaline hydrolyzed SRM proteins, respectively. These results clearly indicate that these hydrolyzed proteins are sufficiently soluble for use as potential feedstock for further chemical processing into value added applications. While these hydrolyzates produced in this work are less soluble than proteins for food applications such as egg protein and whey protein concentrate, they are more soluble than soy protein isolate [30], a feedstock that has been successfully used in several value-added materials.

3.4. Conclusions

This research demonstrates that two hydrolysis protocols approved by the CFIA and FDA can be used to process waste animal tissues into safe proteinaceous feedstock for non-food, bio-based applications. Both hydrolytic methods cleaved the rendered proteins, improving their water solubility and reducing molecular size and distribution. Molecular size distribution and chemical profiling of the released free amino acids showed the presence of intact proteins (>10 kDa) and peptides with functional groups amenable to further processing into value-added applications such as bioplastics, biocomposites and bio-adhesives that are already underway in our laboratory. Residues remaining after extraction were mainly ashes and insoluble protein fractions that can be used in organic fertilizer applications.

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Chapter 4

Subcritical hydrolysis, biorefining and characterization of hazardous waste biomass for value added applications[3]

4.1. Introduction

The societal demand for chemical building blocks and products recovered or produced from low-value or waste streams, non-petroleum based renewable and sustainable resources is rapidly and consistently increasing. Furthermore, the need to reduce waste driven by expensive and restrictive waste disposal legislation and escalating cost of raw materials constitute the necessity of recovering value from waste [1, 2]. The Non-edible portions of slaughtered animals have been collected and processed by the rendering industry to recover fats and proteins for oliochemical and animal feed applications, respectively. However, the emergence of bovine spongiform encephalopathy (BSE) led to the ban of certain cattle tissues from entering the human food and animal feed supply in many countries including European Union, Canada and the USA. These specific banned tissues, also known as specified risk material (SRM), includes the skull, brain, trigeminal ganglia, eyes, spinal cord, and dorsal root ganglia from cattle over 30 months of age and the distal ileum and tonsils from cattle of all ages [3]. These SRM tissues are categorized as hazardous waste because they are the highest risk tissues to contain BSE causing prion proteins. The BSE crisis also restricts the use of non-SRM tissue derived rendering products such as blood meal and meat and bone meal.

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Alkaline hydrolysis, thermal (subcritical) hydrolysis , incineration, and landfilling at designated sites are government approved methods of hazardous SRM control in Canada [4] and the US [5]. However, rendering of SRM to recover fats and landfilling the remaining fractions is by far the most common SRM disposal method in North America. This creates considerable environmental challenges associated with the risk of hazardous prion protein leaching into the aquifer systems, and significant economic strain due to landfilling and tipping fees associated with storage, transportation and disposal fees. Thermal hydrolysis at a temperature of at least 180 °C and pressure of 1200 kPa is shown to destroy the notoriously resistant prion proteins [6], and the hydrolyzates recovered are safe for industrial utilization. While several studies have been conducted on the inactivation potential of such hydrolysis method, comparatively little attention has been placed on characterizing the hydrolysis products composition or evaluating these materials as feedstock for value added application.

The use of subcritical water as a hydrolyzing medium is attractive because it non-flammable, non-corrosive, non-toxic, and relatively cheaper than other possible solvent systems. The properties of subcritical water extend to the unique alterability of ionic products, dielectric constant, and density, making it an interesting and promising reaction medium [7]. As the temperature and pressure increase from the boiling point (100 °C and 0.1 MPa) to the critical point (374 °C and 22 MPa), dissociation of water molecules into hydroxonium ions (H_3O^+) and hydroxide ions (OH^-) increases by a combination of oxygen–hydrogen stretching within each molecule and liberation vibrations between molecules [8]. Water at

subcritical conditions has been demonstrated by several studies to effectively convert biomasses into useful products [9, 10].

Rendered SRM biomass, previously sold as an animal feed ingredient, is mainly composed of proteins (48.7%) and ashes (10.38-34.4%); and relatively small quantity of residual lipids (7.7-14%) left after the rendering [11]. The use of ingredients of this waste protein biomass as renewable industrial feedstock does not only avoid the perceived competition with food that is already a challenge with the use of other proteins, but it is also a way of generating alternative income to the rendering industry and reducing environmental burden. The use of protein hydrolyzates in functional utilization has been extensively reported. Small peptides and amino acids may be used as foliar fertilizers [12], while hydrolyzates with high amino acid content can enhance plant growth, climate tolerance, chlorophyll concentration, and chelation transport of micronutrients [9]. It is reported that protein concentrates and hydrolyzed proteins can also be utilized as feedstock for bioplastics, wood adhesives [13, 14], renewable flocculants [15], surfactants [16], organic fertilizer and nitrogen source for microbes [12].

The demand for reducing waste [1] and the potential industrial utilization of hydrolyzed SRM proteins has prompted the basis for the present investigation as our laboratory continues to pursue new ways of hydrolyzing SRM with more control on the molecular size of the hydrolyzates. Thus, conducting a controlled subcritical hydrolysis of SRM by varying the hydrolysis temperature within the domain of Canadian Food Inspection Agency (CFIA) and Food and Drug Administration (FDA) of the US approved protocol, recovering and characterizing

the hydrolyzate products as a potential industrial resource is the main objective of this study.

4.2. Materials and methods

4.2.1. Materials

Rendered SRM, composed of 44.05 % protein, 8 % fat and 27.2 % ash on dry weight basis was obtained from Sanimax Industries, Inc. (Montreal, QC, Canada). The following chemicals were obtained from Sigma-Aldrich (St. Louise, MO, USA): NaOH (99.6 %); acetonitrile (99.9 %); methanol (99.9 %); acetic acid (99.7 %); 1-hexanol (99.5 %), volatile acid mix (HPLC grade), molecular size calibration kit (HPLC grade) and amino acid solution mix (HPLC grade). HCl (37 %), hexane (99.9 %) and 1-hexanol (99 %) were sourced from Fisher Scientific (Fair Lawn, NJ, USA). Filter paper (Whatman 4, diameter 11 cm, and pore size 20-25 µm) was purchased from Whatman (Cambridge, United Kingdom) and used as received.

4.2.2. Methods

4.2.2.1. Subcritical hydrolysis of SRM

CFIA and FDA approved techniques of SRM subcritical hydrolysis as a method of BSE destruction has to be conducted at a temperature of at least180 °C and 1200 kPa pressure for a period of at least 40 minutes per cycle in an enclosed pressure vessel [3, 5]. In this research, subcritical hydrolysis of SRM was conducted in a batch reactor at a temperature of 180, 200, 220, 240 and 260 °C for a period of 40 minutes per cycle after purging the reactor with nitrogen gas to achieve a pressure of at least 1200 kPa in triplicate under stirring at 200 rpm. For

all reaction conditions, two third of the 5.5 L stainless steel reactor vessel (Parr 4582) was filled with rendered SRM and distilled water at a weight ratio of 1:1. The hazardous SRM sample was handled in a biosafety cabinet located in a government approved biosafety level II containment lab at the University of Alberta. Decontamination of reactor vessel, biosafety cabinet and all other equipment that came in contact with the unhydrolyzed SRM was carried out with 5% Environ LpH for 30 min followed by 70% ethanol.

4.2.2.2. Recovery and biorefining of hydrolyzates

The biorefining of hydrolyzed SRM, mass balance and yield of protein hydrolyzate was conducted as follows: about 100 g of hydrolyzate from each hydrolysis was solubilized in 250 mL of Milli-Q water and agitated at 200 rpm in a shaker (Innova lab shaker, New Brunswick, Canada) for 30 min followed by centrifugation at 7000 × *g* for 30 min. The supernatant was then collected and vacuum filtered to collect the filtrate. Insoluble tissues, bone fragments, and other solid particles were collected from the residue of the centrifugation and filtration. The collected filtrate was then extracted with 750 mL of hexane to recover lipids and lipid fragments in the hexane phase. The proteinaceous material rich raffinate was lyophilized and the total nitrogen was quantified according to Dumas method. The total nitrogen obtained as such was then multiplied by Jones factor of 6.45 [17] to calculate total protein content. The mass of each fraction at each step was carefully measured for the overall mass balance and recovery yield of protein hydrolyzates calculation.

4.2.2.3. Molecular size distribution

Size exclusion high performance liquid chromatography (SEC-HPLC) was used to evaluate the level of protein cleavage and molecular weight distribution of SRM hydrolyzates as a function of the hydrolysis temperature. Samples were prepared from each hydrolysis by solubilizing the lyophilized protein hydrolyzates in the mobile phase to yield 30 mg/mL protein. The solubilized hydrolyzate solutions were then centrifuged at 15,400 × g for 5 min, pH neutralized, and filtered through a 0.22 µm filter (Millipore, Bedford, MA, USA). The prepared samples were then loaded into a Varian Prostar 210 HPLC with a variable wavelength UV detector. The injection volume was 100 µL. Two size exclusion columns in series (Superdex 200 10/300 GL and Superdex Peptide 10/300 GL, GE Healthcare Biosciences AB, Uppsala, Sweden) were used for hydrolyzate size separation. The chromatographic system was calibrated with the following external standards dissolved in buffer solution: albumin (66 kDa), carbonic anhydrase (29kDa), cytochrome C (13.6 kDa), aprotinin (6.5 kDa), vitamin B-12 (1.36 kDa) and glycine (75 Da). The mobile phase was 0.15 M Na_2HPO_4 (adjusted to pH 9 with 1 N NaOH) in HPLC grade water (Milli-Q System; Millipore, Bedford, Massachusetts) containing 5 % acetonitrile at a flow rate of 0.5 mL/min at room temperature. The eluted compounds were detected by UV absorbance at 210 nm.

4.2.2.4. Free and total amino acid quantification

Free and total amino acid quantification of the recovered protein hydrolyzate was accomplished with an HPLC (Agilent 1200 series, Agilent technologies, Inc., CA,

USA) equipped with an Agilent 1260 fluorescence detector (excitation 340 nm emission 450 nm). In order to quantify the free amino acids produced as a result of the subcritical hydrolysis, the recovered protein fractions were solubilized in Milli-Q water and directly derivatized using the O-phthaldialdehyde in accordance with Agilent ZORBAX Eclipse AAA protocol [18]. The derivatized samples were eluted with a gradient composed of 40 mM NaH_2PO_4 pH 7.8 (eluent A) and acetonitrile: methanol: water (45:45:10, v/v/v) (eluent B) for 35 min. An amino acid solution containing 2.5 µmol/mL of each amino acid was used as an external standard. Furthermore, an internal standard solutions consisting of 25 µmol/mL β-amino-n-butyric acid (BABA), was included in each prepared samples. For total amino acid quantification, unhydrolyzed MBM protein extract obtained from non-SRM tissues was adopted as a benchmark *in lieu* of the unhydrolyzed SRM to obviate safety concerns arising from using unhydrolyzed SRM on HPLC. The MBM protein extract and biorefined protein hydrolyzates obtained from subcritical hydrolysis were further hydrolyzed with 6 N HCl to achieve full hydrolysis of protein and peptides into amino acids and quantified using a similar method as that of the free amino acids.

4.2.2.5. Organic acid production in the aqueous phase

The production of low molecular weight organic acids as decomposition product of the SRM hydrolyzate at 180, 200, 220, 240 and 260 °C were analyzed on a Varian 3400 gas chromatograph (Palo Alto, CA) coupled with a flame ionization detector (GC-FID). The protein rich aqueous phase fraction after hexane extraction was centrifuged at 15,700 × g for 15 min, and filtered on 0.22 µm filter

paper. 1-hexanol internal standard and external standard of volatile organic acid composed of formic acid (C1), acetic acid (C2), propionic acid (C3), butyric acid (C4), isobutyric acid (C4), valeric acid (C5), isovaleric acid (C5), isocaproic acid (C6), hexanoic acid (C6) and heptanoic acid (C7) was used. The prepared samples and standards were then transferred to a GC vial and loaded to the GC-FID with to a 30 m long Stablilwax – DA column from Restek (Bellefonte, PA, US).

4.2.2.6. Thin Layer Chromatography (TLC) study of lipids hydrolysis

The possiblility of lipid hydrolysis into glycerol and fatty acids in some of the hydrolysis conditions was tested using thin layer chromatography (TLC). Hexane extract of unhydrolyzed meat and bone meal (MBM) as a control sample of the hydrolyzed SRM and hexane extracts from the 180 °C, 200°C, 220°C, 240°C and 260 °C hydrolyzed SRM were separated on a TLC. Dried extracts were diluted in hexane to 10 mg/mL concentration. About 5µL of these diluted extracts were spotted onto a flexible silica gel TLC plates (Whatman Ltd. Kent, England). External standard solutions composed of single component triglyceride (TG), diglyceride (DG), mononglyceride (MG), and fatty acid (FA) were also spotted and eluted beside the sample solutions. An eluting mobile phase composed of 80:20:1 hexane/ethyl ether/acetic acid (v/v/v), and a developing solution of 10% phosphomolebdic acid in ethanol were used.

4.2.2.7. Statistical analysis

Statistical evaluation of data was performed using the statistical software package Minitab version 15. Data are reported as mean value ± standard deviation of three replicates. Single factor analysis of variance (ANOVA) was conducted to

differentiate significant differences among mean values of the data, according to the least significant difference criteria with a 95% confidence level ($P < 0.05$).

4.3. Results and discussion

4.3.1. Subcritical hydrolysis and biorefining

Figure 4.1 shows the biorefining concept of value recovery from processing of hazardous SRM. The hydrolyzates collected were dark-brownish, tacky and viscous with cooked protein odor. The subcritical hydrolysis conditions used affected the yield, molecular size distribution and composition of the hydrolyzed protein recovered from the SRM (Table 4.1) as expected. Variation of the subcritical hydrolysis temperature resulted in significant changes of the molecular weight and molecular weight distribution and hence free and total amino acid profile of the resulting protein hydrolyzates. Some of the subcritical hydrolysis conditions were shown to simultaneously hydrolyze the lipids fractions as well (Figure 4.3) discussed in later sections.

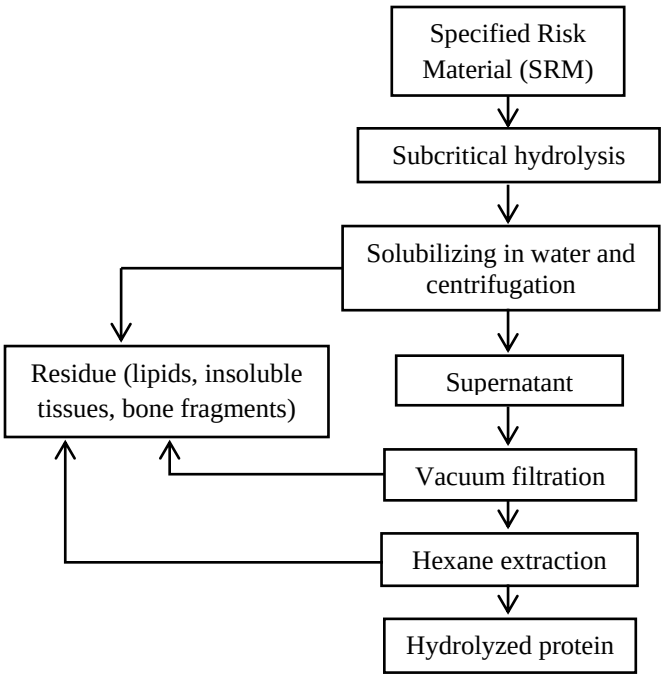

Figure 4.1. Biorefining of SRM into safe industrial feedstock.

Each fractions of the biorefining can be used for different applications. For example, ash residues composed of bones, rich in calcium and phosphorus [19, 20], can be used as a low cost phosphorus source with no heavy metal content, or as an organic fertilizer. The lipid fraction biorefined as hexane extract can be utilized in the oliochemical industry whereas hydrolyzed lipids mainly composed of fatty acids can be further pyrolyzed [21], or transesterified to ethyl/methyl esters for fuel applications [22]. The protein fragments can be used as a resource for applications including bioplastics, flocculants, adhesives, etc. [13, 20]. An overall mass balance, conducted with the aim of calculating the yield of protein hydrolyzate, was computed and presented in Table 4.1. The yield of protein hydrolyzates was lowest for the higher end hydrolysis temperatures (240 and 260 °C), possibly as a result of more severe decomposition of proteins and peptides

into volatiles, such as ammonia, organic acids, and other nitrogenous gases as discussed in the later sections.

Table 4.1. The molecular size distribution, yield and protein concentration of biorefined hydrolyzates at the studied subcritical hydrolysis temperatures.

Hydrolysis temperature (°C)	Molecular size distribution (Da)	Yield (%)*	Protein concentration dry weight basis (%)**
180	< 49,317	87.6 (38.6)	91.0 ± 1.7
200	< 24,638	85.8 (37.8)	89.2 ± 3.0
220	< 8,978	84.2 (37.1)	83.8 ± 4.1
240	< 5,420	77.6 (34.2)	82.7 ± 3.7
260	< 3,485	71.6 (31.7)	84.2 ± 2.2

* Yield was calculated by conducting mass balance of protein concentration in the SRM and biorefined protein hydrolyzates. Values in bracket are yield of hydrolyzates per rendered SRM.
** Protein concentration was calculated by multiplying the Dumas nitrogen with Jones factor of 6.45 [17].

Crude protein concentration in the hydrolyzates calculated based on Dumas nitrogen was higher in the lower range subcritical hydrolysis temperature (180, 200, 220°C) than the 240 and 260 °C (Table 4.1). This observation could also be ascribed to the more severe protein cleavage and decomposition by the higher hydrolysis temperatures (240 and 260°C). One common mechanism of protein decomposition is deamination that results in the loss of nitrogenous compounds. The Dumas method, and its limitation of calculating all nitrogen as protein, obviously takes into account all such compound loss as protein loss and may have resulted in the observed lower protein concentration by the higher temperatures.

4.3.2. Molecular weight of hydrolyzed proteins

Molecular weight and molecular weight distribution of proteins and peptides are important parameters that affect some of the physico-chemical properties, such as solubility, foaming and gelling capacity and stability etc. The SEC-HPLC technique separates molecules on the basis of their hydrodynamic volume and is widely used in the determination of molecular weight distribution of polymers and more particularly proteins and peptides [23]. The chromatogram of an external standard and molecular weight distribution of the extracted hydrolyzates for each hydrolysis temperature is shown in Figure 4.2 (a and b). A calibration of the external standard, approximated by a linear curve of the logarithm of the molecular weight (W) against the distance travelled by the analyte molecule (r), was obtained to calculate weight distribution starting point as follows:

$$Log_{10}^{M} = ar + b$$

Where a and b were determined by fitting the external standard data run on the same column. The starting point of M distributions is reported in Table 4.1. The SEC-HPLC clearly showed a gradual shift of peak chromatograms towards the smaller size peptides with increasing hydrolysis temperature. The subcritical hydrolyzed of SRM at 180, 200, 220, 240 and 260 °C resulted in peptides with molecular size distribution below 49.3, 24.6, 9.0, 5.4, and 3.5 kDa, respectively. The molecular weight distribution pattern as seen from Figure 4.2 appears similar for 180 and 200 °C hydrolyzates with the 180°C showing larger protein hydrolyzates than the 200 °C. Similarly, hydrolyzed protein extracts from

hydrolyses conducted at 220, 240, and 260 °C exhibited another similar distribution pattern.

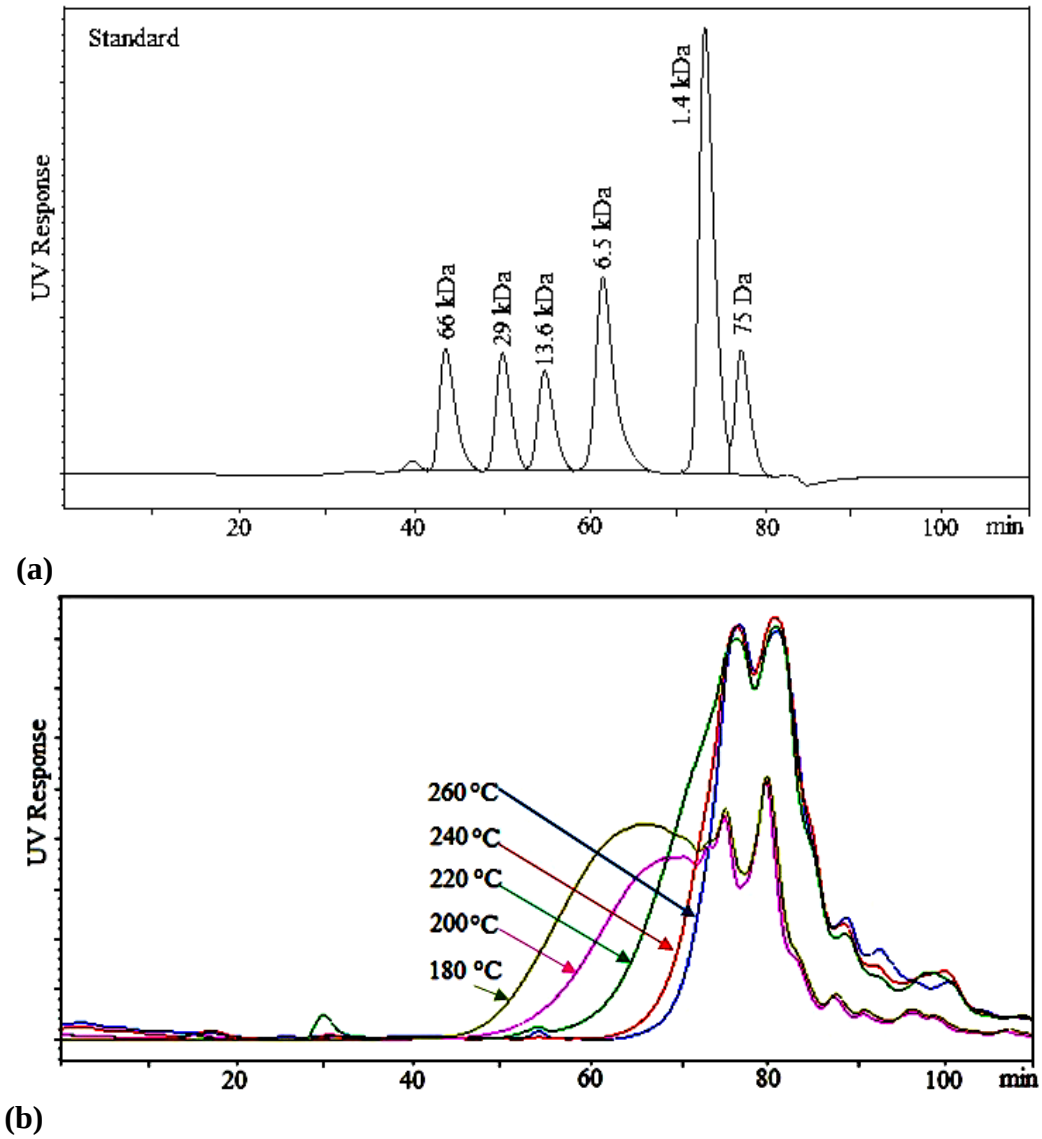

Figure 4.2. SEC-HPLC chromatograms of (a) external standard and (b) hydrolyzed SRM protein extract at temperatures above CFIA and FDA approved protocol.

Higher hydrolysis temperature resulted in narrower molecular size distribution of the resulting hydrolyzates. Compounds that appear to have molecular weight

below the smallest amino acid (75 Da) were also detected in all the hydrolysis cases. These compounds could be attributed to thermal decomposition products of peptides and amino acids such as hydrocarbons, organic acids, aldehydes, primary and secondary amines and imines [24]. Gel electrophoresis and size exclusion chromatography studies reported earlier [20] showed that proteins in unhydrolyzed SRM protein extract were distributed over the range of 75 Da to 400 kDa. This shows that severe cleavage of proteins happened during the subcritical hydrolysis. In addition, the molecular weight distribution was much narrower in the hydrolyzed proteins than the original protein extracts and a clear decreasing pattern with an increase of hydrolysis temperature. Narrow molecular weight distribution or lower polydispersity is a potential advantage for functional utilization [25] because lower polydispersity generally corresponds to greater feedstock uniformity.

Since the hydrolysis changed the molecular weight average and molecular weight distribution, it is expected that it will ultimately change several properties of the extracted proteins and protein hydrolyzates. For instance, variation in metal chelating behavior of proteins and peptides with change in the molecular size as an organic fertilizer was reported in the literature [26, 27]. Additionally, variation of surface hydrophobicity, protein solubility, and emulsification activity with hydrolysis was reported [28]. Prior to hydrolysis, SRM or any of the rendered animal proteins including meat and bone meal [25] have poor solubility and heterogeneity that limit their utility in functional utilization. However, the hydrolyzed SRM extracts showed good solubility and uniformity [20]. As we

reported earlier on epoxy curing with hydrolyzed SRM, activation energy decreased as the molecular weight of protein hydrolyzates decreased [29]. This means that the additional energy needed for hydrolysis at higher temperatures can be compensated for by lower energy needed for subsequent conversion into value-added products.

4.3.3. Free and total amino acids

Quantitation of amino acid is widely accepted method to describe the intensity of the hydrolysis techniques in breaking down the peptide bond of the SRM protein to release free amino acids. The release of free and total amino acids as a result of the subcritical hydrolysis at 180, 200, 220, 240 and 260 °C is displayed in Tables 4.2 and 4.3, respectively. The free amino acid present in rendered meat and bone meal protein extract prior to hydrolysis was below the detection limit of HPLC. As shown in Figure 4.3 and Table 4.2, the greatest amount of total free amino acids produced was 66.7 mg/g at 240°C.

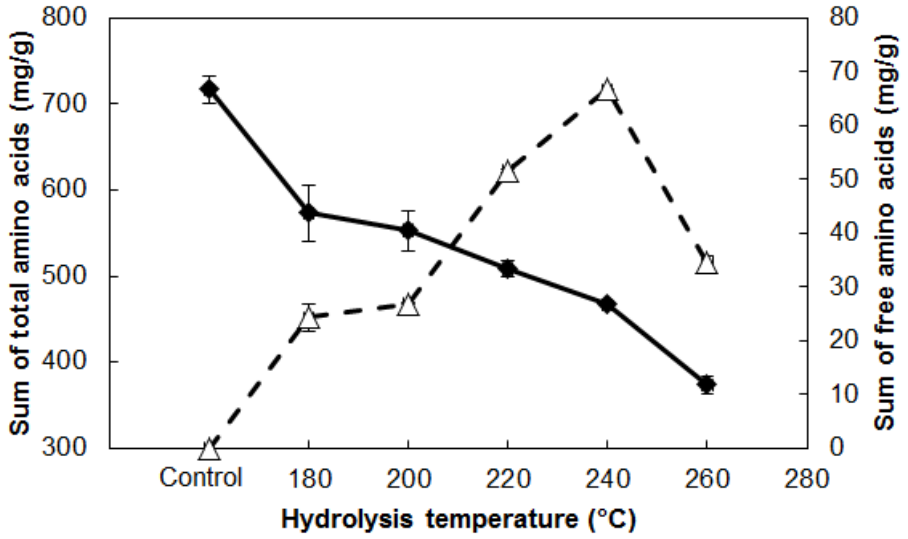

Figure 4.3. Sum of free amino acids (◆) and total amino acids (△) released from control MBM protein extract,180, 200, 220, 240 and 260 °C protein hydrolyzates extracts.

Table 4 2. Free amino acid profile of hydrolyzate extract hydrolyzed at temperatures of 180, 200, 220, 240 and 260 °C in mg/g.

	Free amino acids (mg/g) in dry weight of the hydrolyzed SRM protein				
Temperature (°C)	180	200	220	240	260
Aspartic acid	1.17 ± 0.0[a]	1.89 ± 0.01 [b]	1.55 ± 0.03 [a]	-	-
Glutamic acid	0.10 ± 0.02	-	-	-	-
Serine	0.84 ± 0.08[a]	0.85 ± 0.00[a]	-	-	-
Histidine	0.58 ± 0.02[a]	0.77 ± 0.00[b]	1.60 ± 0.01[c]	-	-
Glycine	6.64 ± 0.01[a]	8.47 ± 0.02[b]	21.33 ± 0.08 [c]	23.02 ± 0.07 [c]	10.81 ± 0.01[d]
Arginine	1.71 ± 0.30[a]	1.80 ± 0.00[a]	2.74 ± 0.01[b]	0.01± 0.00[c]	0.01± 0.00[c]
Alanine	7.01 ± 1.20[a]	7.11 ± 0.10[a]	12.38 ± 0.13[b]	23.43 ± 0.02[c]	12.67 ± 0.01[d]
Tyrosine	0.65 ± 0.11[a]	0.62 ± 0.00[a]	1.09 ± 0.01[b]	1.55 ± 0.00[c]	0.66 ± 0.00[a]
Valine	1.00 ± 0.17[a]	0.87 ± 0.00[a]	2.21 ± 0.02[b]	3.76 ± 0.02[c]	2.23 ± 0.02[b]
Methionine	0.86 ± 0.15[a]	0.82 ± 0.01[a]	1.41 ± 0.01[b]	1.30 ± 0.06[c]	-
Phenyalalanine	0.50 ± 0.09[a]	0.55 ± 0.00[a]	1.21 ± 0.0[b]	1.87 ± 0.00[c]	0.68 ± 0.01[d]
Isoleucine	0.40 ± 0.07[a]	0.37 ± 0.00[a]	0.82 ± 0.02[b]	1.79 ± 0.01[c]	0.78 ± 0.02[b]
Leucin	1.00 ± 0.17[a]	1.05 ± 0.00[a]	2.34 ± 0.02[b]	3.74 ± 0.00[c]	1.41 ± 0.00[d]
Lysine	1.88 ± 0.30[a]	1.74 ± 0.01[a]	2.88 ± 0.01[b]	6.30 ± 0.04[c]	5.39 ± 0.03[d]
Total	24.34 ± 2.53[a]	26.91 ± 0.15[a]	51.56 ± 0.26[b]	66.73 ± 0.18[c]	34.61 ± 0.12[d]

Value – mean ± standard deviation

[a-d] Means with the same superscript letters within a row are not significantly different at $P < 0.05$ level.

Generally, released free amino acids increased as the hydrolysis temperature was increased with the exception of 260 °C hydrolysis (Figure 4.3) possibly as a result of degradation of the released amino acids into other decomposition products.

Among the 15 quantified amino acids, five amino acids at 260 °C, four amino acids at 240 °C, two amino acids at 220 °C and one amino acid at 200 °C hydrolysis were below the detection limit of HPLC (Table 4.2).

As expected, results of the molecular weight distribution and released free amino acid quantification all showed an incremental hydrolysis as the subcritical hydrolysis temperature was increased. The increase in the degree of hydrolysis by a temperature increment was much more pronounced than the increase observed by increasing the hydrolysing water concentration reported by Mekonnen *et al* [20]. These results could be due to increased solubility of protein at high temperature. However, the decrease in density of water at elevated temperatures that contributes also to other property changes, such as an increase in dissociation constant (K_w) or dielectric constant of water may have attributed to the increased hydrolysis of the protein [10]. For example, K_w increases from 1×10^{-14} at 25 °C to 7×10^{-12} at 220 °C, and thus the concentration of hydronium and hydroxide ions increases. In the presence of hydronium and hydroxide ions, the water acts as an acid/base precursor to break down peptide bonds into smaller molecules of soluble protein or amino acids. Low density and low viscosity of water together with complete miscibility with many substances, provides subcritical water with an enhanced transport properties and an excellent reaction medium for fast and efficient reactions [30] that may have resulted in the observed protein cleavage.

Table 4 3. Total amino acid profile of hydrolyzate extract hydrolyzed at temperatures of 180, 200, 220, 240 and 260 °C in mg/g.

	MBM	SRM hydrolysis temperature (°C)				
		180	200	220	240	260
Aspartatic acid	61.9 ± 0.8 [a]	23.7 ± 1.8 [b]	21.4 ± 0.4 [b]	24.9 ± 0.8 [b]	-	-
Glutamic acid	119.4 ± 1.1 [a]	113.9 ± 6.9 [a]	102.4 ± 1.6 [b]	93.3 ± 2.0 [b]	138.3 ± 1.3 [c]	151.6 ± 3.4 [d]
Serine	31.5 ± 0.3 [a]	9.9 ± 0.6 [b]	8.1 ± 0.2 [b]	7.8 ± 0.2 [b]	-	-
Histidine	18.4 ± 0.2 [a]	11.8 ± 0.6 [b]	11.6 ± 0.3 [b]	10.3 ± 0.2 [b]	7.1 ± 0.1 [b]	0.5 ± 0.1 [c]
Glycine	136.1 ± 3.8 [a]	108.2 ± 6.5 [b]	108.4 ± 2.0 [b]	102.7 ± 1.6 [c]	101.3 ± 0.9 [c]	64.0 ± 1.6 [d]
Threonine	24.9 ± 0.2 [a]	7.8 ± 0.5 [b]	6.8 ± 0.1[c]	5.3 ± 0.1[d]	-	-
Arginine	66.4 ± 3.3 [a]	38.2 ± 2.0 [b]	39.2 ± 0.6 [b]	40.8 ± 1.1 [b]	1.1 ± 1.9 [c]	-
Alanine	72.3 ± 0.6 [a]	68.6 ± 4.2 [a]	67.8 ± 1.1 [a]	60.0 ± 1.0 [b]	54.3 ± 0.4 [c]	46.4 ± 1.1 [d]
Tyrosine	22.3 ± 0.3 [a]	19.0 ± 1.0 [a]	22.8 ± 0.4 [a]	19.0 ± 1.8 [a]	26.5 ± 0.1 [a]	24.5 ± 2.0 [a]
Valine	29.1 ± 1.1 [a]	32.6 ± 1.9 [a]	32.2 ± 0.6 [a]	27.5 ± 0.5 [a]	30.9 ± 0.5 [a]	23.4 ± 0.7 [b]
Methionine	15.3 ± 5.1 [a]	11.2 ± 0.5 [a]	8.2 ± 3.5 [b]	7.6 ± 2.1 [b]	8.3 ± 0.8 [b]	4.8 ± 0.3 [c]
Phenylalanine	22.4 ± 3.2 [a]	25.5 ± 1.5 [a]	25.0 ± 0.4 [a]	21.6 ± 0.4 [a]	20.4 ± 1.4 [a]	17.2 ± 2.3 [a]
Isoleucine	21.3 ± 0.2 [a]	22.0 ± 1.3 [a]	21.7 ± 0.5 [a]	18.6 ± 0.4 [a]	18.0 ± 0.2 [a]	11.9 ± 0.3 [b]
Leucine	38.9 ± 0.2 [a]	47.1 ± 2.8 [b]	45.3 ± 0.8 [b]	39.8 ± 0.8 [a]	35.4 ± 0.3 [a]	21.2 ± 0.6 [c]
Lysine	36.5 ± 2.5 [a]	33.7 ± 3.2 [a]	32.2 ± 0.4 [a]	29.0 ± 1.0 [a]	24.4 ± 1.6 [b]	13.9 ± 1.2 [c]
Total	716.7 ± 16.4 [a]	573.2 ± 33.1 [b]	553.1 ± 23.5 [b]	508.2 ± 9.9 [c]	466.7 ± 4.15 [d]	373.4 ± 9.4 [e]

Value – mean ± standard deviation (n=3),

[a-e] Means with the same superscript letters within a row are not significantly different at $P < 0.05$ level.

Comparison of a previous study result [20] and the findings obtained here indicate that for applications that require short peptide segments, increasing the temperature was more efficient than increasing the water concentration. It is also plausible that increasing the temperature might be better justified than increasing the hydrolysing water concentration from energy consumption perspective.

The total amino acid quantification conducted by complete acid hydrolysis of the protein hydrolyzates obtained from biorefining of the subcritical hydrolyzates is shown in Table 4.3. It can be observed that some of the amino acids are completely destroyed during the hydrolysis. This is more pronounced particularly for the 240 and 260 °C hydrolyses. Such severe hydrolysis by the might also cause degradation and generation of other organics that could have inflated the amino acid quantification. For example, an increase in glutamic acid concentration at hydrolysis temperatures beyond 220 °C (Table 4.3) could be an artifact due to the detection of some other peptide/amino acid decomposition product having the same fluorescence as glutamic acid. This claim is further substantiated by thermal lability of glutamic and aspartic acid under subcritical hydrolysis as reported by Espinoza *et al*. [9].

Near complete decomposition of aspartic acid, serine, threonine and arginine was observed at a hydrolysis temperature between 220 and 240 °C. The only thermostable amino acids that did not show any significant change over the hydrolysis temperature range in this study were tyrosine and phenylalanine (Table 4.3), perhaps because of the stable benzene ring. Generally, a significant number of the studied amino acids showed a pronounced degradation above 220 °C. The

sum total amino acids released at each temperature against hydrolysis temperature is plotted and shown in Figure 4.4. The 180 °C subcritical hydrolysis resulted in a sum total amino acids reduction of 20 % whereas the 260 °C hydrolysis resulted in 48 % reduction from the control MBM. It is worth pointing out here that the acid hydrolysis conducted following the subcritical hydrolysis for total amino acid quantification might have enhanced the decomposition of some of the amino acids observed in Table 4.3. Furthermore, co-existence of multiple amino acids together may negatively influence the overall stability of amino acids [31]. In recapitulation, the free and total amino acid analysis indicated that the effect of subcritical hydrolysis was strongly marked when the temperature increases.

4.3.4. Lipids hydrolysis

Some of the subcritical hydrolysis conditions may hydrolyze the rendering left over lipids/tallow TG into DG, MG, FA, and glycerol in addition to the proteins. This suspicion was on the basis of the drying (lyophilization and oven drying) difficulties observed on the 240 and 260 °C hydrolyzed protein extracts. Since glycerol is hydrophilic and has a high boiling point (292 °C), it is likely that it remains with the hydrolyzed proteins during the biorefining process and could change the drying thermodynamics and increase water retention of the protein fragments to cause the observed drying challenge. Levine *et al*. [22] also reported the possibility of lipid hydrolysis starting from as low as 225 °C for 15 min under subcritical water. The possibility of lipid hydrolysis into TG, DG, MG, and FA at subcritical hydrolysis conditions was investigated here using TLC and results are shown in Figure 4.4. Comparison of the TG, DG, MG, and FA markers with the

hexane extracts of the hydrolyzates clearly exhibited that at 240 and 260 °C, the TG band has completely disappeared, and FA appeared to be the dominant band on the chromatogram. This was a clear indication of the TG hydrolysis possibility at the higher end of the subcritical hydrolysis investigated in this research. On the other hand, it was observed that the 180, 200 and 220 °C hydrolyzate hexane extracts contained triglycerides in significant quantities. Moreover, the significant increase in total volatile organic acid concentration as the temperature increases may be attributed to peptide and amino acid degradation (discussed in section 4.3.5).

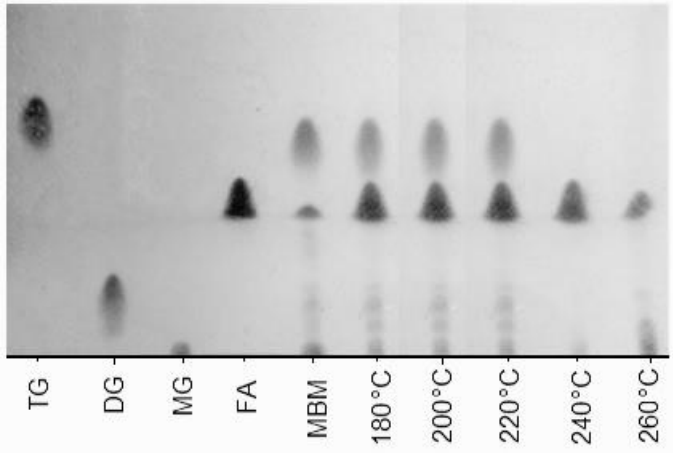

Figure 4.4. Thin layer chromatography of fat hydrolysis: Triglyceride (TG), Diglyceride (DG), monoglyceride (MG) and fatty acids (FA) marker. The samples are hexane extract of MBM, and hexane extract recovered from 180, 200, 220, 240 and 260 °C hydrolyzates.

High temperature subcritical hydrolysis (e.g. 260 °C) might have an advantage, because it results in simultaneous hydrolysis of both the protein and fats/triglycerides. Fatty acids obtained as a result of fat hydrolysis can

subsequently be either transesterified or pyrolyzed to produce renewable chemicals or fuels [21]. In summary, the observed challenges in drying the 240 and 260 °C hydrolyzed protein fractions could be attributed to the presence of glycerol as hypothesized earlier. Such drying challenges could be solved through modification of the biorefining process. For example, isoelectric precipitation of the hydrolyzed protein followed by centrifugal separation prior to the oven drying or lyophilization (Figure 4.1) that will leave the glycerol in the water phase, is expected to solve the observed drying challenge.

4.3.5. Low molecular weight organic acid

Low molecular weight organic acids are useful industrial chemicals and feedstock for manufacture of a wide range of useful chemicals and polymers. For example, acetic acid and propionic acids are a feedstock and an intermediate for the production of cellulose acetate, respectively. The formation of these low molecular weight organic acids, and other organics, such as amines, aldehydes, ammonia, and other organic compounds due to deamination, decarboxylation, hydroxylation and oxidation of amino acids produced as a result of the hydrothermal decomposition was reported in the literature [32]. These organic acids could also be produced from the hydrolysis of lipids [33]. Among the identified organic acids (Table 4.4), acetic acid showed the highest yield over all the hydrolysis conditions.

Table 4 4. Low molecular weight organic acids in mg/g of aqueous extract

	Average organic acids (mg/g) DWB				
Hydrolysis temp	180	200	220	240	260
Acetic acid	9.3 ± 0.3^{a}	9.0 ± 0.3^{a}	9.9 ± 0.3^{b}	10.2 ± 0.1^{b}	24.7 ± 3.2^{c}
Propionic acid	2.0 ± 0.1^{a}	1.8 ± 0.2^{a}	3.1 ± 0.0^{b}	3.9 ± 0.0^{c}	8.7 ± 0.8^{d}
Isobutyric acid	0.6 ± 0.1^{a}	0.5 ± 0.1^{a}	0.4 ± 0.2^{a}	0.8 ± 0.1^{b}	1.8 ± 0.1^{c}
Butyric acid	1.8 ± 0.3^{a}	3.1 ± 0.3^{b}	4.8 ± 0.1^{c}	8.9 ± 0.2^{d}	8.8 ± 0.7^{d}
Isovaleric acid	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}	0.6 ± 0.0^{b}	1.2 ± 0.1^{c}	5.3 ± 0.4^{d}
Valeric acid	0.7 ± 0.2^{a}	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	1.5 ± 0.0^{b}	2.1 ± 0.1^{c}
Isocaproic acid	0.3 ± 0.2^{a}	0.4 ± 0.0^{a}	1.0 ± 0.0^{b}	1.5 ± 0.0^{c}	5.7 ± 0.4^{d}
Hexanoic acid	0.6 ± 0.2^{a}	0.7 ± 0.1^{a}	1.3 ± 0.0^{b}	4.1 ± 0.1^{c}	2.8 ± 0.6^{d}
Heptanoic acid	0.2 ± 0.3^{a}	0.1 ± 0.0^{a}	0.5 ± 0.2^{a}	1.1 ± 0.1^{b}	3.2 ± 0.2^{c}
Total	15.9 ± 1.6^{a}	16.6 ± 1.2^{a}	22.2 ± 0.6^{b}	33.1 ± 0.4^{c}	62.2 ± 6.3^{d}

Value – mean ± standard deviation (n=3).

[a-d] Means with the same superscript letters within a row are not significantly different at $P < 0.05$ level.

Similar observation of high acetic acid yield from sewerage sludge and waste fish entrails was reported by Quitain *et al*. [32]. Butyric and propionic acids were the next observed acids in fairly high quantities. The total organic acids produced at 180 and 200 °C hydrolysis was not statistically significant. However, a significant increment was observed as the hydrolysis temperature went beyond 200 °C (Table 4.4). This is aligned with the previous observation documenting that amino acids start to degrade above 220 °C and at the same time more fatty acids with components of low molecular weight organic acids were also produced from the lipid hydrolysis observed at 240 and 260 °C. Yoshida *et al*. [34] reported that

lactic acid and pyroglutamic acid are the other common organic acids produced during proteinacious biomass hydrolysis. The pyroglutamic acid could be produced from decomposition of glutamic acid through lactamization processes [31]. Some of the longer chain organic acids, in particular the C5 and longer organic acids [33] might be contributed by the fat and fatty acid fractions of the lipid hydrolysis.

4.4. Conclusions

This work demonstrated that subcritical hydrolysis followed by biorefining is a technically feasible and controlled pathway to convert an otherwise hazardous SRM waste into valuable industrial feedstock such as hydrolyzed proteins and peptides, amino acids, fatty acids, volatile organic acids and mineral rich ashes. Such integrated bio-refinery approach could also eliminate the accumulation of hazardous waste in the landfill, reduce risk of aquifer contamination with prion, and also reduce the greenhouse gas emission as a result of incineration of SRM in some places. Results of this study could be extended to other non-hazardous waste biomasses including food waste, blood meal, meat and bone meal, chicken feathers etc. The temperature ranges studied had a substantial effect on the molecular weight and weight distribution, amino acid profile and other ingredients of the SRM. Although the higher temperature ranges (240 and 260 °C) resulted in severe protein cleavage and degradation of the amino acids, the molecular weight distribution of the biorefined protein hydrolyzates obtained as such were more uniform. The lower end hydrolysis temperatures (180, 200 and

220°C), on the other hand, produced protein hydrolyzates with wide molecular size distributions.

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Chapter 5

Thermosetting Proteinacious Plastics from Hydrolyzed Specified Risk Material[4]

5.1. Introduction

Prior to the emergence of bovine spongiform encephalopathy (BSE), the primary use of proteinacious fraction from rendering industry was as feed ingredients in the form of meat and bone meal, meat meal and blood meal. The recent outbreaks of BSE in North America and Europe prompted the widespread ban of certain cattle tissues, known as specified risk materials (SRM), from use as animal feed, pet food and fertilizers [1]. BSE causing infectious agents, prions, concentrate in these SRM tissues that include the skull, brain, trigeminal ganglia, eyes, spinal cord, and dorsal root ganglia from cattle over 30 months of age and the distal ileum and tonsils from cattle of all ages [1]. The economic strain on the entire livestock sector and on rendering operators, and the environmental risks posed by several million tons of such materials already deployed in landfills, demand immediate economically viable solutions. In Canada more than three hundred thousand tonnes/year [2, 3] of such materials are landfilled.

Prion proteins, infectious agent causing BSE, are extremely resistant to conventional decontamination techniques [4, 5]. Thermal hydrolysis at a temperature of at least 180 °C, a pressure of at least 1200 kPa for at least 40 min is among the Canadian Food Inspection Agency (CFIA) [1, 3], Food and Drug

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Administration of the US (FDA) [6] and European Union approved techniques of handling prohibited material including SRM. Such hydrolytic protocols destroy the prions that cause BSE while improving the solubility of the proteinaceous material, a major limiting factor to the functional utilization of rendered animal proteins such as blood meal, [7] meat and bone meal [8] and SRM [9].To our knowledge there are no commercially viable applications of protein material extracted from SRM.

Significant investment has driven research efforts aiming to identify technology protocol to produce plastics using renewable feedstock in an attempt to reduce energy consumption costs and mitigate environmental challenges posed by the production of conventional synthetic polymers. Proteins are among the most suitable renewable candidates as a feedstock to develop bioplastics and plastics. A wide range of proteins have been investigated as a possible gateway bioplastics: casein [10], corn zein [11, 12], wheat [13], egg albumin [14], soy protein [15], feather meal [16] and blood meal [17]. Nevertheless, most of these proteinacious plastics remain poorly utilized on the commercial scale because of their limited resistance to moisture [18], and poor mechanical properties [19]. The high moisture sensitivity and poor mechanical property shortcomings of most protein based plastics could be solved by chemical crosslinking of the protein molecules and modifying the molecular structure. The abundant functional groups of protein on the side chain of each amino acid or the end of each main chain including amine, carboxyls, sulfhydryls and carbonyls give an excellent opportunity for such crosslinking conversion into thermoset hard plastics.

Thermoplastic protein films can be prepared through solvent casting or dry process such as hot pressing, compression moulding as well as melt and extrusion techniques [10-12]. However, poor mechanical property and high hydrophilicity of protein limits the thermoplastic processing techniques for many applications. Thus, chemical crosslinking that involves the formation of covalent bond bridges between proteins chains by using multifunctional reactive agent, that target the reaction between protein functional groups—such as primary amines, carboxyl, hydrolxyl and sulfhydryls—of amino acid residues may provide the desired mechanical property and moisture resistance. The most used agents for chemical crosslinking of proteins are aldehydes (formaldehyde, glutaraldehyde and glyoxal) [20-22]. However, concerns related with toxicity, especially formaldehyde, release of monomer after crosslinking [21], and relatively poor performance of the crosslinked polymer limits their wide use. Epoxy resins, obtained from epoxidation of triglycerides or from the petro-chemical industry, have epoxide end groups that are known to be very reactive with primary and secondary amines, hydroxyls and carboxyl [23, 24] groups of chemicals. This work investigated, for the first time, how epoxide groups of epoxy resin react with hydrolyzed proteins having all the aforementioned functional groups in a similar pattern.

The primary goal of this research was to develop a novel technology platform to convert a hazardous waste protein stream into a value added material. The protocol developed in this research encompasses three sequential steps: thermal hydrolysis, proteinacious material extraction and chemical crosslinking. This

approach may provide an environmentally and economically rewarding alternative of utilizing SRM for non-food/feed industrial applications of plastics than the current land filling option.

5.2. Materials and methods

5.2.1. Materials

Samples of SRM in this study were provided exclusively by Sanimax Industries, Inc. (Montreal, QC, Canada). The samples were shipped and received following the Transport of Dangerous Goods (TDG) regulations and the receipt and use of SRM was documented. The received SRM samples were handled in a biosafety level II laboratory, according to Canadian Food Inspection Agency (CFIA) protocol[1] for safe handling, hydrolysis and disinfection of SRM material. The total protein content of the SRM was reported by Sanimax as 44.05% on dry weight basis. Diglycidyl Ether of Bisphenol A (DGEBA) epoxy resin (Araldite 506 epoxy resin, number average molecular weight <= 700 g/mol), HCl (37%, mol wt. 36.46 g/mol), NaOH (99.6 %, mol wt. 40 g/mol), NaCl (99 %, mol wt 58.44g/mol), Na_2HPO_4(99.6 %, mol wt. 268.07g/mol), KH_2PO_4(99.8%, mol wt 136.09 g/mol) and $MgCl_2$ (99%, mol wt. 95.21 g/mol) were obtained from Sigma-Aldrich, St. Louise, MO, USA. Hexane (99.9% HPLC grade, mol wt. 86.18 g/mol) was purchased from Fisher Scientific Fair Lawn, NJ, USA. KBr (>99%, mol wt. 119 g/mol, FTIR grade) was purchased from Pike technologies, Madison, WI, USA and filter paper (Whatmann 4, diameter 11cm, pore size 20-25 µm) was purchased from Whatmann, UK and used as received.

5.2.2.. Hydrolysis of SRM material and proteinacious material extraction

5.2.2.1 Thermal hydrolysis of the SRM

Hydrolysis reactions were conducted at a temperature of 180 ºC, pressure of 1200 kPa and agitation of 200 rpm for 40 minutes in aqueous solution to inactive prions in SRM according to CFIA approved techniques of disposal [1]. A commercial high-pressure and high-temperature batch reactor (Parr Instrument, Moline, IL, USA) [25] was used for this work. The mass ratio of SRM to water during hydrolysis was set to one. Under these conditions, detailed in our previous study of SRM hydrolysis [9] the proteinaceous material recovered post hydrolysis is characterized by high water solubility. Each hydrolyzed sample (100 g) was treated with 450 mL salt solution consisting of 18 g NaCl, 0.23 g $MgCl_2$, 4.1 g KH_2PO_4 and 4.3 g Na_2HPO_4 according to the method used by Park *et al* [26] by agitating at 200 rpm for 30 min in a shaker (Innova lab shaker, New Brunswick, Canada). The supernatant was separated from the residue through centrifugation (7000 x g for 30 min) on Beckman Centrifuge followed by hexane extraction to remove fats and lipid residues. The raffinate was then collected, vacuum filtered (Whatman no 4), freeze-dried, and grinded to particle size below 100 μm.

5.2.2.2 Chemical crosslinking and plastic preparation

Protein hydrolyzate prepared from thermal hydrolyzed SRM was used as a curing agent for Diglycidyl ether of bisphenol A epoxy resin (DGEBA). The epoxy resin was heated to 60 ºC to reduce viscosity, and mixed under vigorous stirring for 10 minutes with 20, 30, 40 and 50 wt % of extracted and dried hydrolyzed SRM protein samples. The weight ratios for these experiments were selected based on

preliminary studies in our laboratory. According to these studies, when the total weight percentage of protein hydrolyzate powder in the formulation is below 20% long curing time (more than 12h) was required. On the other hand, formulations with over 50% protein hydrolyzate are characterized by high viscosities that impede adequate mixing. The formulated mix was then degassed at 100 ºC for 1h in a vacuum oven to remove solubilized air bubbles and as a result to avoid pore formation during casting. The prepared mix was then casted on a silicon mold. Curing took place at 160 ºC for four hours followed by post curing at 180 ºC for an hour.

5.2.3. Fourier Transform Infrared Spectroscopic (FTIR) Analysis

Fourier transformed infrared (FTIR) spectra of protein hydrolyzate, epoxy resin and cured plastics were carried out on a Shimadzu FTIR-8400S spectrometer fitted with a germanium attenuated total reflection (ATR) with a high sensitivity pyroelectric detector. Samples of fine milled protein hydrolyzate and plastic powders were mixed with KBr salt while the liquid DGEBA were placed on KBr salt disc for analysis. A total of 20 scans were performed at 4 cm^{-1} resolution and the measurements were recorded between 4500 and 400 cm^{-1} under the same condition as the background. IRsolution software (version 1.10) was used for instrument control and data analysis.

5.2.4. Thermal Properties

5.2.4.1. Differential Scanning Calorimetry and Thermogravimetric Analysis

Differential Scanning Calorimetry (DSC) analysis experiments were conducted to investigate thermal transition temperatures of the plastics with a thermal analyzer ($Q_{100,}$ TA Instruments, USA) under nitrogen atmosphere. 5 mg of the polymer samples at the various protein concentration were packed in an aluminum pan and heated at a rate of 10 ºC/min from an equilibration temperature of 0 ºC to 175 ºC. The change in heat flow vs. temperature was recorded by the instrument. Furthermore, to investigate the cure behavior of the epoxy/protein resin system, DSC studies were performed on a pre-cured epoxy-protein mix sample. For this study, the samples were heated over a temperature range of 25 to 250˚C at a heating rate of 5˚C/min and a flow rate of 40 ml/min.

Thermogravimetric (TGA) analysis was also conducted using a thermogravimetric analyzer (Q_{500} series, TA instruments) to study the thermal degradation behavior of the hydrolyzed protein based plastics. About 8 mg of the plastic samples were heated at 10˚C/min over a temperature range of 20 to 800 ˚C in Nitrogen atmosphere.

5.2.4.2. Dynamic Mechanical Analysis (DMA)

Dynamic mechanical properties were studied in multifrequency strain mode at an oscillatory frequency of 1 Hz with an applied deformation of 0.05 mm during heating on TA DMA, ensuring that the materials were still in the linear

viscoelasticity region (Q_{800} DMA, TA instruments). Analyses were performed on rectangular specimen of 20 x 10 x 2 mm dimensions clamped with single cantilever. Specimen dimensions were measured with digital calipers at three different places and averaged. Each sample was analyzed in triplicate. Temperature scans between 0 and 175 °C were performed at 2 °C/min heating rate and the storage modulus (G′), loss modulus (G″) and tan δ ($tan\ \delta = G''/G'$) were recorded as a function of temperature.

5.2.5. Mechanical Properties

Test specimens for mechanical property testing was prepared by machining specimens using water jet cutters (Omax 2652) at a pressure of 50,000 psi from casted sheets according to the standard ASTM D638 – 10 [27]. The specimens were then conditioned in a humidity chamber (ETS 5518, Glenside, PA, USA) at 23 ± 2 °C and 50 ± 5 % relative humidity for 40 h prior to the test. Mechanical property tests (tensile strength, breaking elongation, and modulus) were conducted at room temperature on an Instron (Instron 5967, Norwood, MA, USA) equipped with a 5 kN load cell at a crosshead speed of 10 mm/min. The data for each sample were obtained from an average of testing five specimens with an effective length of 3.18 mm and width of 9.53 mm.

5.2.6. Moisture Resistance

The moisture absorption and weight loss of the cured plastics in water, weak acid and base were studied according to ASTM Standard D 570 [28]. This is because moisture absorption and weight loss to water and solvents are among the major drawbacks of protein based polymers. About 1g of weighed (W_1) specimen of

each casted sheet was submerged in 20 mL distilled water (pH 7); weak acid (pH 3, 0.01N HCl solution) and weak base (pH 9, 0.01 N NaOH solution) in a controlled environment of 23 °C for 24 h. After removal from the solutions, the extra moisture on the surface of the sheets was removed with a paper towel, and the sheets were weighed again (W_2). The absorbed moisture content (*Wabs*) was calculated as follows:

$$W_{abs} = ((W_2 - W_1)/W_1) \times 100$$

The wet sheets were dried in a conventional oven at 70 °C for 24 h and weighed again (W_3); the weight loss of the sheets (*W loss*) was calculated as follows:

$$W_{loss} = ((W_1 - W_3)/W_1) \times 100$$

5.3. Results and discussion

Hydrolysis and protein extraction

Thermal hydrolysis breaks down long chain molecules, such as proteins into shorter protein molecules and peptides and destructing bacteria and other microorganisms. Several studies have also shown that thermal hydrolysis at such temperature and pressure combination not only inactivates BSE causing prions [29], [3] but also cleaves the protein molecules into short protein molecules and peptides. Hydrolyzed proteinacious material was then extracted from the hydrolyzed SRM according to a previously optimized method [9]. The protein yield from the hydrolyzed SRM was about 42% on dry weight basis.

Protein crosslinking reactions

The major active functional groups in protein and protein hydrolyzate include amines ($-NH_2$), carboxyls (-COOH), sulfhydryls (-SH), hydroxyl (-OH) and carbonyls (-CHO) associated with side chain of the amino acids and the end groups of each protein chain. Such abundant functional groups of protein and protein hydrolyzate provide excellent opportunity to modify through a technologically viable route to produce marketable, high-value plastics and biopolymeric materials. Several studies [10-13] have reported thermoplastic processing and chemical crosslinking pathway of processing proteinacious material of various sources into plastics. However, methods and pathways developed for proteins may not be suitable for hydrolyzed proteins due to variation in average molecular size, size distribution, and amino acid composition.

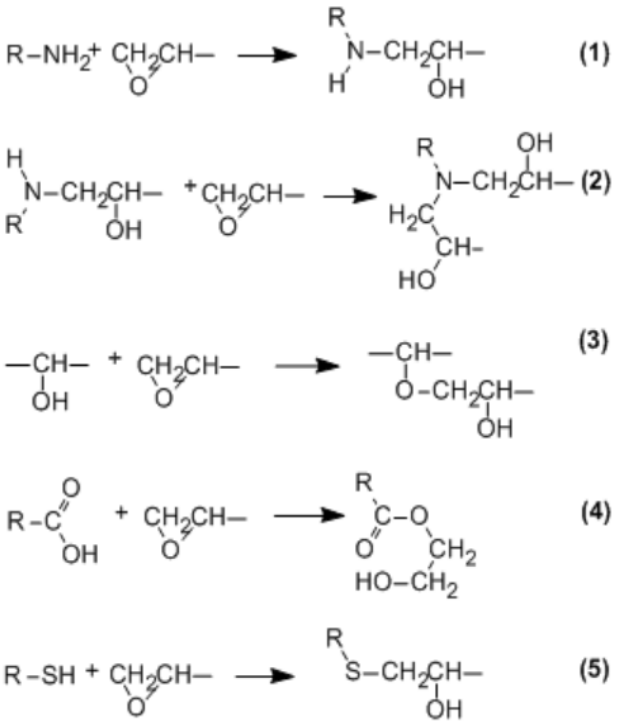


Figure 5.1. Possible reaction between (1) primary amine, (2) secondary amine, (3) hydroxyl, (4) carboxyl and (5) sulfhydryl functional groups of protein hydrolyzate and epoxide group of DGEBA.

Epoxy resins are widely used as a base polymer in adhesives, coatings, casting, laminates and matrix for composite materials. This is because the epoxide functional group of epoxy resin can react with a wide variety of functional groups such as hydroxyl groups, amine groups, and carboxylic acid groups [23], [24]. In this research the hydrolyzed protein, with an average molecular weight of about 13kDa [9] was used as a base polymer at 20, 30, 40 and 40 wt. % and the relatively shorter epoxy resin (700g/mol) were used as crosslinking agent to the hydrolyzed protein. Reaction between the hydrolyzed protein and epoxy resin could go through two main curing reaction mechanisms, polyaddition [23, 24] and

homopolymerization [24] reported for synthetic diamines and other curing agents. Both mechanisms are expected to increase the molecular weight and crosslinking density of the cured polymer [24].

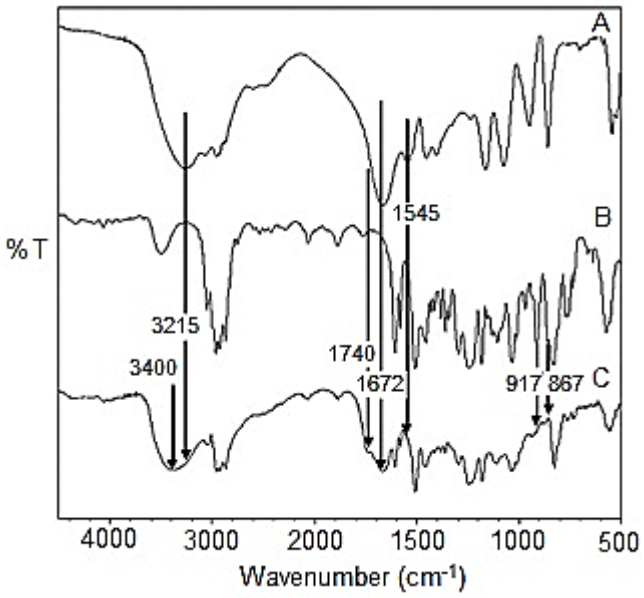


Figure 5.2. Typical FTIR spectra of (A) Protein hydrolyzate, (B) Epoxy resin and (C) cured plastic made by incorporating 30 wt % hydrolyzed Protein hydrolyzate protein and 70 wt. % epoxy resin.

Curing of reactive prepolymer, in this case epoxy resin with hydrolyzed SRM protein , involves the transformation of reactive substances from liquid to viscous rubbery and solid states as a result of the formation of a polymeric network by chemical reaction of some groups in the system [30], and as a result the final cured polymer can no longer melt to flow or solubilize in solvents. Epoxide functional group, characteristic group in the epoxy resin, open up under the attack of amine molecule [31] during the curing reaction of epoxy resins with hydrolyzed protein , resulting in drastic reduction of epoxide groups content.

Possible reaction mechanism between the primary and secondary amine [32, 33] hydroxyl and carboxyl groups [33] of protein hydrolyzate with epoxide group of epoxy resin are depicted in Figure 5.1. Active hydrogen of primary amine reacts with an epoxide of epoxy resin to form secondary amine, and the secondary amine reacts with epoxide group again to cure. IR spectra for protein hydrolyzate, epoxy resin and cured plastic are presented in Figure 5.2. The phenomenon of epoxide group dwindling as a result of the reaction of epoxide groups of the resin with the various functional groups of hydrolyzed protein was observed through intensity reduction at 915 and 863 cm^{-1}. The IR spectra also show intensity increase at 1740 cm^{-1} in the polymer possibly due to the formation of new ester linkages as shown in equation 4 of Figure 5.1. The carboxylic acid groups observed at 3050 cm^{-1} in the protein sample shifted in the crosslinked plastic, and intensity amplification was observed at 3450 – 3300 cm^{-1} that might be attributed to hydroxyl stretching indicating the possibility of equation 4. The hydrolyzed protein and plastics exhibit common peaks at wave numbers of 1672 cm^{-1} corresponding to amide I (C=O). The peak at 1545 cm^{-1} corresponding to secondary amine (N-H) has disappeared. The broad band between 3600 -3200 cm^{-1} corresponding to free and bound –OH and –NH groups didn't allow primary amine group intensity reduction observation in the plastic than the protein hydrolyzate as proposed in equation 1 of Figure 5.1.

Thermal properties

Differential scanning calorimetry (DSC)

DSC is an effective technique of measuring the onset of cure and thermal transitions such as glass transition temperature of thermosetting plastics by measuring the heat flow into or from a sample as it is heated, cooled or held isothermally. Figure 5.3 shows typical DSC exothermic peaks of four different formulation of epoxy resin cured with hydrolyzed protein under nitrogen atmosphere at 5 ˚C/min. Curing was observed for all protein/epoxy formulations in the temperature window 120-160˚C. These peaks correspond to the exothermic reaction involving the epoxide group of epoxy resin and the reactive functional groups of the hydrolyzed protein [34]. Figure 5.3 reveals that the onset, completion of curing and the exothermic peak where the maximum rate of curing takes place was affected by the amount of hydrolyzed protein and epoxy resin in the formulation.

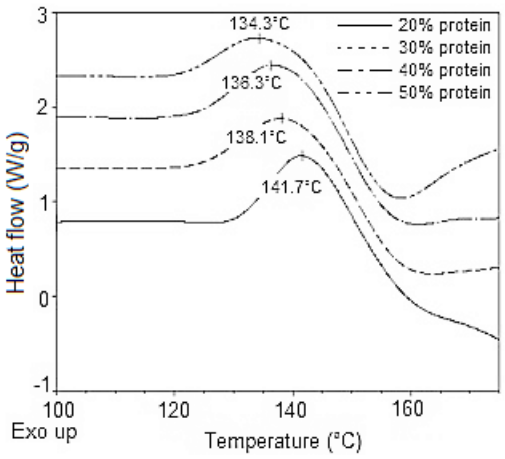


Figure 5.3. DSC cure curves of protein with epoxy resin at 20, 30, 40 and 50 wt % concentration of protein hydrolyzate.

The onset, the peak and completion of curing were observed to decrease as the amount of protein hydrolyzate content in the system increases continuously from 20% to 50% in the studied ranges. Such reduction of all the curing onset, completion and peak cure temperatures might be due to the relative availability of functional groups in the formulations. Similar observation of curing behavior changes with curing agent variation was reported by Zhao *et al* [34] for epoxy resins crosslinked with synthetic hardeners. DSC experiments indicate that, within the studied range of formulation, curing rate improved as the protein hydrolyzate content increased. The curing reactions of protein hydrolyzate with epoxy monomers require high temperature and relatively long curing times. Industrially viable applications may encompass catalysts and accelerators to address these issues.

Glass transition temperature of the plastic network

The glass transition temperature (T_g), the temperature where polymers goes from a hard glass like state to rubber like state, provides important information about the property and structure of polymeric materials. The glass transition temperatures of the cured protein hydrolyzate formulated plastics were studied using a combination of DSC and DMA measurements. Figure 5.4 presents typical DSC thermogram of the formulated epoxy-protein hydrolyzate plastics discussed in this work. The figure shows a broad endothermic depression in all the formulation ranges studied likely due to a glass transition temperature. The inflection point of the endothermic depressions, T_g, decreases as the protein hydrolyzate concentration increases from 20 wt % to 50 wt % in the formulation.

Glass transition temperatures corresponding to 95, 87.5, 77.5 and 66.5 °C were observed on DSC thermogram of 20 %, 30 %, 40 % and 50 % protein hydrolyzate formulations, respectively.

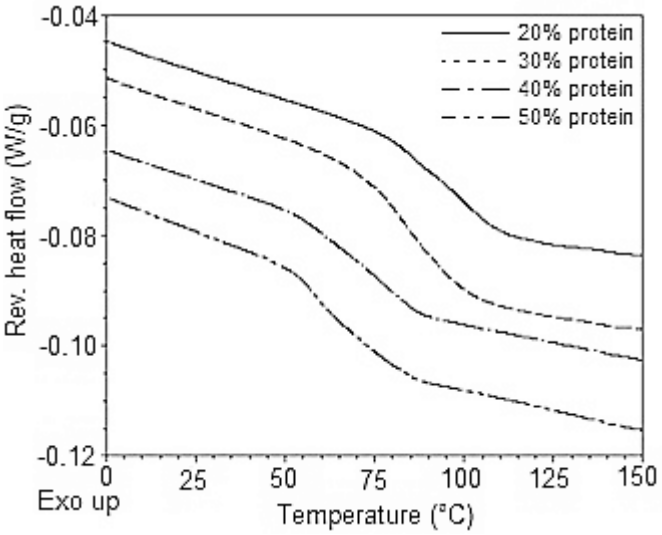


Figure 5.4. DSC thermograms for 20%, 30%, 40% and 50% protein based polymers for T_g determination.

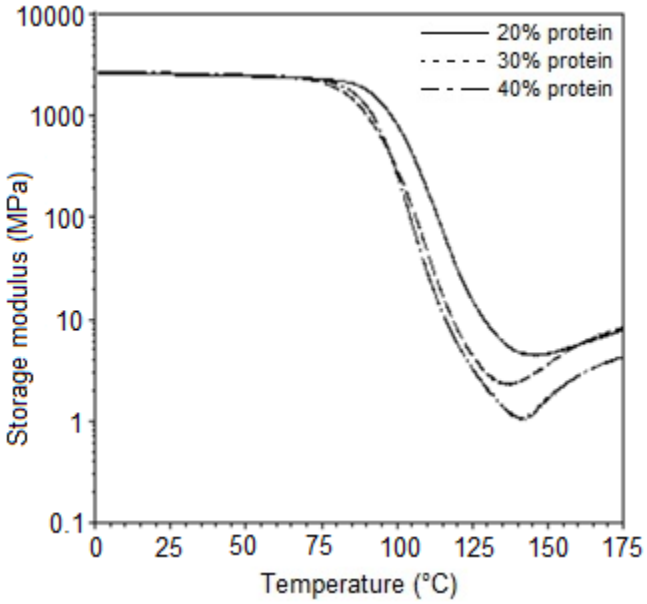
This variation of T_g of the different formulations may be attributed to differences in the crosslink density [35]. It may also be due to the more plastic nature of the proteinacious material backbone chain contributing to the plasticity of the plastic as its content increases in the overall formulation. Similar reduction of T_g as protein concentration increases was observed form the DMA studies as shown in Figure 5.5.

Dynamic mechanical analysis (DMA)

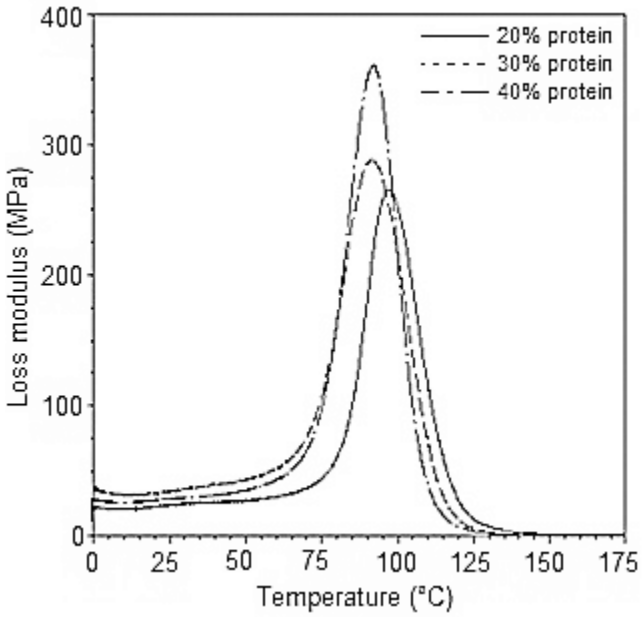
Dynamic mechanical analysis was used here to study the change in storage modulus (G′), loss modulus (G″) and tan δ ($tan\ \delta = G''/G'$) as the cured plastic is heated from 0 to 175 °C and glass transition temperature (T_g) of the cured

plastics. The storage modulus and tan δ at 20, 30 and 40 % hydrolyzed protein concentrations are shown in Figure 5.5 (a-c). The 50% protein formulation reported in the other sections was found to be very brittle for clamping and oscillatory shear strain of the method as described in the materials and methods section, hence results were very variable and not reported here. At lower temperature ranges between 0 to 68 °C, the storage modulus ranges from 2700 to 2400 MPa for the 20, 30 and 40% hydrolyzed protein formulations which shows their load bearing capacity in the temperature range. As the testing temperature was increased through the glass transition temperature, the storage modulus drops and the loss modulus increases in all formulations. The loss modulus increases with increasing temperature. This is because higher temperature levels are correlated to greater molecular mobility of polymer segments which, in turn, increases the heat energy dissipation. The glass transition temperature, T_g, was observed as the maximum peak of tan δ from DMA (Figure 5.5c). The T_g observed here for 20, 30 and 40 % formulations were 118.4, 114.3 and 109.2 °C, respectively. The drop of T_g with proteinacious material increment in the plastic system observed from DMA is in agreement with the DSC observation. It was observed that the value of the T_g observed on DMA is off by about 20 °C from the DSC. Although the T_g of polymers has a kinetic dependence and viewed as a second-order phase transition, it cannot be considered as a true thermodynamic phase transition parameter because it is not a sharp transition from one thermodynamic equilibrium to another [36]. Hence, such offset between the two methods might be attributed to variation in the heating rates used for DMA (2

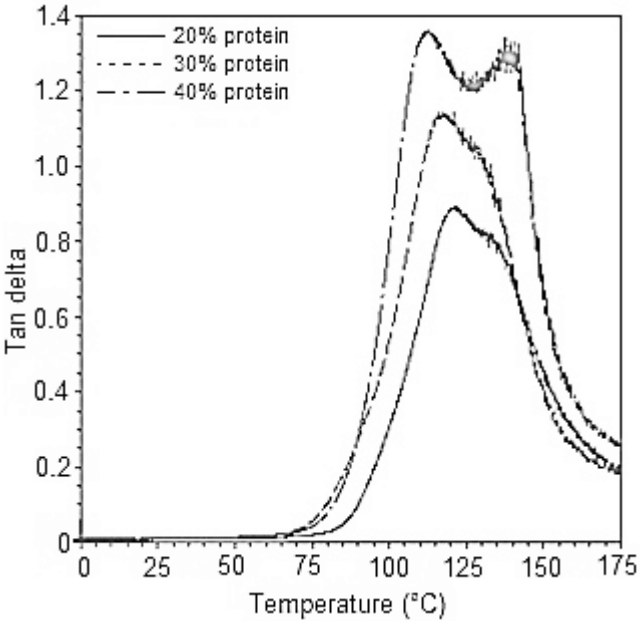
°C/min) and DSC (10 °C/min) and other variations in the measurement conditions [37].



(a)



(b)



(c)

Figure 5.5. (a) Storage modulus of plastics (b) loss modulus and (c) tan δ of plastics at 20%, 30%, and 40% protein hydrolyzate formulation as the cured plastic is heated from 0 to 175 °C in an oscillatory mode of DMA.

The 20 and 30 % formulations exhibited a shoulder after the peak of tan δ (Figure 5.5c), and the 40 % formulation clearly exhibited a secondary peak of tan δ indicating presence of a second T_g . This could be attributed to the high viscosity and relatively poor miscibility of protein hydrolyzate and epoxy resin components that may have resulted in a separated network during curing with a second glass transition temperature [38] [39]. Contrary to the DMA (tan δ) observation, second glass transition temperatures were not detected on the DSC curves (figure 5.4). These observation could be due to difference in the quantities of the phase separated components in the network, one of which being minor to the other leading to very small ΔCp to be detected on DSC. Sircar *et al*. [39] reported a

secondary T_g from DMA and similar difficulty of observing a secondary T_g for elastomers with separated phase.

Thermal stability

Thermogravimetric analysis (TGA), one of the commonly used techniques for rapid evaluation of thermal stability of materials, indicates the thermal degradation of plastics at different temperatures. The thermal stability and degradation pattern of the cured plastics were evaluated between 30-800˚C under nitrogen atmosphere using TGA and a typical thermal degradation pattern of protein hydrolyzate formulated plastic is presented in Figure 5.6. The onset decomposition temperature for the 20, 30, 40 and 50 wt % protein hydrolyzate formulations was observed at 352˚C, 332, 326 and 317 °C, respectively. Generally, the thermograms showed single stage decomposition and an onset decomposition temperature above 317 ºC in all formulations under nitrogen, indicating good thermal stability of the synthesized plastics. Becker *et al* [40] reported 358.3 °C onset degradation temperature for DGEBA epoxy resin cured with commercial hardener. The plastic samples exhibited a 10 % weight decomposition temperature of 387, 368, 353 and 347˚C for 20, 30, 40 and 50 % formulations, respectively. It is noted from the onset and 10 % weight degradation temperatures that the thermal stability of the plastic gradually decreased with increasing protein concentration in the formulation.

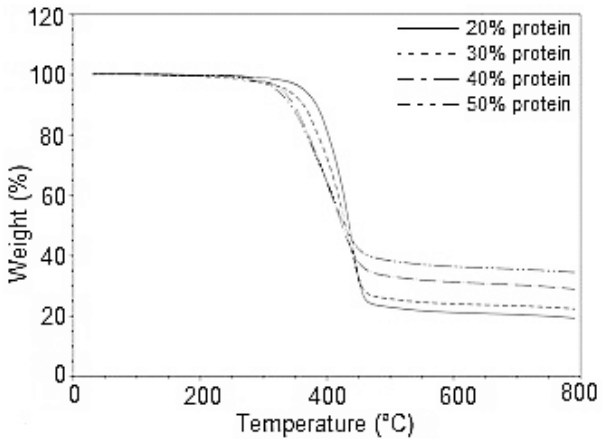


Figure 5.6. TGA thermograms of epoxy resin cured with different ratios of protein under nitrogen atmosphere.

This reduction might be associated with variation in crosslinking density of the different formulations. Furthermore, variability of thermal decomposition temperature with curing time and temperature change is plausible [31]. The char left over after the 800 °C was highest (32 %) for 50 % protein hydrolyzate formulation and lowest (17%) for 20 % formulation. More char yield with 50 % protein hydrolyzate formulation was because of the presence of more inorganic salts used during salt extraction and other inorganic ashes associated with the protein hydrolyzate that survive 800 °C than a formulation with more epoxy and less hydrolyzed protein (20% formulation).

Mechanical properties

Mechanical properties of plastic material provide an indication of expected polymer integrity under stress conditions that would occur during processing, handling, usage and storage. Stress-strain profiles from tensile tests are used to characterize mechanical behavior of polymeric materials [41]. A typical tensile

stress versus strain curve of 20 % hydrolyzed protein plastic is shown in Figure 5.7. The plastic exhibited a rapid increase of stress with increasing strain up to the point of failure. The tensile strength, elongation at break and modulus for each formulated plastic is shown in Table 5.1 below. It is depicted from Table 5.1 that as the protein hydrolyzate content increases in the formulation, the elongation at break and tensile strength decreases, whereas the modulus increases monotonically.

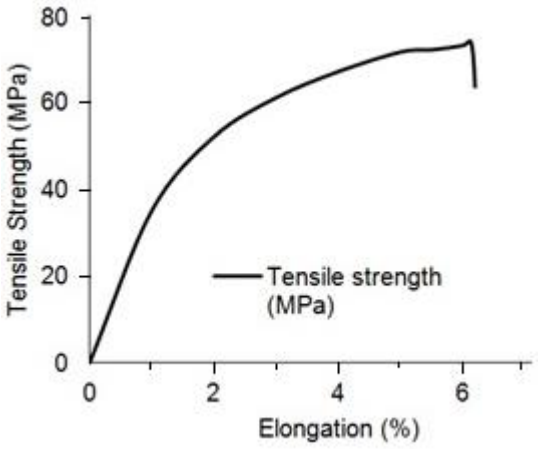


Figure 5.7. Typical tensile stress versus elongation curve of 20% SRM protein polymer.

Table 5 1. Tensile strength, elongation at break and modulus of the proteinacious plastics at the different formulations.

Protein concentration in epoxy resin (wt %)	Tensile Strength (MPa)	Elongation at break (%)	Tensile modulus (GPa)
0	73.75 ± 4.91[a]	6.13 ± 0.11[a]	1.335 ± 0.12[a]
30	58.56 ± 6.82[b]	4.34 ± 0. 07[b]	1.463 ± 0.17[b]
40	34.93 ± 2.37[c]	1.95 ± 0.05[c]	1.498 ± 0.11[b]
50	12.88 ± 1.29[d]	1.47 ± 0.04[d]	1.567 ± 0.15[c]

Average ± standard deviation

Mean values with the same superscript letters within a column are not significantly different at $P < 0.05$ level.

Mechanical properties such as tensile stress, elongation and modulus are directly influenced by crosslinking density[42] and network structure of the resulting thermoset. The decrease of tensile strength with an increase in protein hydrolyzate concentration in the formulation might be due to reduced crosslinking density owing to lack of sufficient diffusion during the curing reactions. All the tested specimens fail at low strains and small elongation, indicating that the polymers had low elasticity. The hydrolyzed protein based plastic developed in this research exhibited high tensile strength and modulus than other protein based systems reported in the literature. [17, 21, 43] The tensile strength of the hydrolyzed protein based plastic specimens was also within the range of other epoxy resin based plastics. [44] Hanoosh and Abdelrazaq [44] reported a tensile strength of

26.86 MPa for an araldite epoxy resin cured with commercial hardener. Generally, mechanical property of the plastic will be affected by several other factors including the degree of cure, curing temperature, thermal history during cure process[45] and catalysis.[46] We point out here that the plastic developed in this research is stiff and brittle – a phenomenon common to epoxy based plastics.[47, 48] Hence, toughening mechanisms such as incorporation of rubbery material [47] in the uncured premix has to be studied.

Moisture absorption and resistance

Plastics are used in broad range of applications including demanding environments. In order to evaluate the possible range of immediate industrial applicability of the system described here, moisture absorption and resistance as a function of pH was evaluated and presented in Table 5.2.

Neutral pH: the water absorption of the plastics was below 1% until 40% by weight of hydrolyzed protein and was not significantly different. The water absorption at 50% protein exhibited statistically significant difference from the rest of the formulations. The weight loss to water on the other hand is not significantly different between 20 and 30 % and between 40 and 50%.

Table 5 2. Water absorption and weight loss of the different plastics in acidic, neutral and basic pH.

(Wt % protein)	pH = 3		pH = 7		pH = 10	
	Water abs. (wt %)	Weight loss (wt %)	Water abs. (wt %)	Weight loss (wt %)	Water abs. (wt %)	Weight loss (wt %)
20	1.12 ± 0.1^{a}	0.77 ± 0.1^{a}	0.44 ± 0.1^{a}	0.52 ± 0.2^{a}	0.97 ± 0.3^{a}	0.66 ± 0.1^{a}
30	2.61 ± 0.1^{b}	1.75 ± 0.3^{a}	0.58 ± 0.1^{a}	1.26 ± 0.4^{a}	2.32 ± 0.3^{a}	1.85 ± 0.2^{a}
40	4.68 ± 0.8^{c}	4.69 ± 0.9^{b}	0.85 ± 0.4^{a}	2.38 ± 0.6^{b}	4.45 ± 0.4^{b}	4.10 ± 0.3^{b}
50	8.51 ± 0.6^{d}	8.72 ± 0.7^{c}	1.43 ± 0.3^{b}	3.03 ± 0.2^{b}	8.35 ± 1.8^{c}	8.33 ± 1.4^{c}

Average ± standard deviation, mean values with the same superscript letters within a column are not significantly different at $P < 0.05$ level.

The high moisture resistance of the plastics observed here is because of crosslinking of the epoxy resin with an otherwise very soluble hydrolyzed protein. Amine crosslinked epoxies are relatively hydrophilic materials able to absorb 1 to 6 % by weight of water in the most frequently used industrial formulations.[49] More importantly, moisture resistance is a major challenge of protein based plastics. [17] Our findings here show that the performance of the protein hydrolyzate plastic towards water is comparable to commercial epoxy based plastics, [49] and it is much more resistant to water than other protein based plastics reported in the literature.[17] For formulations corresponding to 50% protein hydrolyzates, it is plausible to anticipate the formation of an incomplete or partial network structures. This could be due to poor chain mobility owing to the high viscosity of this formulation and/or presence of hydrolyzed protein in quantities above the stoichiometric condition. This in fact could be a reason for a

lower crosslinking density and as a result for the comparatively lower moisture resistance, tensile strength and Tg marked in this 50% formulation. Such incomplete network chains may embed themselves in-between the crosslink nods as filler, resulting in lower elasticity and higher modulus of this formulation shown in Table 5.1.

Acid (pH 3) and base (pH 10) resistance: The water absorption in acid (pH 3) was found to be higher in each formulation compared to the water absorption at neutral pH and in base of the same formulation. The weight loss to acid is also higher than the weight loss to water. With the exception of the 20% protein hydrolyzate formulation, the water absorption is significantly higher in acid than at neutral pH. Water absorption and weight loss at alkaline pH is not significantly different between 20% and 30% and between 40 and 50% formulations. The water absorption at pH 10 didn't show significant difference from the water absorption at neutral pH up to 30% formulation. The weight loss at alkaline pH has a similar pattern of increase as the weight loss in acid pH for each corresponding formulation. The relatively higher water absorption and weight loss of the plastic at low and high pH might be due the hydrolyzing effect of the acid and base to certain bonds such as the peptide or ester bonds [50] of the network resulting in opening up of the structure for water penetration and as a result more absorption and losing of some chains to the solvent observed as weight loss.

Our finding showed that as the concentration of protein hydrolyzate in the formulation increased, increase in both water absorption and weight loss was observed in each of the studied pH ranges. This may be attributed to the lower

crosslinking density of the formulation as more protein hydrolyzate content was incorporated during the curing reaction owing to limited molecular diffusion. The water absorption and solubility property of the protein based plastic exhibited promising characteristics, because solvent resistance was a major challenge associated with most protein based plastics.

5.4. Conclusion

This work demonstrates a new plastics technology platform that utilizes SRM proteins as feedstock. Our group developed a three-step protocol to convert this waste, environmentally hazardous material through thermal hydrolysis, protein extraction and crosslinking with epoxy resin into safe plastics with desirable performance characteristics. DSC and DMA investigations showed that the glass transition temperature of the developed plastic increased in the order 50 < 40 < 30 < 20% hydrolyzed protein formulation. The mechanical properties of the developed plastic were also dependent on the composition of epoxy resin and protein hydrolyzate. Moreover, it was found that all the plastic formulations had good moisture resistance at neutral, acid and basic pH in all formulations. Overall, the 20% protein hydrolyzate formulation exhibited the highest tensile strength and T_g, low temperature curing and better solvent resistance. Crosslinking techniques developed in this study may be extended to unhydrolyzed, long molecular chains proteins of animal source (such as blood meal and meat and bone meal) and plant source (such as soy protein, corn zein or canola protein). However, molecular size variation, amino acid composition and orientation are expected to affect the diffusion of molecules during chemical crosslinking reaction. Hence, the plastic

prepared here by crosslinking of protein hydrolyzate of SRM with epoxy resin has been shown to have suitable properties to be used for applications such as adhesives, matrix of composites and biocomposites, concrete and flooring.

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Chapter 6

Biocomposites from hydrolyzed waste proteinaceous biomass: Mechanical, thermal and moisture absorption performances[5]

6.1. Introduction

Over the past two decades, substantial research efforts have been devoted to developing technologies to incorporate renewable fibers as reinforcing fillers in polymeric composite materials. The use of natural fibers as a mechanical reinforcement material in place of fiberglass for composite materials has many marked advantages. Low cost, low specific density and high specific strength, natural fibers are renewable material and are less energy intensive to produce (grow) than glass fibers [1]. The reduction in fiber density and thus composite density is favorable in certain engineering applications. For instance, biocomposites for automotive parts where vehicle weight and fuel economy are often a concern, lower density is very desirable.[2] Moreover, glass fibers are abrasive to tooling and can cause irritation and discomfort to personnel during manufacturing [3]. Thus, natural fibers such as hemp and flax fibers can potentially compete with E-glass fibers, which serve as a reference because of their great importance in composite technology [4].

Considerable progress has resulted in a broad range of polymeric matrices reinforced with plant fibers such as hemp, jute, bamboo, flax and sisal that are

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now commercially available. [5, 6] During the same period, concerns about the growing environmental footprint and dwindling availability of fossil fuels reserves have driven research and development aimed at developing biodegradable polymeric matrices that could be reinforced with natural fibers. Thermosetting and thermoplastic materials have been synthesized using a broad range of biomass feedstock, from starch [7] to plant protein [8] to animal protein [9]. The growth and commercial success of the global bio-based plastic market, which is expected to reach a total production of approximately 3.5 million metric tons in 2020 [10], remain challenged by biomass processing challenges and by high feedstock costs.

The outbreaks of bovine spongiform encephalopathy (BSE) have resulted in legislations that limited the use of certain cattle tissue known as specified risk material (SRM) as human or animal foodstuffs or fertilizer applications in Canada, the US and European Union [11-13]. While the fats recovered during the rendering process find a market in the oliochemical industry, large quantities of SRM, mainly composed of protein and ash are either presently incinerated or landfilled in regulated facilities in some European Union countries, Canada and the US [14]. The environmental risks posed by such practices and the associated massive costs have inflicted significant economic stress on the rendering industry with profound ramifications to the whole livestock sector. Such waste animal proteins recovered at rendering facilities presently constitute a low-value commodity that can be used as feedstock for the production of a wide array of renewable materials, including plastics [9, 15, 16] [17].

Our laboratory has recently demonstrated that a thermal hydrolysis protocol for the destruction of BSE prions approved by Canadian Food Inspection Agency (CFIA) and Food and Drug Administration (FDA) of the US can be used as a platform to convert SRM into hydrolyzed protein fragments that can be fractionated for industrial functional utilization [17]. These fragments bear reactive functional groups such as primary and secondary amines, hydroxyls, carbonyls and sulfhydryls associated with the side chain of each amino acid or the end of each main hydrolyzed protein chain [9, 18]. Therefore, the short chain length of the hydrolyzed proteins coupled with the abundant functional group make them a unique crosslinking agent for epoxy resins [9] replacing petroleum based crosslinking agents such as diamines to constitute the matrix of biocomposites. As such, the reported toxicity and contact dermatitis [19, 20] resulting from the residual amines used as hardeners of epoxy resins could be eliminated. Other renewable epoxy co-reactant crosslinkers such as dimerized fatty acids (e.g. pripol 1008, 1009) [21, 22] and phenalkamine [23] prepared from cashew nuts are under investigation. However, cost, some performance limitations and demand for waste utilization motivated for further research of examining alternative curing agents.

The goal of this study was to investigate the compatibility and performance of the renewable thermosetting plastic platform developed in our laboratory with both glass and natural fibers. While the primary aim of this work was to provide technically viable utilization route for SRM in order to mitigate pressing societal and economic issue, it also sought to contribute with information that could lead

to a greater understanding of the interaction between hydrophilic natural fibers and hydrophobic polymer matrices. Weak interactions between these two phases often result in poor compatibility [24]. The central proposition of this study was that the incorporation of hydrophilic hydrolyzed proteins could improve the hydrophilicity of the epoxy matrix and thus better interaction with natural fibers.

6.2. Materials and methods

6.2.1. Materials: Two types of E-glass fiber mats and hemp mat were used as reinforcing fiber mats. The glass fibers were randomly oriented chopped strand mat (CSM) of 450 g/m^2 and woven roving (WR) of 200 g/m^2, respectively purchased from Ashland Inc. (Ohio, US). Wet laid randomly oriented hemp (HE) mat of 300 g/m^2, prepared through in-house filtration method, was obtained from Alberta Innovates Technology Futures, Edmonton, AB. Diglycidyl Ether of Bisphenol A (DGEBA) epoxy resin (Araldite 506 epoxy resin, number average molecular weight <= 700 g/mol), 4-aminophenyl sulphate (APS) (97 %, mol wt. 248.3 g/mol) were obtained from Sigma-Aldrich, St. Louise, MO, USA. SRM was exclusively provided by Sanimax Industries, Inc. (Montreal, QC, Canada). SRM samples were hydrolyzed, and proteinaceous fragments extracted and dried in the laboratory according to [17]. SRM transportation and receiving were conducted according to Transport of Dangerous Goods (TDG) regulations. Handling and hydrolysis of SRM were conducted according to CFIA protocol [25].

6.2.2. Methods

6.2.2.1. Hydrolysis of SRM and biorefining

Hydrolysis of the SRM was performed using a 2 L batch reactor (Parr 4530, Parr Instrument, Moline, IL, USA) at a temperature of 180 ºC, pressure of at least 1200 kPa and agitation of 200 rpm for 40 minutes in aqueous solution to inactive prions in SRM according to CFIA approved techniques of disposal [25]. The mass ratio of SRM to water during hydrolysis was kept to one to one, according to our previous study of SRM hydrolysis [17]. The biorefining of hydrolyzed SRM, focused on separation of salt soluble fractions from the insoluble ash and lipids of the SRM, was conducted as follows: 100 g hydrolyzed SRM was extracted with 450 mL salt solution consisting of 18 g NaCl, 0.23 g $MgCl_2$, 4.1 g KH_2PO_4 and 4.3 g Na_2HPO_4 according to the method used by Mekonnen *et al*. [26] by agitating at 200 rpm for 30 min in a shaker (Innova lab shaker, New Brunswick, Canada). The supernatant was separated from the residue through centrifugation (7000 x g for 30 min) on Beckman Centrifuge followed by triple hexane extraction to remove lipids and other organic residues. The salt soluble hydrolyzed proteins were then lyophilized and grinded to particle size below 100 μm. The molecular sizes of the extracted hydrolyzates were also studied using size exclusion high performance liquid chromatography (SEC-HPLC) according to the method reported by Mekonnen *et al*. [17] and compared with an external standard.

6.2.2.2. Composite specimen preparation

Polymer baselines without reinforcement were prepared by curing calculated quantities of epoxy resin with curing agents (i.e. hydrolyzed proteins and APS) as

shown in Table 6.1. Prior to curing, the polymer premix was degassed in a vacuum oven for an hour at 100 °C and cured on silicon molds (22.5 cm x 22.5 cm) at 185 °C for four hours [9] to make polymer baselines. Fixed 20 vol. % pre-cut fiber mats were used to reinforce 80 vol % resin matrix composed of calculated quantities of epoxy resin/curing agent and cured (Table 6.1). A silicon mold with its bottom covered with polytetrafluoroethylene (PTFE) to prevent sticking of the resin on the mold surface was used to prepare the composites.

About 20 % wt. of resin mix was first poured on the PTFE sheet covered mold and spread out with hand rollers. A fiber mat was then applied on the top of the resin and rolled with the hand rollers to allow the resin to soak in the matrix. More resin was poured on top of the mat and distributed evenly with the hand rollers. More layers of fiber mats, depending on the type of fiber, were added by alternating the fiber and the resin layers and squeezing the resin into the fiber with the hand rollers. Finally, another PTFE sheet was placed on the top of the composite sandwich to get good surface finish.

The number of fiber mat layers varied with type of the fiber: three layers of CSM, eight layers of WR, and two layers of HE fiber to keep the volume fraction shown in Table 6.1. The composite sandwiches were degassed and then transferred to PTFE sheet and then to a French press. The sandwich composites were then pressed at a temperature of 185 °C and clamping pressure of 7 ton for 2 h in the French press to squeeze out trapped air bubbles entrapped in the mat layers and also to overcome springiness of fibers. 20 % fiber volume fraction and 3mm thickness of the plates were achieved by using 3mm shims and the applied

pressure to the plates. Each of the APS/resin and hydrolyzed protein/resin composites were post cured for 2 h at 185 °C and 200 °C, respectively. The prepared polymer sheets and composites were then prepared for property evaluation.

Table 6 1. Quantities of epoxy resin, curing agent and fiber mat

Label	Curing agent	Curing agent (wt. %)	Epoxy (wt. %)	Fiber (vol. %)	Resin (vol. %)
APS20	APS	20	80	0	100
P20	hydrolyzed protein	20	80	0	100
P30	hydrolyzed protein	30	70	0	100
APS20CSM	APS	20	80	20	80
P20CSM	hydrolyzed protein	20	80	20	80
P30CSM	hydrolyzed protein	30	70	20	80
APS20WR	APS	20	80	20	80
P20WR	hydrolyzed protein	20	80	20	80
P30WR	hydrolyzed protein	30	70	20	80
APS20HE	APS	20	80	20	80
P20HE	hydrolyzed protein	20	80	20	80
P30HE	hydrolyzed protein	30	70	20	80

APS20, P20, P30 – 20 wt. % APS, 20 wt. % and 30 wt. % hydrolyzed protein, respectively.

6.2.2.3. Thermal Analysis

Thermogravimetric analysis (TGA) was conducted according to ASTM 2550-11 (Standard Test Method for Thermal Stability by Thermogravimetry) with TA Instruments Q600 analyzer. 10 mg of each sample specimens were heated at 5

°C/min from 23 °C to 450 °C under a nitrogen atmosphere to prevent sample oxidation at a flow rate of 100 mL/min. All thermogravimetric studies were replicated 5 times.

6.2.2.4. Mechanical property testing

The polymer sheet used as controls and the composites were cut into tensile dog bones and flexural bars for mechanical property evaluation. The tensile dog bones and flexural bars were prepared by water jet cutters (Omax 2652) at a pressure of 50,000 psi from the prepared polymer and composite sheets. Tensile strength tests were conducted according to ASTM D638-08 (Standard Test Method for Tensile Properties of Plastics) [27] with a crosshead speed of 5 mm/min on an Instron (Instron 4302, Norwood, MA, USA) equipped with a load cell of 10 kN. The flexural strength and modulus was also measured at a crosshead speed of 1.39 mm/min in accordance with ASTM D790-07 (Standard Test Methods for Flexural Properties of Unreinforced and Reinforced Plastics and Electrical Insulating Materials) [28]. Specimens were conditioned at 23 °C and 50 % relative humidity for 48 hours and tested according to the respective ASTM procedures.

6.2.2.5. Water absorption tests

Water absorption performance of the composites was assessed in both short-term (one day and seven days) and long-term (thirty days). The short-term water absorption tests were performed as per ISO 62:2008 (determination of water absorption in plastics). At least five samples of each specimen were cut from the molded composite sheets into 5cm x 2cm dimensions and dried to constant

weight. The dried specimens (W_1) were then submerged in 100 mL distilled water at room temperature for one day (24 h) and seven days. After removal from the water, the extra water on the surface of the specimens were wiped with a clean dry cloth, weighed immediately (W_2). The percentage of water uptake (Water absorption) was calculated by weight difference between the samples immersed in water and dry samples using equation (1):

$$Water\ absorption = ((W_2 - W_1)/W_1) \times 100 \quad (1)$$

The long-term water absorption tests, on the other hand were investigated in accordance with ASTM D570-98 method [29], in such a way that the tensile and flexural properties would also be studied after the long-term moisture conditioning. Tensile and flexural bar samples that were dried to constant weight (*W1*) were immersed in deionized water for 30 days. The extra water on the surface of the soaked samples was then wiped with a clean dry cloth and immediately weighed (*W2*). The water absorption, tensile and flexural properties were then evaluated in accordance with ASTM D570-98 [29] (equation 1), ASTM D638-08 [27] and ASTM D790-07 [28], respectively.

6.2.2.6. Scanning Electron Microscopy (SEM)

Fracture surfaces form tensile tests before and after water soaking (1 month) were imaged using a Hitachi S-3000 N scanning electron microscope (SEM) at accelerating voltage 15 kV with a tungsten filament. Fracture surfaces were gold coated prior to imaging using an Edwards S150 Sputter Coater.

6.2.2.7. Statistical analysis

Statistical analyses of data were conducted using the statistical software package Minitab version 15. Single factor analysis of variance (ANOVA) was used to identify significant differences among mean values, according to the least significant difference (LSD) criteria with a 95 % confidence level ($P < 0.05$).

6.3. Results and discussion

6.3.1. Specified Risk Material hydrolysis and characterization

One method of adding value to SRM that otherwise is an industrial waste is hydrolysis with the aim of breaking down BSE causing prion proteins into short protein chains and peptides. [9, 15] Studies have shown that thermal hydrolysis mentioned in the method section at a temperature, pressure and time of at least 180°C, 450 kPa, 40 min, respectively, inactivates BSE causing prions [13, 25]. The molecular size of representative non-biohazardous unhydrolyzed meat and bone meal (MBM) protein extract and biorefined TH SRM extract studied using gel permeation chromatography (GPC) is presented in Figure 6.1.

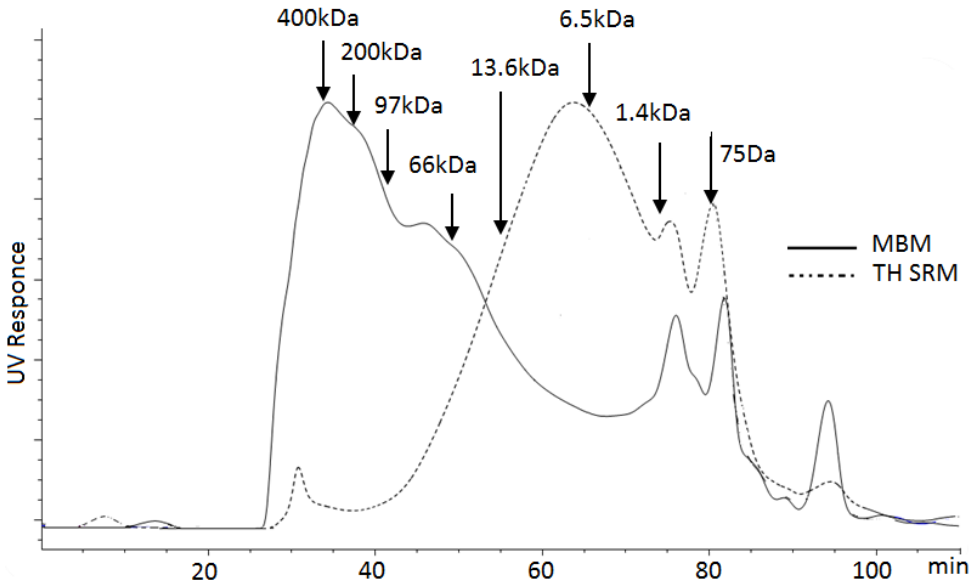


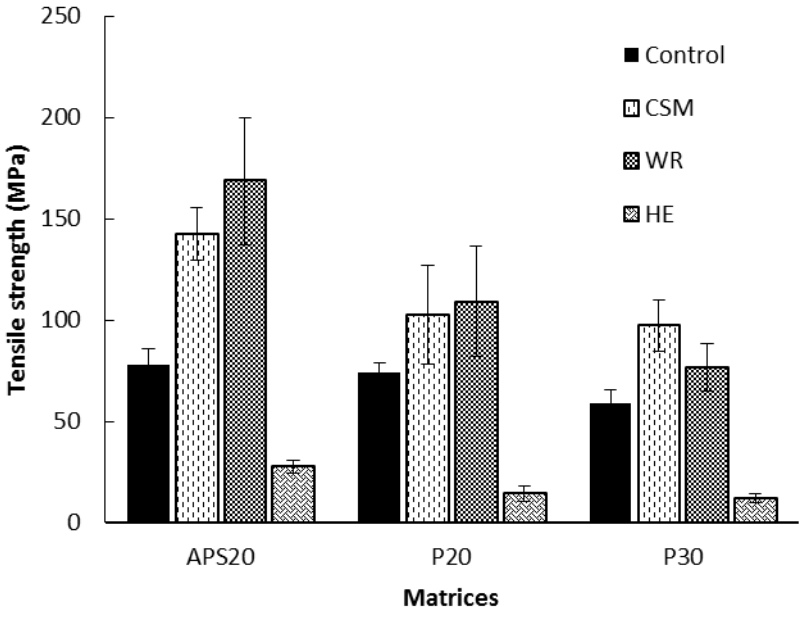
Figure 6.1. GP-HPLC chromatograms of meat and bone meal (MBM) protein extract prior to hydrolysis and hydrolyzed protein extract of thermal hydrolyzed specified risk material (TH SRM).

The molecular size of unhydrolyzed MBM protein extract was broadly distributed over a wide range of molecular sizes. On the other hand, the hydrolyzed proteins obtained from the hydrolyzed SRM had relatively narrower size distribution. The majority of these hydrolyzates lied below 66 kDa, and the average was about 13 kDa. This showed that the thermal hydrolysis severely chopped the SRM protein molecules into short protein and peptide molecules. Moreover, the narrower molecular size distribution imparted by the hydrolysis offers more uniformity and hence resembling behavior and functionality than a broadly distributed unhydrolyzed protein. Previous works reported that hydrolyzed SRM protein fractions were large enough to possess most of the functional groups of proteins [17]. Additionally, the reactivity of the hydrolyzed

protein functional groups with glutaraldehyde [15] and epoxy resin [9] to make thermosetting polymers was also reported.

6.3.2. Mechanical properties

The mechanical strength of composites is an intrinsic property of the constituents, i.e. reinforcement used, the nature and formulation of the matrix [30] and of the nature of the interaction between these two phases [31]. Tensile strength and modulus of composites made of epoxy resin cured with 20% APS and 20 and 30% hydrolyzed protein, reinforced with CSM, WR and HE fibers and the respective control matrices are summarized in Figures 6.2 and 6.3. In the following section, the effect of the matrices and reinforcing fibers used on the mechanical properties of the composites are discussed.



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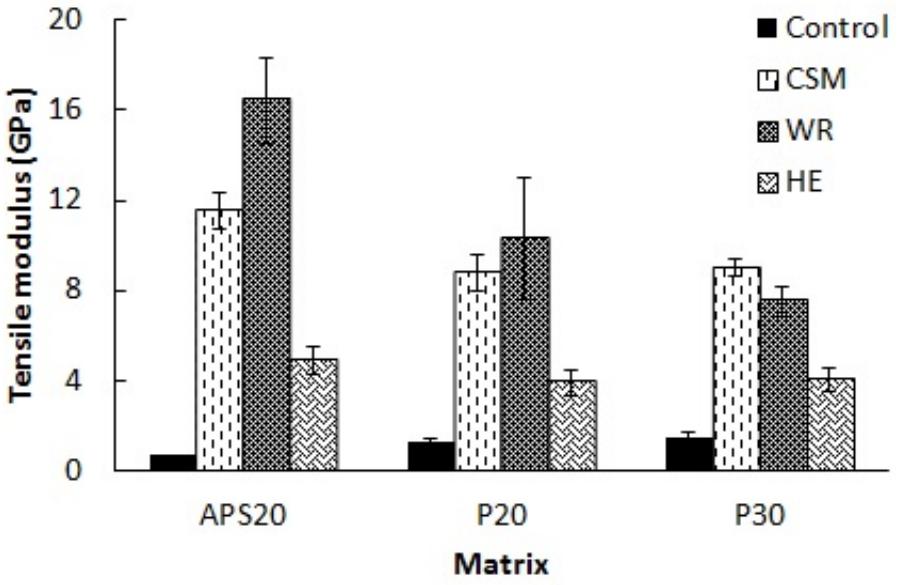
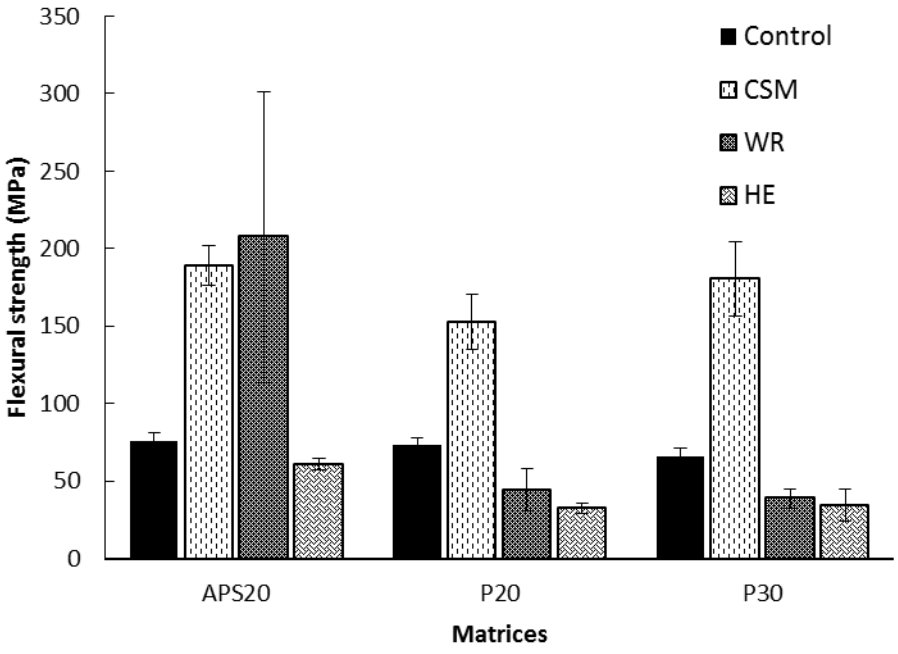


b

Figure 6.2. Tensile strength (a) and tensile modulus (b) of 20APS, 20 and 30P based matrices and their counter composites reinforced with CSM, WR and HE fibers reinforced with CSM, WR and HE fibers



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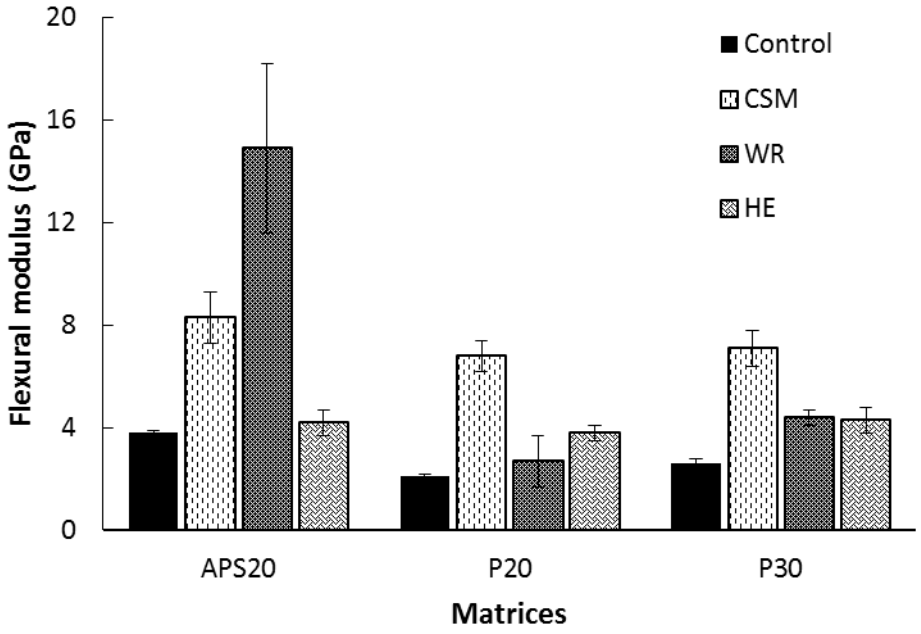


b

Figure 6.3. Flexural strength (a) and flexural modulus (b) 20APS, 20 and 30P based matrices and their counter composites reinforced with CSM, WR and HE fibers reinforced with CSM, WR and HE fibers.

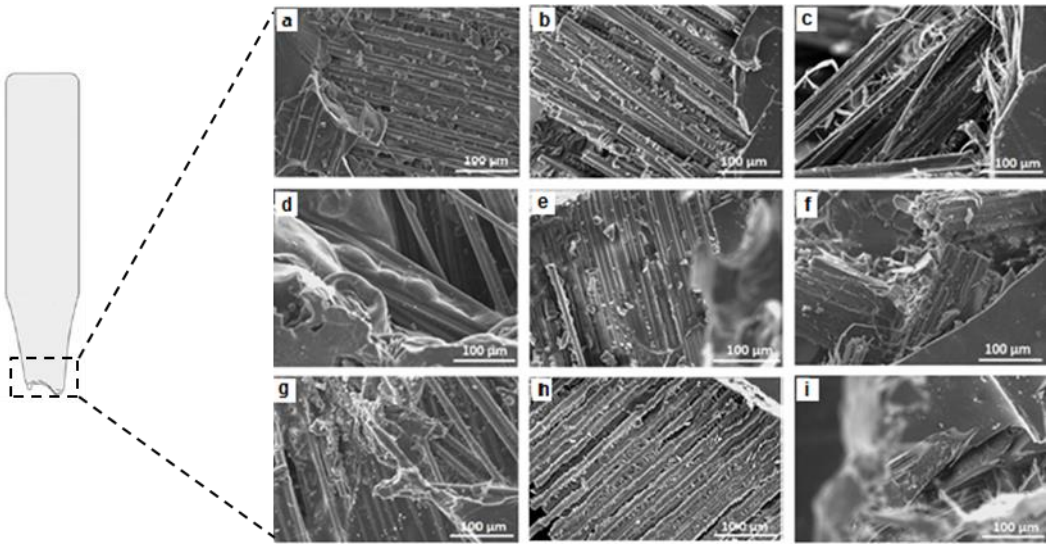


Figure 6.4. SEM images of fractured surfaces after tensile testing for (a) APS20CSM (b) APS20WR (c) APS20HE (d) P20WR (e) P20WR (f) P20HE (g) P30CSM (h) P30WR (i) P30HE.

6.3.2.1. Effect of Matrix Type

The matrix in a fiber-reinforced composite holds the fiber together, transfers mechanical loads applied to those fiber and protects them from mechanical damage and other environmental factors. [4] In this research a thermosetting resin based systems were used as matrices for the composites. The resin systems were (i) synthetic resin composed of epoxy resin cured with APS and (ii) bio-based resin composed of epoxy resin cured with hydrolyzed proteins. The tensile and flexural property of the composites made with each matrix and reinforcing filler combination is presented in Figures 6.2 and 6.3, respectively. The epoxy polymers cured with APS, P20 and P30 generally exhibited higher tensile strength and modulus compared to many polyethylene terephthalate [32] and polypropylene [33] owing to their thermosetting behavior [34]. The matrices used in this research did not exhibit significant difference ($p>0.05$) of tensile strength and modulus with each other. Whenever APS cured epoxy was used as the matrix polymer, the composites exhibited significantly higher tensile strength and modulus with all the fiber types. Since the matrices did not exhibit significant difference of either tensile strength or modulus between each other, the only plausible explanation here would be better interaction of the synthetic APS cured polymer with each type of fiber than the hydrolyzed protein based biopolymeric systems. Weak interaction of fiber with the matrix usually results in slippage and segregation of fiber from the matrix during testing or application that may result in poor mechanical property. The P20 and P30 matrix based composites did not exhibit tensile strength difference; however the P30 composite with WR

reinforcement exhibited significantly lower tensile modulus. This behavior may be attributed to the higher viscosity of P30 compared with P20, which may have resulted in limited spreading of the matrix on the fiber and therefore weaker interactions between the two phases.

The flexural strength of the synthetic and bioresin polymeric matrices (controls) also did not exhibit significant difference ($p>0.05$) among each other. Matrices Reinforced with CSM resulted in significantly improved flexural strength and modulus of all systems. Nevertheless, the flexural strength of the P20 and P30 polymeric matrices, were significantly reduced with WR and HE reinforcement. This apparent contraction can be attributed to the difference in number of layers (eight) used in the WR mat, compared with the HE and CSM mats (two). As discussed above, this difference was motivated by the choice to maintain constant volume fraction of fibers. The use of such multiple layers obviously resulted in more interfaces between the reinforcement and polymeric matric. These interfaces are known weak spots between the matrix and reinforcement, and as a result delamination was visually observed when the tensile and flexure bars were machined (Figure 6.4 (b), (e) and (h)). The delamination occurred as a result of poor bonding and consolidation, resulted in comparatively weak flexural strength of WR reinforced hydrolyzed protein cured polymers (P20WR and P30WR). Improved flexural strength of the composites was observed when synthetic resin was used than the bioresins, which might be due to better interfacial interaction and consolidation. The flexural modulus on the

other hand did not change when the resins are changed, with the exception of the WR reinforced polymers.

6.3.2.2. Effect of reinforcement

The principal goal of reinforcing polymers is to improve the strength and stiffness of the resulting composites [35]. The tensile and flexural strengths (Figures 6.2 and 6.3) of the experimental materials revealed that large disparity exists between the natural fiber containing composites and glass fibers. The tensile strengths of the composites were improved when either of the CSM or WR glass fibers was used as reinforcing filler. However, the tensile strengths of all the composites were significantly reduced from the base polymers during HE reinforcement. Such reduction in tensile strength for composites compared to the unreinforced polymers could be attributed to stress concentration caused by the presence of less strainable fibers in conjunction with a brittle matrix [36]. Sawpan *et al*., [36], Fuqua and Ulven [37] and Karnani *et al*. [38] also observed a similar trend of inferior tensile strength of composite than the pure polymer reinforced with lignocellulosic fibers.

The tensile strength of the composite made of APS based matrix and either of the glass fiber mats (WR and CSM) exhibited the highest tensile strength and modulus. Stiffness is closely related to consolidation of the matrix with the reinforcing fillers [31]. In all cases studied here, the stiffness of the composites was unanimously improved when compared with the control polymers (Figure 6.3b): 1) HE reinforcement improved the modulus of the APS20, P20 and P30 matrix polymers by about 600, 202 and 170 %, respectively; 2) WR improved the

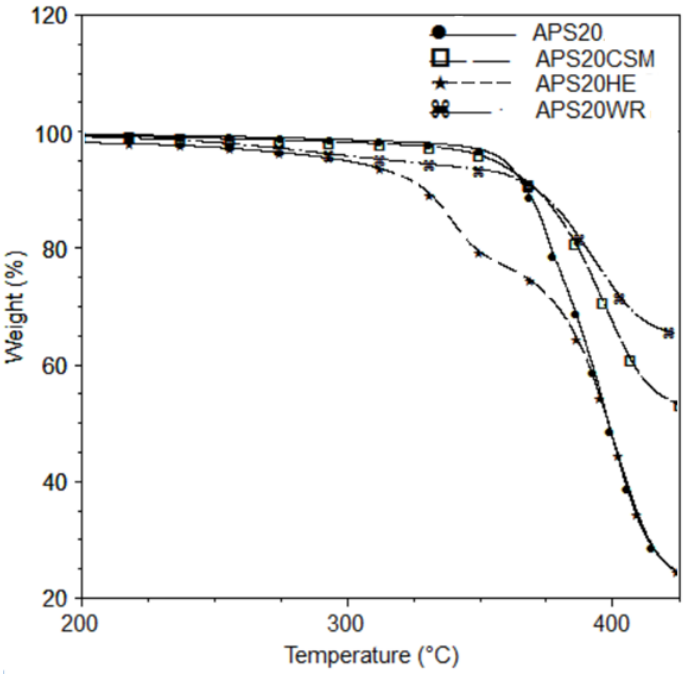
modulus of APS20, P20 and P30 matrices by 2242, 692 and 400 %, respectively; 3) CSM also improved the modulus of APS20, P20 and P30 matrices by 1542, 576, and 500 %, respectively.

With respect to flexural property, all reinforcing efforts improved the flexural moduli; despite the improvement by HE is only marginal. CSM improved the flexural strength of all its respective composites; while WR reinforced APS resin (APS20WR) exhibited the highest flexural strength (207.6 MPa). A decrease was observed when WR was compounded with the bioresins (P20 and P30) as a result of delamination of layer of the composite, probably because of poor interfacial adhesion of the smooth woven surface of WR with the hydrolyzed protein based bioresins (P20 and P30). Furthermore, since eight layers of WR were used in all WR based composites, it obviously formed more interfaces with the matrix than the other two (HE and CSM) based composites that used only two layers. It can be anticipated that the interface is the weakest link in comparison to the body of the polymer matrix or the fiber itself that results in failure under external force. Thus, the use of eight layer mats in WR reinforcement that resulted in formation of more interfaces might be the reason for the relatively poor flexural strength of WR reinforced composites. Preferential adsorption of resin components onto the surface of the fibers, usually result in a gradient of cure [39, 40] and difference in effectiveness of the interface in transferring stress, that may have led to the observed variation. Reinforcement with HE did not result in greater flexural strength improvement of any of the composites. The presence of pectins and waxes in native HE may lead to the formation of ineffective interface between the

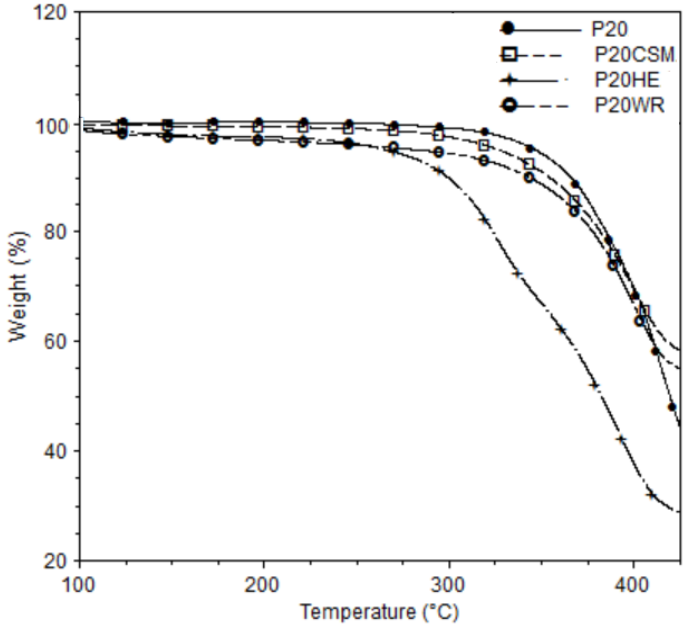
fiber and matrix, with subsequent problems such as debonding and voids in the resulting composites [36]. Therefore, surface treatment of natural fibers using enzymatic, chemical and physical techniques before introducing it into the polymeric matrix material is a common practice to improve the adhesion between the fiber and the matrix and as a result the mechanical properties of the resulting composite [40]. In addition, the use of coupling agents and compatibilizers to reduce hydrophilicity and enhance compatibility with different matrices is another area of research [41].

6.3.2. Thermal stability

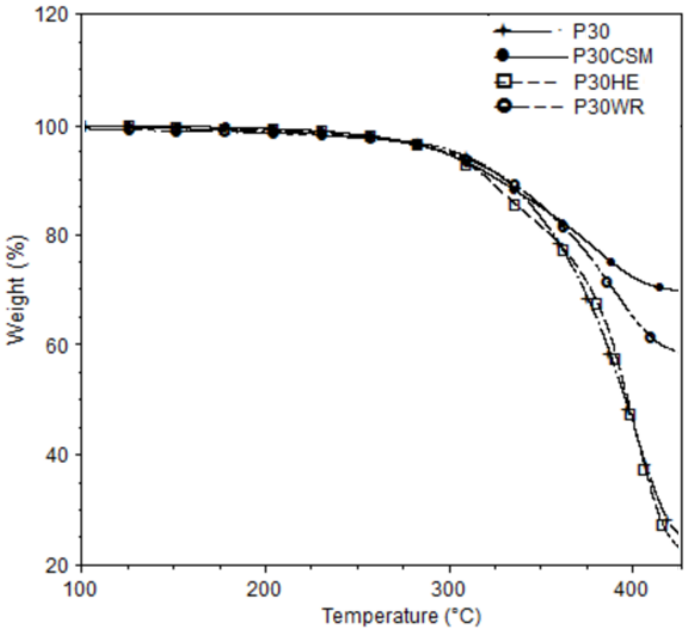
Thermal stability data are crucial to design and develop composites. This is because it is one of the limiting factors in the selection of curing temperature in the case of thermosets, extrusion and injection molding temperature in thermoplastic matrix composites and it determines a possible range of user application. The thermal stability data of the polymeric matrices and their counter composites reinforced with CSM, WR and HE based mats investigated with thermogravimetric analysis (TGA) is presented in Figure 6.4.


APS20
APS20CSM
APS20HE
APS20WR
Weight (%)
Temperature (°C)
120
100
80
60
40
20
200
300
400

(a)


P20
P20CSM
P20HE
P20WR
Weight (%)
Temperature (°C)
120
100
80
60
40
20
100
200
300
400

(b)



(c)

Figure 6.5. TGA thermograms of (a) 20% APS based composites (b) 20% hydrolyzed protein based composites (c) 30% hydrolyzed protein based composites, reinforced with each of the CSM, WR and hemp fiber mats. Each experiment was replicated 5 times.

The composites prepared using hemp, i.e. APS20HE, P20HE and P30HE were characterized by two stages of degradations as shown in Figure 6.5. These two stages were likely attributed to the difference in thermodegradibility of the cellulose and hemicellulos components of the hemp fiber. Previous studies demonstrated that the hemicellulose degrades at lower temperature than cellulose and lignin of lignocellulosic materials in non-oxidative environment [33, 42]. Based on these observations, the first and second degradation peaks of HE based composites may be attributed to hemicellulose and cellulose degradation of the hemp fiber, respectively. This is in line with data obtained by Panthapulakkal and

Sain [33] for hemp reinforced polypropylene. Five percent weight loss (T_5), twenty five percent weight loss (T_{25}) and the residue left after 425 °C degradation is summarized in Table 6.2. The onset of degradation occurred between a wide temperature windows of 296 – 371 °C. Five percent weight loss (T_5) happened between 248 - 295 °C for HE reinforced composites and above 305 °C for WR and CSM reinforced composites, demonstrating the dependence of degradation on the specific reinforcing fibers. The T_5 and T_{25} temperature's apparently depended on the matrix type as well (Table 6.2). The APS20 based composites exhibited higher degradation temperature, while the P30 based composites exhibited lower temperature because hydrolyzed protein and some of their constituent amino acids start to degrade above 230 °C [43]. The residue left was found to be dependent mainly on the type of reinforcing fiber. The glass fiber composites (CSM and WR) showed more left over residue than the hemp based composites – owing to higher thermal resistivity of glass fibers than hemp.

Table 6 2. Five and twenty five percent weight loss temperatures and residue left after 425 °C degradation.

Label	T_5 (°C)	T_{25} (°C)	Residue left after 425 °C (%)
APS20	356.3 ± 1.1[a]	379.5 ± 0.3[a]	24.9 ± 0.3[a]
P20	286.3 ± 0.8[b]	355.0 ± 0.9[b]	27.8 ± 0.4[a]
P30	308.2 ± 2.3[c]	369.2 ± 1.3[a]	25.5 ± 0.4[a]
APS20CSM	358.8 ± 1.0[a]	393.1 ± 1.5[c]	55.7 ± 1.7[b]
P20CSM	329.4 ± 7.6[d]	391.5 ± 2.0[c]	57.9 ± 0.6[b]
P30CSM	305.0 ± 5.1[b]	380.7 ± 3.4[ac]	58.6 ± 0.2[b]
APS20WR	351.9 ± 6.4[a]	398.6 ± 3.5[c]	66.3 ± 2.6[c]
P20WR	328.9 ± 16.7[d]	399.6 ± 8.0[c]	62.8 ± 3.5[c]
P30WR	303.4 ± 17.9[b]	378.1 ± 5.7[a]	54.4 ± 3.5[c]
APS20HE	295.0 ± 17.0[b]	356.3 ± 10.2[b]	24.4 ± 0.5[a]
P20HE	247.6 ± 17.7[e]	331.1 ± 1.9[d]	25.6 ± 3.4[a]
P30HE	284.2 ± 10.3[b]	347.3 ± 18.1[b]	27.3 ± 3.6[a]

Data in this table are means ± standard deviation with a sample size of at least 5 for each group. [a-e] Means with the same superscript letters within a column are not significantly different at $P < 0.05$ level.

Thermal stabilities of all the biocomposites achieved here were acceptable for end user application purpose. Thermal stability was shown here to be not a concern at all as far as curing temperature is concerned, as the curing took place at a maximum temperature of 200 °C. Catalysts and/or accelerators can also be included to cut down the curing temperature and the energy consumption as well.

6.3.3. Water Resistance

6.3.3.1. Water absorption

Water absorption is an important parameter to study the degradation of polymers and polymer composites. Most polymers and polymer composites absorb moisture in humid atmosphere and when immersed in water [9, 44], resulting in the deterioration of fiber-matrix interface region, diminished stress transfer efficiencies and therefore poorer mechanical and dimensional properties [44, 45]. Triplicate determination of water absorption of composites after one, seven and thirty days of water immersion, calculated by weight gain is displayed in Figure 6.5.

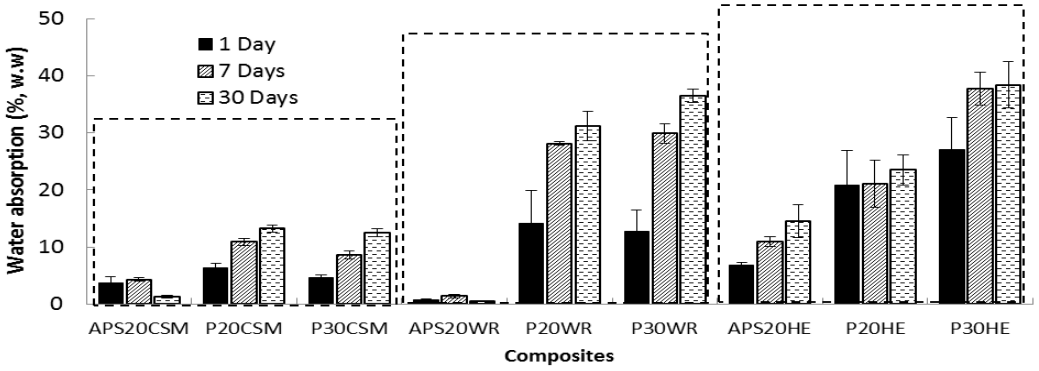


Figure 6.6. Water absorption of the APS, 20% and 30% hydrolyzed protein composites reinforced with CSM, WR and HE fibers at day 1, 7 and 30. Error bars are standard deviation of quintuplicate determination.

It is observed that both the matrix type and fiber type had an effect on the water absorption of the composites. The synthetic matrix, composed of epoxy resin cured with APS, was more resistant to moisture than the hydrolyzed protein (P20 and P30) cured matrix. This behavior signifies that presence of a single

functional group ($-NH_2$) in APS that crosslinks with the epoxy resin effectively may not leave behind any residual groups to associate with water. On the other hand, hydrolyzed proteins have several functional groups such as primary and secondary amine ($-NH_2$, -NH), carboxyls (-COOH), sulfhydryls (-SH) and hydroxyl (-OH) associated with side chain of the amino acids and the end groups of the hydrolyzed protein chain that would react with epoxy to cure [9]. Even though such abundant functional groups of hydrolyzates provide an excellent opportunity to crosslink with epoxy resin and bond with the fibers, it is plausible to expect that some hydrophilic unreacted left over functional groups may associate with water leading to higher water absorption. Presence of incomplete network chains is another possibility that can lead to leaching out of matter into the water. Such migration of matter leaves behind voids or pores that would drive more water diffusion into the composites resulting in higher moisture absorption of hydrolyzed protein cured matrices.

It was also observed (Figure 6.6) that HE and WR reinforced composites absorbed more water than their counterpart CSM based composites in most cases. WR reinforced composites also exhibited high moisture absorption when compounded with both of P20 and P30. This is consistent with the delamination behavior as a result of the poor interfacial interaction of hydrolyzed protein with WR observed and discussed in the mechanical property section. Glass fibers including WR and CSM absorb negligible amounts of water [44] while cellulosic fibers such as hemp is well-known to absorb water and swell owing to its hydrophilic nature. [46] It has been demonstrated that lower moisture absorption

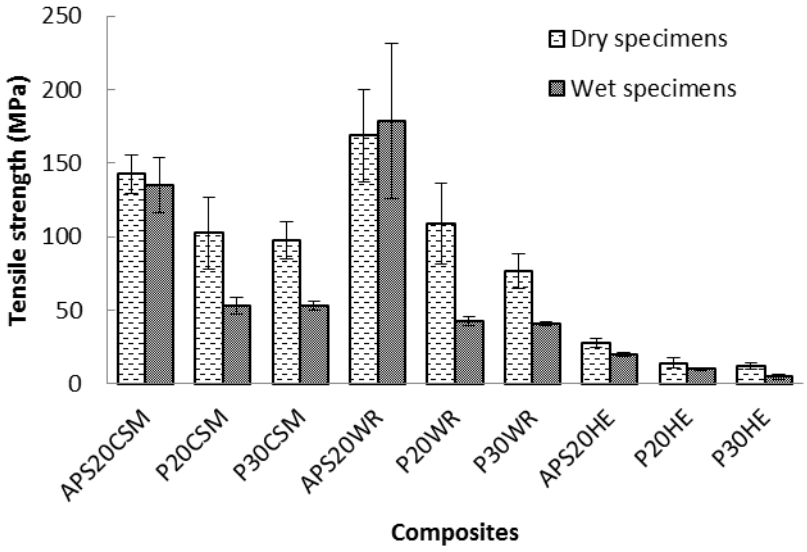
can be attributed to better adhesion between matrix and fibers, leading to fewer and smaller gaps in the interfacial region and hence less diffusion of water into the biocomposites [46]. For instance, the water absorption difference observed between WR and CSM reinforced composites might be completely attributed to the adhesion bonding variation.

The highest water absorption was observed when HE is compounded with hydrolyzed protein based polymeric matrices (P20HE and P30HE) because of the absorption contributed by both the matrix and fiber. For hemp reinforced composites, a maximum water gain of 38% for P30HE and a minimum of 14.5% for APS20HE were observed. It was also shown in Figure 6.6 that more than half of the total water absorbed in the thirtieth day was absorbed in the first day; its absorption rate was then decelerated until the seventh day and further deceleration of the rate until the final testing day, thirtieth day. Espert *et al*. [46] and Dhakal *et al* [45] reported similar trend of rapid water absorption of biocomposites in the first 20 h, then reduced rate until it reaches a saturation point where no more water was absorbed and the content of water in the composites remained the same.

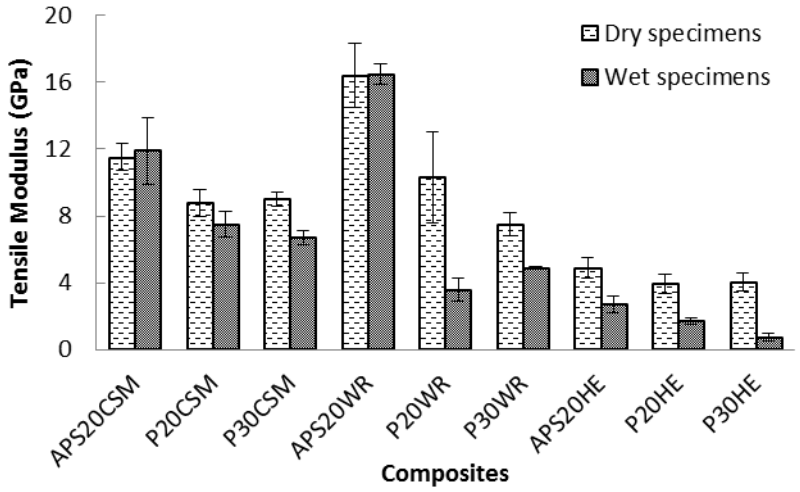
6.3.3.2. Influence of water absorption on mechanical properties of the biocomposites

Moisture absorption is one of the main concerns for use of natural fiber reinforced composite materials and the effect on its performance [47]. Moisture absorption, attributed to diffusivity of water into the material, leads to moisture

induced interfacial cracks as a result of degradation in the fiber-matrix interface region [45]. The tensile and flexural properties of the APS and hydrolyzed protein based composites reinforced with each of the CSM, WR and HE fibers were tested after complete soaking of the respective tensile dog bones and flexural bars for 30 days. The influence of water soaking on mechanical properties of the specimens and comparison with the original dry specimen is shown in Figure 6.7 and 6.8.

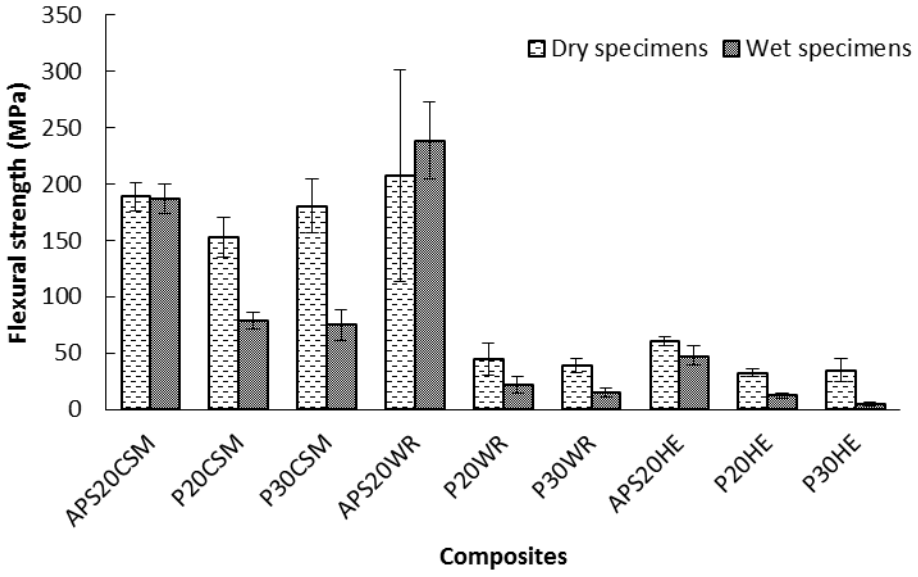


a

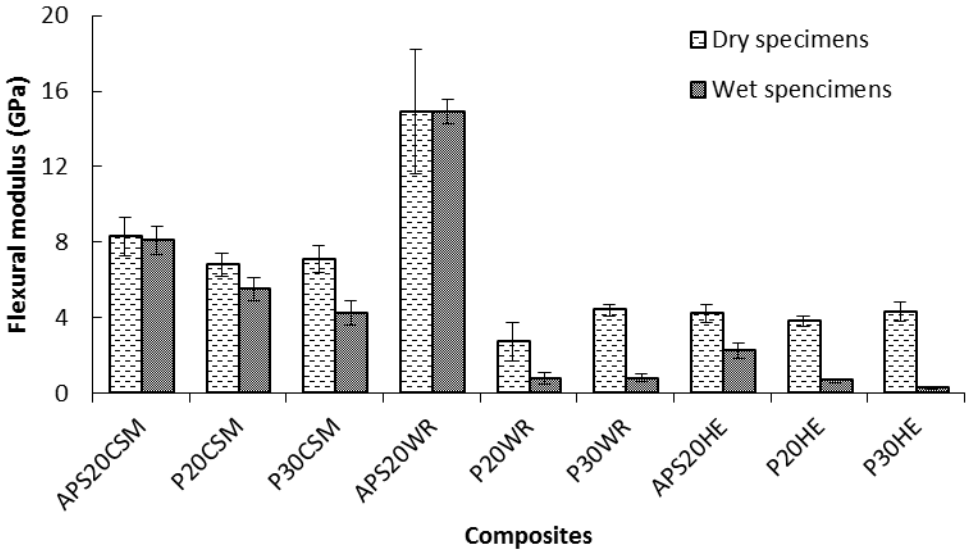


b

Figure 6.7. Comparison of (a) tensile strength and (b) modulus of dry composite specimens and wet composite specimens. Error bars are standard deviations from at least quintuplicate determinations.



a



b

Figure 6.8. Comparison of (a) flexural strength and (b) flexural modulus of dry and wet composites. Error bars are standard deviations from at least quintuplicate determinations.

All composite specimens showed significant reduction of tensile strength when tested after 30 day soaking with the exception of APS20CSM and APS20WR. The most pronounced tensile strength deteriorations were observed for the hydrolyzed protein based matrices. P30HE, P20WR and P30WR exhibited 61, 60 and 46 % tensile strength reduction, respectively. This is not surprizing, because it was these same composites that exhibited the highest water absorption (Figure 6.6) signifying that higher water absorption resulted in deterioration of tensile strength. With respect to tensile modulus, significant reduction was observed for all composites made of P20 and P30 matrices regardless of the fiber type, and HE reinforced composites regardless of matrix type used. The influence of water absorption resulted in a similar pattern of flexural strength and modulus

reduction as that of the tensile strength and modulus (Figure 6.8) with reductions in flexural strength and modulus observed when either the matrix was hydrolyzed protein based or fibers used were HE, attributed to high water affinity of both ingredients. The highest reduction of flexural strength and modulus was observed when hydrolyzed protein cured epoxy matrices were reinforced with HE fiber.

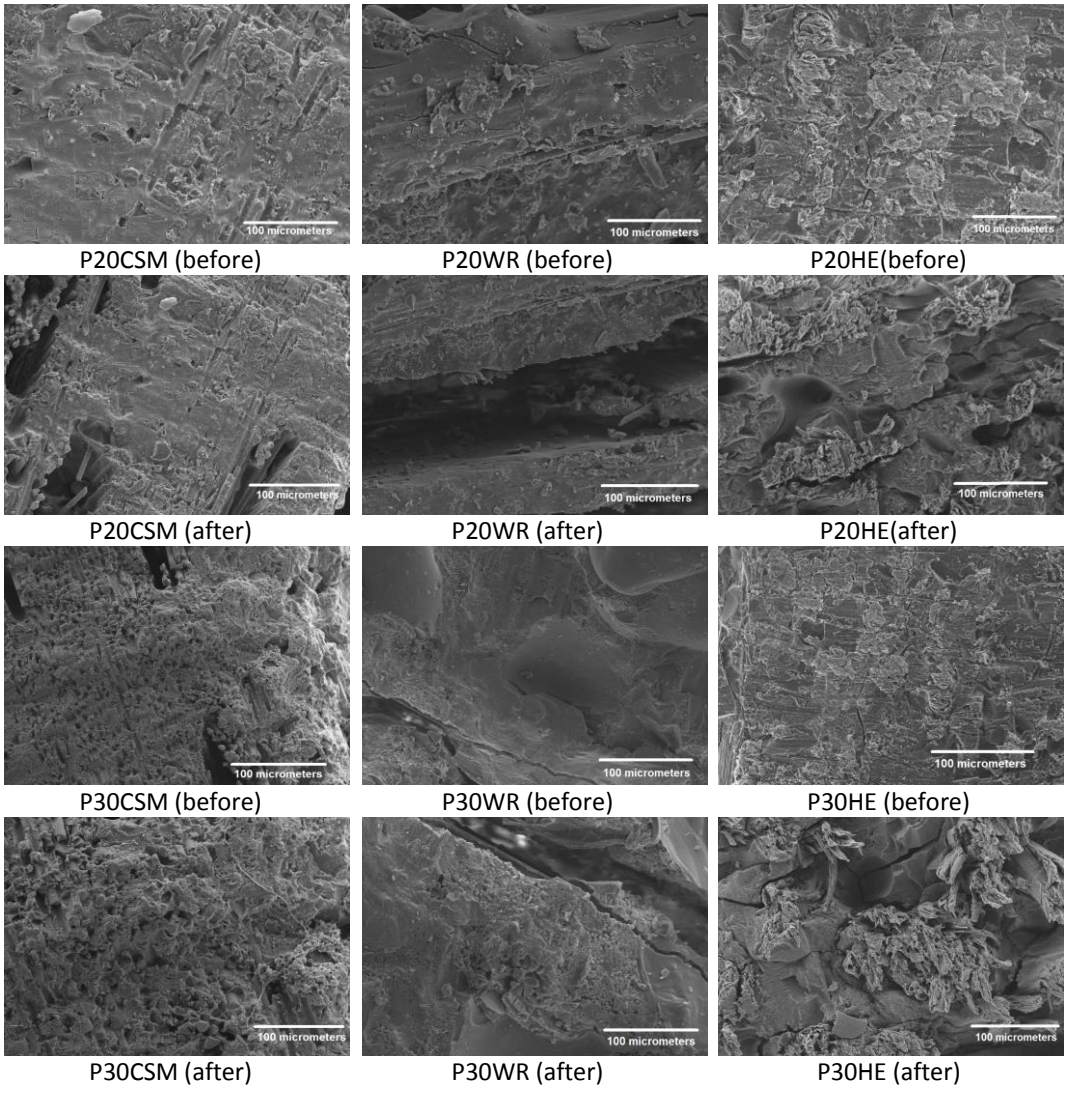


Figure 6.9. Scanning electron microscopy of fractured surfaces of protein based composites before and after 1 month soaking in water.

Water absorption in composites may alter the structure, mechanical and thermal properties of the matrices [48], fibers [49], and the interface between the two. High water absorption of the hydrolyzed protein based matrices (P20 and P30) may shrink and deteriorate the chain orientation resulting in the observed relatively poor mechanical performance. Furthermore, higher moisture absorption of either the matrices or fibers causes swelling, resulting in developing gradient shear stress at the interface and initiating micro-cracks and debonding of fiber from the matrix and ultimately deteriorating the integrity of the composite. More micro-cracks and debonding of water soaked specimens (after) compared to the original specimens (before) were observed on SEM micrographs (Figure 6.9). As such, the composite developed may not serve for purposes that require long term moisture exposure. Nevertheless, the good mechanical performance in dry environment could allow it for other applications including indoor construction, furniture adhesives, degradable construction practice, mulching trays etc.

In addition, some components of the hydrolyzed protein based matrices (P20 and P30) were observed to be released into the immersion tank. Analysis of the leachate (result not shown here) exhibited that salts used during hydrolyzed protein extraction and minor quantities of incompletely networked hydrolyzed proteins were released during the prolonged soaking. Similar leaching of natural fibers components were expected as natural fibers themselves commonly contain high levels of polar extractives [50]. It is plausible that the release of such components can result in formation of voids and porosity as shown in Figure 6.9,

which may act as epicenters of stress concentrators leading to tensile and flexure failure of the composite specimens.

Table 6.3. Tensile and flexural strain at break of dry and wet composite specimens.

	Tensile strain at break (%)		Flexural strain at break (%)	
	Dry	Wet	Dry	Wet
APS20CSM	1.6 ± 0.2^{a}	1.5 ± 0.2^{a}	2.6 ± 0.3^{ab}	2.6 ± 0.2^{ab}
P20CSM	1.6 ± 0.3^{a}	0.8 ± 0.1^{b}	2.5 ± 0.3^{ab}	1.5 ± 0.1^{a}
P30CSM	1.5 ± 0.2^{a}	0.9 ± 0.1^{b}	2.7 ± 0.3^{ab}	1.8 ± 0.1^{a}
APS20WR	1.4 ± 0.1^{a}	1.4 ± 0.4^{a}	1.4 ± 0.4^{a}	1.7 ± 0.1^{a}
P20WR	1.4 ± 0.2^{a}	1.2 ± 0.2^{a}	1.9 ± 0.3^{a}	3.2 ± 0.9^{d}
P30WR	1.6 ± 0.1^{a}	1.0 ± 0.1^{b}	1.0 ± 0.2^{b}	3.6 ± 1.2^{d}
APS20HE	0.8 ± 0.1^{c}	1.1 ± 0.2^{b}	2.1 ± 0.3^{ab}	4.0 ± 0.5^{d}
P20HE	0.6 ± 0.2^{c}	0.9 ± 0.1^{b}	1.0 ± 0.1^{a}	3.3 ± 0.4^{d}
P30HE	0.4 ± 0.1^{c}	1.1 ± 0.5^{a}	1.0 ± 0.3^{a}	4.1 ± 0.7^{d}

Data in this table are means ± standard deviation with a sample size of at least 5 for each group.

Means with the same superscript letters within adjacent column are not significantly different at $P < 0.05$ level.

It has been reported that water molecules act as a plasticizing agent in composite materials exposed to moisture [46, 49, 51], which would lead to an

increase of the maximum strain after water absorption. The tensile strain and flexural strain at break of dry and wet composite specimens are presented in Table 6.3. An increase in both tensile and flexural strain rate was observed for all HE reinforced composites. The mechanism of water plasticizing fibers is that water that penetrated the cellulose network of natural fibers will attach itself by chemical links such as hydrogen bonding to hydroxyl (-OH) groups in the cellulose molecules. The attached water molecules then swell the fiber and force the cellulose molecules apart destroying the rigidity of the cellulose structure. This allows the cellulose molecules to move more freely and hence water here acted as a plasticizer [45, 46]. Moisture absorption has also resulted in flexural strain enhancement of the P20WR and P30WR composites. Since WR fiber barely absorbs any moisture the tensile and flexural strain improvement in these specimens can be due to the plasticization of the hydrolyzed protein cured matrices attributed to left over hydrophilic sites.

6.4. Conclusion

Biocomposites were successfully developed with hemp and two types of glass fiber mats to reinforce hydrolyzed protein-cured and APS cured epoxy resin polymers using French Press compression. It was observed that all the reinforcing fibers improved the tensile and flexural stiffness of the biocomposites made. The thermal stability study also displayed that all composites prepared were stable until a temperature close to 230 °C. The use of waste protein hydrolyzate extracts, hydrolyzed proteins, as crosslinking agent of epoxy resins in making biocomposites was novel and promising and results could be insightful that can be

extended for uses other proteinaceous biomasses as curing agent of epoxy resins. The study also showed that the mechanical performance of the biocomposites was negatively affected by the water absorption. Generally, the conjuncture of hydrolyzed protein cured epoxy with hemp fiber exhibited comparatively lower water resistance. The influence of water absorption was in such a pattern that water-saturated samples presented poor mechanical properties. Water absorbed HE reinforced composites specifically exhibited an improvement in tensile strain due to plasticization effect of the water molecules on the cellulose component. These results highlight the importance of interfacing the matrix and reinforcing fiber phases may be needed to improve the mechanical performance and degradation behavior under moisture sorption.

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Chapter 7

Development of renewable waste protein based adhesives for oriented strand board applications[6]

7.1. Introduction

Oriented strand board (OSB) is an engineered structural panel manufactured from thin wood strands bonded together with adhesives under heat and pressure [1]. The remarkable development in manufacturing practices of these systems has resulted in mechanical properties of commercial OSB that make them suitable for a wide range of applications. The construction industry (e.g., wall sheathing, roof panels, subfloors, single layer floors, structural insulated panels, floor joints or rim boards), packaging, and the furniture sector [2, 3] are among the major applications areas of OSB.

A key element in all modern industrial production technologies of OSB and other wood composites is the use of petrochemical adhesives such as urea-formaldehyde (UF), methyl urea formaldehyde (MUF), phenol-formaldehyde (PF), and isocyanates (MDI). The main advantage of isocynates-based adhesives, one of the major commercial adhesive on the market at present, is their intrinsic rapid polymerization in wood and the formation of bonds with the hydroxyl group of the cellulose and hemicellulose in wood [4, 5]. However, isocyanates may also react with water in the wood competing with desired reactions with the wood [4].

[6] A version of this chapter has been accepted for peer reviewed publication as: Tizazu Mekonnen, Paolo Mussone, Phillip Choi, David Bressler. Macromolecular Materials and Engineering.

Isocyanates can react rapidly with many compounds present in human bodies, posing safety issues related to handling during manufacturing [4, 6]. Additional major concerns have been raised in regard to the deleterious effects related to possible emission of some of the main components of these adhesives [7]. For example most isocynates are known to act as asthma inducers or sensitizers [6] while formaldehyde, a key component in the manufacture of UF, PF or MUF, has been classified as a human carcinogen by the International Agency for Research on Cancer [8]. For these reasons, substantial efforts have been placed into complementing and substituting such ingredients with safe and renewable feedstock derived from biomass feedstock.

Isocynate functional groups contain monomeric diphenylmethane diisocyanate (MDI) and emulsion-polymer isocynate (EPI), which are highly reactive towards a variety of nucleophile functional groups including hydroxyl, carboxyls and primary and secondary amines across the C=N double bond of the NCO group [5]. Such high reactivity of isocyanates, usually associated with the low electron density of the central carbon, is the basis for the common use of polymeric isocynates (MDI and EPI) as wood adhesives. These chemical properties are particularly suited for protein crosslinking because the latter has all these functional groups. Proteins have been investigated as possible replacements of petroleum based synthetic adhesives of engineered wood [9][10]. However, most of the investigated proteins are useful in the food industry, in addition to their known limitations in terms of water resistance and adhesion performance compared to the commercial synthetic adhesives. Hence, it is important to

evaluate waste protein resources that does not compete with food production and investigate property improvement strategies.

The emergence of bovine spongiform encephalopathy (BSE) in the late nineteen nineties and early two thousands has resulted in the implementation of an enhanced ban of specific cattle tissues from entering the human food and animal feed supply in many countries including European Union, Canada and the USA. These tissues, also known as specified risk material (SRM), are categorized as hazardous waste because they are the animal portion at the highest risk tissues of harboring the prions that are suspected to cause the BSE. Other non-SRM tissue derived rendering products such as blood meal and meat and bone meal have also been restricted from their traditional animal meal use. Thermal hydrolysis at a temperature of at least 180 °C and pressure of 1200 kPa for 40 minutes has been shown to destroy the notoriously resistant prion proteins [11]. The hydrolyzates recovered are safe for industrial utilization. While several studies have been conducted on the inactivation potential of such hydrolysis method, comparatively little attention has been placed on the biorefining and developing value added application from this protein rich biomass. Our laboratory has recently reported the utilization of thermal hydrolysis for the destruction of BSE causing prions in SRM according to Canadian Food Inspection Agency (CFIA) [12] and Food and Drug Administration (FDA) of the US [13] and valorization platform [14, 15]. We also reported the successful development of bioplastics [16-18] and biocomposites [19] using the valorized material as feedstock.

The main goal of this work was to investigate how isocynate functional groups of MDI react with hydrolyzed proteins recovered from waste proteinacious biomass for an industrially viable adhesive application. The primary goals are valorization of an otherwise hazardous waste protein into a useful OSB adhesive that could contribute to tackling some of the challenges of petroleum based adhesives.

7.2. Materials and methods

7.2.1. Materials

Rendered SRM composed of 44.05 % protein, 8 % fat and 27.2 % ash on dry weight basis was obtained from Sanimax Industries, Inc. (Montreal, QC, Canada). 4, 4-diphenylmethane diisocyanate (MDI) (viscosity 200 mPa.S at 25 °C; MW = 360 g/mol) and petroleum wax (58 % solid) were purchased from BASF chemical company (Mississauga, ON, Canada). Aspen poplar wood strands obtained from Alberta Innovates Technology Futures (AITF) (Edmonton, Alberta, Canada) were used to make the OSB. Hot melt ethylene vinyl acetate adhesive for internal bond strength testing of the OSB was purchased from Henkel Corporation (Bridgewater, NJ, USA).

7.2.2. Hydrolysis of SRM and recovery of hydrolyzates

SRM was hydrolyzed in aqueous solution at a temperature of 180 °C for a period of 40 minutes per cycle in a 5.5 L batch reactor to inactivate infectious misfolded prion proteins, according to CFIA and FDA approved SRM disposal techniques [11, 12]. The hydrolysis reactor (Parr 4582, Parr Instrument, Moline, IL, USA), equipped with an impeller, was purged with nitrogen gas to achieve a pressure of

at least 1200 kPa and continuously agitated at 200 rpm. The reaction time, temperature, and pressure were recorded during the course of the hydrolysis starting from the moment the required temperature was achieved. Prior to hydrolysis, the SRM sample was handled according to bio-hazardous protocols in a biosafety cabinet located in a government approved biosafety level II containment lab at the University of Alberta, Edmonton, Canada. Decontamination of reactor vessel, biosafety cabinet and all other equipment that came in contact with the unhydrolyzed SRM was carried out with 5% Environ LpH for 30 min followed by 70 % ethanol [20].

The recovery of hydrolyzate proteins material was conducted as per a previously optimized protocol [15]: 1 kg of hydrolyzate from each hydrolysis was solubilized in 2 L of Milli-Q water and agitated at 200 rpm in a shaker (Innova lab shaker, New Brunswick, Canada) for 30 min followed by centrifugation (7000 × *g* for 30 min). The residue consisting of insoluble tissues, bone fragments, and other solid particles were discarded and the supernatant was collected. The lipids and other organic components of the supernatant were extracted out with hexane. The water soluble raffinate fraction, rich in hydrolyzed protein fragments was then vacuum filtered (Whatman filter paper no 4) and lyophilized under reduced pressure, and grinded to powder of particle size 100 μm.

7.2.3. Formulation of binder and OSB fabrication

Adhesives based on hydrolyzed proteins were formulated by first solubilizing the hydrolyzates in distilled water at various concentrations and then mixing with an MDI crosslinking agent to constitute 40, 50, 60, 70 and 85 % by weight of TH

SRM with the aforementioned crosslinking agent on dry weight basis (shown in Table 7.1). The wood strands were pre-conditioned to a moisture content of 2 % and blended with water repellant wax and the formulated binders at a concentration of 1% and 3% (wt. /wt.), respectively. A drum (20.32 cm diameter × 10.16 cm deep, Coil manufacturing, Surrey, BC, Canada) equipped with a spinning disk atomizer with was used to blend the wax and formulated adhesives with the wood strands at drum speed of 24 rpm and spray nozzle speed 12,000 rpm. The blended materials were then immediately collected and formed into homogenous mats and pressed at an applied pressure of 5000 kPa and surface temperature of 204 °C for 4 min, in accordance with a preliminary study of curing. The press used was 450 Ton Lab Press, 86.4 cm × 86.4 cm platen area (Dieffenbacher North America Inc., Tecumseh, ON, Canada). The pressing cycle was monitored and controlled by AITF's PressMAN © Press Monitoring Software System. Triplicate OSB panels of dimension 611 mm × 611 mm, average density 624 kg/m^3, and thickness 11.1 mm was obtained for each binder formulation.

Table 7.1. Composition of formulated adhesive and the ingredients.

Adhesive type by peptide conc. (DWB)	MDI (Wt. %)	TH SRM (Wt. %)	Water (Wt. %)
Control	100	0	0
40% TH SRM	30	20	50
50% TH SRM	25	25	50
60% TH SRM	20	30	50
70% TH SRM	15	35	50
85% TH SRM	7.5	42.5	50

7.2.4. Fourier Transform Infrared (FTIR) spectroscopy study of reaction

The reaction between hydrolyzed protein and MDI was studied using FTIR spectra scans of protein hydrolyzate, MDI and cured adhesives carried out on a Shimadzu FTIR-8400S spectrometer fitted with a germanium attenuated total reflection (ATR) with a high sensitivity pyroelectric detector. Samples of fine milled protein hydrolyzate and cured adhesive powders were mixed with KBr salt. The liquid MDI was placed on KBr salt disc for analysis. A total of 100 scans were performed at 4 cm^{-1} resolution and the measurements were recorded between 4500 and 400 cm^{-1} under the same condition as the background. IR solution software (version 1.10) was used for instrument control and data analysis.

7.2.5. Performance of OSB panel

The performance of the OSB panel including static bending strength, internal bond strength, and bond durability test were conducted according to ASTM (D1037-12) [21] and Canadian Standard Association protocol for fiberboards (CSA O437.0-93) [1]. All test specimens were first conditioned to a constant mass and moisture content prior to test, by exposing the specimens to a relative humidity of 65 % and a temperature of 20 °C.

7.2.5.1. Static bending test: Static bending tests were conducted to measure the flexural properties including the modulus of rupture and modulus of elasticity. For such static bending test six specimens per test panel of size 75 mm (in width) × 50 mm (in length) were prepared. A three point bending test was then conducted with an Instron (Instron 4204, Norwood, MA, USA) with 25 kN load cell, at a head speed of 5 mm/min. A load/deflection curve was generated as the output of the continuous testing. For the calculation of the modulus of elasticity (MOE) and modulus of rupture (MOR), the maximum load at the linear range of the curve and the complete failure curve, respectively, were used as shown in equations (1) and (2).

$$MOR = {}^{3P_{max}L}/_{2Wt^2} \quad (1)$$

$$MOE = ({}^{L^3}/_{4Wt^3}) \times ({}^{\Delta P}/_{\Delta Y}) \quad (2)$$

Where:

P_{max} = modulus of failure load (N)

L = span between centers of supports (mm)

W = width of test specimens (mm)

t = average thickness of test specimens (mm)

ΔP = increment in load (N) on the straight line portion of the load/deflection curve

ΔY = increment in deflection at mid-span (mm) corresponding to the P increment in load

7.2.5.2. Bond durability (Two hour boil test)

Modulus of rupture specimens prepared as aforementioned above (Section 7.2.5.1) were submerged in boiling water for 2 h and then immediately submerged in cold water at 23 °C for 1 h and tested for modulus of rupture as per the method described above.

7.2.5.3. Internal bond strength (IB)

Internal bond strength is a tensile measurement conducted perpendicular to the surface to determine cohesion of panel along the thickness of the panel. Six test specimens from each formulation (Two test specimens from each triplicate OSB board formulations) of dimension 50 mm × 50 mm, were tested for internal bond strength using an Instron (Instron 4204, Norwood, MA, USA) with 10 kN load cell. Each face of the prepared test specimens was glued to aluminum alloy block (50 mm × 50 mm) fixtures of the Instron. The loading of the test specimens was then carried out by separating the loading fixtures until failure at a crosshead

speed of 0.88 mm/min. The average IB of the specimens was then calculated in accordance with the following formula:

$$IB = {Failing\ load\ (N)}/{(length\ (mm)\ \times\ width(mm))} \quad (3)$$

7.2.5.4. Thickness swell test and water resistance of adhesive

Thickness swell test

The thickness of six dried test specimens of dimensions 150mm × 150mm was measured to an accuracy of 0.05 mm with digital varnier caliper at four points midway along each side 25mm in from the edge of the specimens. The specimens were then horizontally submerged under 25 mm of distilled water for 24 h maintained at 23 °C. After the 24 h submerging, the specimens were suspended to drain for ten minutes, and excess surface water was also removed carefully using a paper towel and the swollen thickness was measured immediately.

The thickness swell was then calculated as:

$$Thickness\ swell = \frac{(\sum_1^4 t_{24} - \sum_1^4 t_0)}{\sum_1^4 t_0} \times 100 \quad (4)$$

Moisture resistance of cured adhesive

The weight loss of the cured adhesive in water was studied according to ASTM Standard D 570 [22]. This is because weight loss/solubility in water is among the major drawbacks of protein- based polymers. About 1 g of weighed (W_1) specimen of cured adhesive sheet was submerged in 20 mL distilled water (pH 7) in a controlled environment of 23 °C for 24 h. After removal from water, the extra

moisture on the surface of the sheets was carefully removed with paper towel, dried in a convection oven at 70 °C for 24 h and weighed again (W_2); the weight loss of the sheets (*W loss*) was then calculated as:

$$W_{loss} = ((W_1 - W_2)/W_1) \times 100 \quad (5)$$

7.2.5.5. Density profile along thickness

Test specimens from each triplicate OSB panels were prepared by cutting into 50 × 50 mm in triplicate. The prepared specimens were then positioned in a cassette holder and loaded into the profiler. The density profile was then measured using QDP X-ray profiler (QDP-01X, Quintek Measurement Systems Inc. Tennessee, USA) where an automated X-ray was transmitted through the specimen along the thickness with a profile step resolution (slit width) of 0.05 mm.

7.2.6. Statistical analysis

Statistical evaluation of data was performed using the statistical software package Minitab version 15. Data are reported as mean value ± standard deviation of three replicates. Single factor analysis of variance (ANOVA) was conducted to differentiate significant differences among mean values of the data, according to the least significant difference criteria with a 95 % confidence level ($P < 0.05$).

7.3. Results and Discussion

7.3.1. Valorization of waste protein biomass

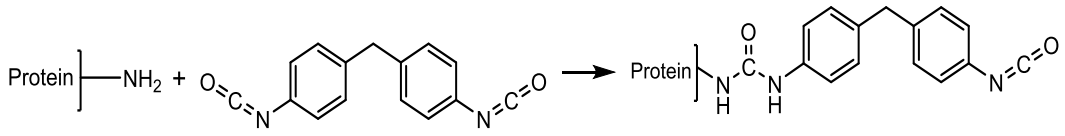
The hydrolysis of SRM material resulted in safe protein hydrolyzate that can be used as an industrial feedstock [11]. As we have previously reported [14, 15], the hydrolysis condition used has affected the yield, molecular size distribution and

composition of the hydrolyzed protein recovered from the SRM. The collected hydrolyzates powders were dark-brownish in color with a mild cooked protein odor, that had an average molecular weight of about 13 kDa [15]. We also showed that the hydrolyzates recovered as a result of the valorization had adequate functionality for further modification, associated with the functional groups of the protein main chain and the side groups from the amino acids. The presences of such functional groups were the basis for the proposed reaction of protein hydrolyzates with the isocynate functional groups of the MDI crosslinking agents and ultimately the interaction and bonding with the wood. Moreover, the recovered hydrolyzed proteins exhibited high solubility in water (72.6 %) [15], less viscosity in resin systems [16] than most long chain native proteins including soy protein isolate, that have a solubility of only about 18.7% at neutral pH [23].

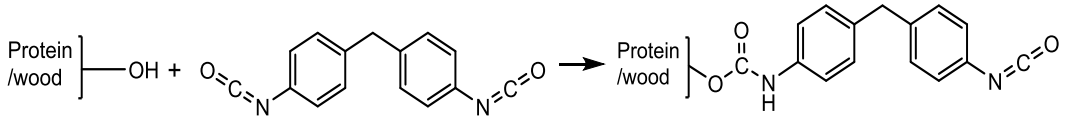
7.3.2. Reaction of hydrolyzed proteins with MDI

It is well known that the isocynate functional group of MDI reacts with chemical compounds that contain active hydrogen groups [24-26]. Such reactions involve attack by a nucleophilic center upon the electrophilic carbon of the isocynate. Proteins are chemical compounds that contain several active hydrogen containing functional groups associated with the side groups of the amino acids that could react with isocynates. Some of these functional groups that are reactive to isocynates include primary and secondary amines, hydroxyls, carboxylic acids and thiols [24, 26]. The hypothesis here was that the reaction of an isocynate group of MDI with amine group of protein results in formation of urea linkage as

shown in Figure 7.1 (a). The reaction of isocynates with hydroxyl group of protein or cellulose from wood, in turn, forms a urethane linkage Figure 7.1(b).



(a)



(b)

Figure 7.1. Possible reaction between (a) amine group of protein (b) hydroxyl group of protein cellulose in wood with isocynate functional group of MDI to form urea and urethane linkages, respectively.

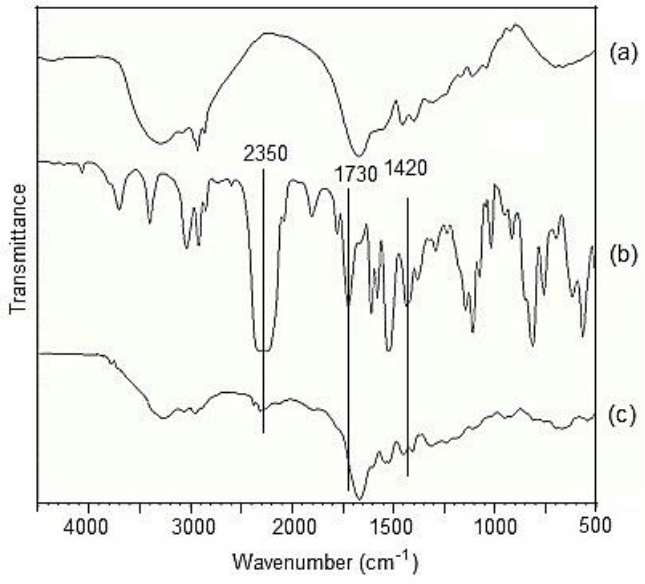
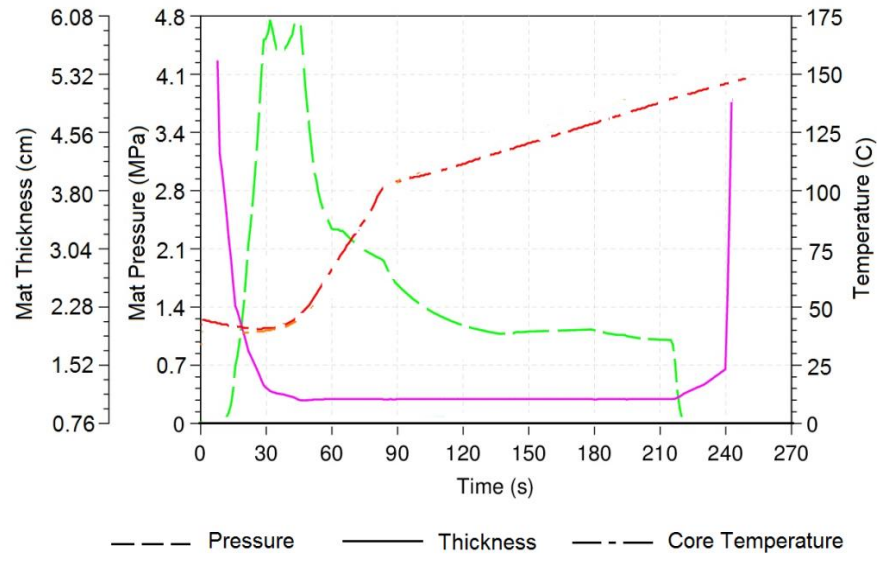
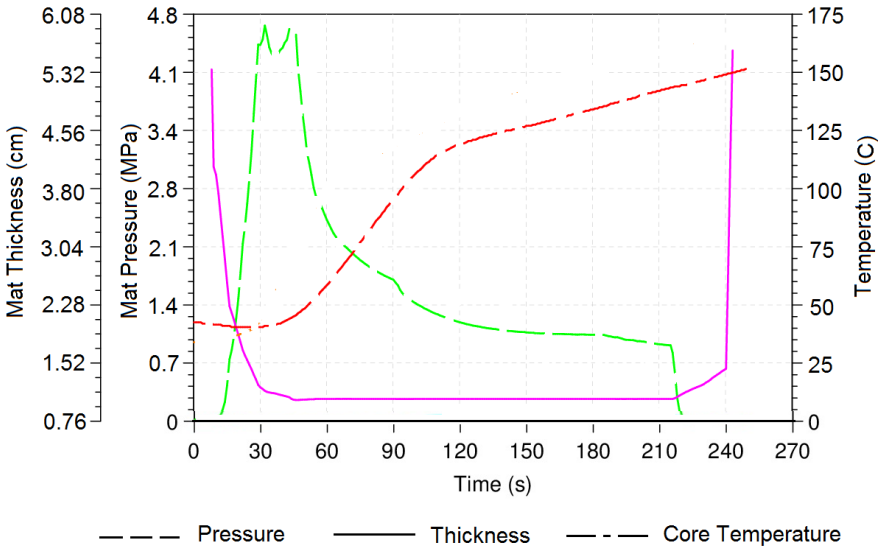


Figure 7.2. FTIR spectra of (a) protein hydrolyzate extract (b) MDI resin (c) cured polymer (1:1 protein hydrolyzate to MDI resin).



(a)



(b)

Figure 7.3. Typical press curve of OSB fabrication (a) MDI (b) 50% TH SRM.

FTIR studies conducted to examine such reaction possibilities were presented in Figure 7.2. The reduction of the isocynate group (-N=C=O) peak, very clearly evident at ~ 2350 and 1420 cm^{-1} (as suggested in Figure 7.1 (a & b)), can be attributed to consumption by the reaction with protein hydrolyzates functional

groups. Furthermore, a curtailed peak was observed on the broad band of the cured polymer, between 3600-3200 cm^{-1} (Figure 7.2c) than the hydrolyzed protein (Figure 7.2a) corresponding to free and bound –OH and –NH groups. A plausible explanation for this phenomenon could be the reaction of hydroxyl, carboxyl and amine group of protein hydrolyzate with the isocynate functional groups. A strong absorption band, characteristic of ester carbonyl, was also observed at ~ 1649 cm^{-1} in the reaction product, suggesting the formation of carbamate esters as a result of the reaction between –NCO and –OH [27] (Figure 7.1b). Close inspection of the carbonyl band at 1730 cm^{-1} (attributed to the isocynate) showed a shift to ~1649 cm^{-1} (Figure 7.2) in the cured plastic, possibly as a result of urea carbonyl formation in the adhesive [28] and thus providing further evidence of reaction with –NCO groups. According to literature [28-30], the IR spectra shift observed for such carbonyls (urea carbonyls) compared with isocynate carbonyls, was due to the formation of hydrogen bonds with the neighboring secondary amines.

7.3.2. OSB panels

Six OSB panels in triplicate with the different adhesives were successfully manufactured at a pilot plant facility (AITF, Edmonton, Alberta, Canada). There was no visual difference among the controls or formulated adhesives. During fabrication of the OSB, the *in situ* mat thickness, core temperature and pressure were automatically monitored. In press fabrication of OSB, a number of factors including the adhesive type and level wax level, pressing temperature, wood type, mat moisture level and distribution and pressure influence the performance. It can be noted here that the only parameter that were varied for this study was the

adhesive formulation. Figure 7.3 shows a typical OSB pressing curve for hydrolyzed protein based (50 %) and control (100% MDI). During the press closing phase, platens came to the target position (target thickness) to form the desired board. During the subsequent stage, the mat thickness was maintained to allow the heat to transfer into the core and cure the adhesive. It was also observed that neither the core temperature and pressure nor the thickness variation appeared to be noticeable in utilizing the two adhesives (Figure 7.3) for the panels.

7.3.3. Performance evaluation of the OSB panels

Mechanical performance

Table 7.2 shows the static bending, internal bond strength and bond durability performance of the panels fabricated using the different adhesive formulations. The panels produced using adhesive formulations with 40, 50 and 60 % (wt. /wt.) hydrolyzed protein had static bending (MOE and MOR) values that satisfied CSA requirement. The internal bond strength requirement of CSA was met by the 40 and 50 % formulations. A commercial OSB adhesive, composed of 100 % MDI adhesive, used here as a control satisfies all the CSA requirements of static bending and internal bond strength. MOE is a mathematical description of the panels' tendency to deform elastically (non-permanently) when an external load is applied to it. MOR on the other hand is the resistance to permanent bending deformation. Hence, larger MOE and MOR quantities reflect stronger resistance of the panel to elastic and permanent deformation, respectively.

The results obtained in this study had a negative correlation between hydrolyzed protein concentration in the formulated adhesives and static bending or internal

bond strength of the OSB panels (Table 7.2). The 70 and 85 % protein hydrolyzate formulated adhesives had a statistically significant MOE, MOR and internal bond strength of the OSB panel from the control panel. The reduction of MOE, MOR and internal bond strength was pronounced for the 85 % formulation with an average reduction of 38.6 %, 60 % and 83 %, respectively compared with the control. However, the bond durability requirement of the CSA (2 hour boil test) was not met by any of the formulations because of the relatively poor water resistance of the hydrolyzed proteins and their well-described tendency to associate with water [16, 18].

The variation in the mechanical performance of the panels as a function of adhesive formulation could be attributed to the degree of curing of the adhesive, the chemical bond formed with the wood strands and the spreadability of the cured adhesive [31]. The control MDI adhesive is an extensively used adhesive for OSB partially because of the capability of rapid polymerization with water in wood. The theory according to which reactions occur between the isocynate functional group of MDI with hydroxyl functional group of the cellulose and hemicellulose in wood is widely accepted in the literature [4, 32]. Nevertheless, newer researches using two-dimensional nuclear magnetic resonance spectroscopy suggested that there may not be detectable reactions of such type between isocynate adhesives and hydroxyl of wood polymers [33, 34]. This could be because of the low mobility of the hydroxyls in wood.

The adhesive formulated in this research contained functional groups of the hydrolyzed protein (amine, carboxyl, amide, hydroxyl, etc.) that could form

hydrogen bonding with the –OH groups of the wood cellulose, hemicellulose and other wood components. Furthermore, residual isocynates left from the reaction of hydrolyzed protein with the isocynates (Figure 7.2), could form covalent bond with –OH groups in the wood. Hence, the possibility of hydrogen bonding and mechanical interlocking between the adhesive and wood strands could be the main reasons for the observed mechanical performance of the OSB panels.

The bond durability, measured as MOR of the OSB after two hour boiling in water, was relatively poor for all the formulated protein-based adhesives. An explanation for this might be the general water resistance challenge observed in most protein-based polymeric materials [35] including adhesives [36]. The association of the hydrophilic functional groups of protein such as primary and secondary amine ($–NH_2$, –NH), carboxyls (–COOH), sulfhydryls (–SH) and hydroxyls (–OH) associated with side chain of amino acids and end group of proteins with water might lead to leaching out and consequently reduced adhesion and poorer bond durability. Moreover, the urethane and carbamic acid linkages formed between the protein and isocynates may not be resistant to hydrolysis during the boiling test. The hydrolysis of similar chemical bonds in polyol based polyurethane is reported in the literature [37-39]. In summary, the adhesives formulations bases on 40, 50 and 60 % hydrolyzed protein met the existing CSA static bending and internal bonding requirements. The bond durability requirement, on the other hand was not met by these formulations owing to the strong water affinity of protein functional groups coupled with the possibility of urethane bond hydrolysis.

Table 7.2. Performance of OSB panel specimens tested according to CSA O437.0-93 and ASTM D1037-12.

Adhesives	Static Bending		Internal Bond	Bond Durability (2 Hour Boil test)
	MOE (MPa)	MOR (MPa)	(MPa)	MOR (MPa)
Control (100% MDI)	4400 ± 322 [a]	28.7 ± 0.7 [a]	0.6 ± 0.1 a	11.5 ± 1.6 [a]
40 % hydrolyzate	3700 ± 357 [a]	21.5 ± 1.0 [b]	0.4 ± 0.1 [b]	3.2 ± 0.0 [b]
50 % hydrolyzate	3500 ± 366 [a]	19.1 ± 2.0 [b]	0.4 ± 0.0 [b]	3.6 ± 0.8 [b]
60 % hydrolyzate	3900 ± 290 [a]	19.0 ± 3.0 [b]	0.3 ± 0.0 [b]	1.8 ± 0.0 [c]
70 % hydrolyzate	3600 ± 351 [a]	16.0 ± 3.0 [b]	0.2 ± 0.0 [b]	-
85 % hydrolyzate	2700 ± 300 [b]	11.5 ± 1.1 [c]	0.1 ± 0.0 [c]	-
Requirement	3100	17.2	0.345	8.6

Data in this table are means ± standard deviation with a sample size of at least 6 for each group. [a-c] Means with the same superscript letters within a column are not significantly different at $P < 0.05$ level.

Thickness swell test

The water resistance of the cured adhesive, measured as weight loss to the water while it was soaked for 24 h in water, showed a near-constant trend at 40, 50 and 60 % hydrolyzed protein concentration. Similarly, swelling of OSB panels manufactured using the 40, 50 and 60 % hydrolyzed protein formulation did not show statistically significant difference of swelling with each other (Figure 7.4). However, when the hydrolyzed protein concentration in the adhesive formulation was increased to 70 and 85 %, a marked thickness swelling and loss of water resistance was observed. An 85 and 102 % increase compared with the control

MDI was measured for formulations that contained 70 and 85 % hydrolyzed protein concentration respectively. Figure 7.4 shows that a similar trend of thickness swelling and loss of water resistance as the hydrolyzed protein concentration in the adhesive formulation was increased. Thus, the thickness swelling could be strongly correlated to the water resistance of the formulated adhesive.

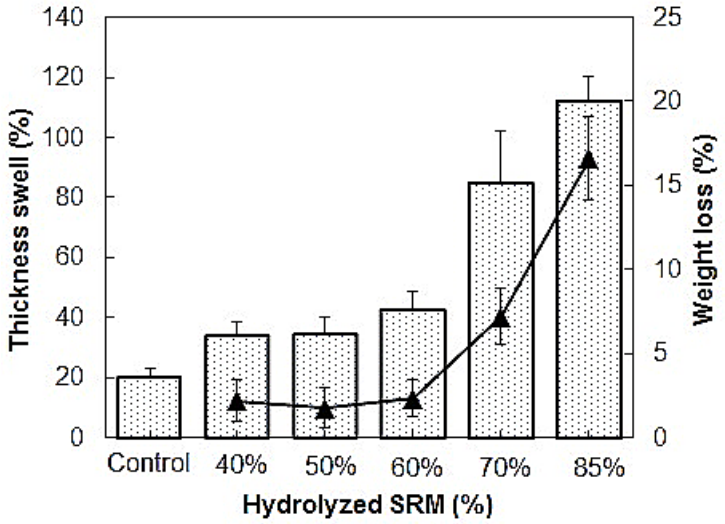


Figure 7.4. The bar shows thickness swell of OSB panels using MDI cured hydrolyzed protein adhesives. The scattered line chart show the weight loss of cured adhesive in water, as the hydrolyzed protein concentration was varied from 0 to 85%.

The association of polar functional group of hydrolyzed proteins with water was the most likely reason for loss of water resistance. Hydrogen bonding provides mechanical performance only in dry state [36]. In the presence of water, the hydrogen bonds formed between the formulated adhesive and wood substrate, are ruptured because of the association with water molecule. The presence of

incomplete protein-MDI network chains could also lead to leaching out of the adhesive into the water. Such migration of adhesive material from the panel would leave behind voids that in turn would drive more water diffusion into the OSB composites. This would ultimately result in higher moisture absorption of the panel and consequently more thickness swelling.

Density Profile

A typical density profile over the thickness of the developed fiberboard with the different adhesive formulations is shown in Figure 7.5. The profile over the different zones of the thickness resembled a symmetric "m-shape", with peak densities appearing near the board top and bottom surfaces (zones 1 and 3), and the lowest density in the core region (zone 2). Similar observations of higher surface and lower core density using commercial adhesives are extensively reported in the literature [40-44]. The presence of this vertical density gradient is known to result in higher bending strength, but lower internal bond strength [40]. Wang et al. [43] investigated and reported that the formation of density profile in OSB was the result of a combination of events that occurred during consolidation of the mat. Because the mat is at high temperature at the end of pressing cycles, the consolidation process and density profile formation are expected to continue until the mat arrive a thermodynamically stable state.

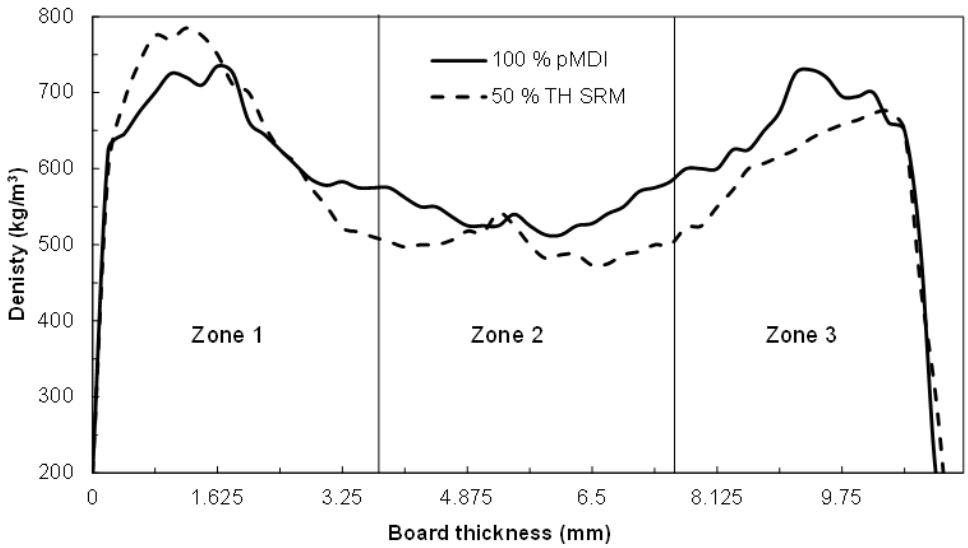


Figure 7.5. Typical density profile of 100% MDI and 50% TH SRM formulated adhesive bonded fiberboard.

Table 7.3. Density profile of formulated adhesives bonded specimen.

TH SRM: MDI concentration (wt. /wt.)	Density (kg/m^3)		
	Zone 1	Zone 2	Zone 3
Control	716.3 ± 15 [a]	599.9 ± 9 [a]	678.5 ± 18 [a]
40%	717.7 ± 23 [a]	579.0 ± 17 [a]	657.1 ± 17 [a]
50%	685.2 ± 22 [a]	568.0 ± 13 [a]	647.5 ± 11 [a]
60%	656.0 ± 13 [a]	529.9 ± 8 [b]	622.8 ± 19 [a]
70%	641.5 ± 4 [b]	491.3 ± 10 [c]	635.2 ± 23 [a]
85%	633.9 ± 17 [b]	493.0 ± 13 [c]	580.0 ± 12 [b]

Data in this table are means ± standard deviation with a sample size of at least 6 for each group. [a-c] Means with the same superscript letters within a column are not significantly different at $P < 0.05$ level.

Factors influencing the density profile include the moisture content, wood fiber type, hot pressing conditions (temperature, closing speed, pressure and duration) and adhesive type. In our case, all parameters were kept constant except the adhesive formulation. The effect of adhesive formulation on the average density profile of OSB panel zones (1-3) is shown in Table 7.3 below. From Table 7.3, it was observed that the 40 and 50 % hydrolyzed protein formulations exhibited no statistical ($P < 0.05$) density profile difference in each zone of the panel compared with the control formulation. At 60 % hydrolyzed protein adhesive formulation, the first and last zones did not exhibit density difference. However, the core zone (zone 2) exhibited statistically lower average density compared with control.

Overall, the density profile of the core zone appears to be the most influenced by the variation of the adhesive formulations. The minimum average density in the core zone, observed for the 85% formulation, exhibited only 20 % reduction compared with the control adhesive. This observation was in line with the mechanical performance of the OSB panels developed with the different adhesives. Hence, the 70 and 85 % formulations with 24.5 and 23.4 % variations exhibited the largest average density variation between the face and core zones. It is plausible that the relatively weaker adhesion strength observed by these two formulations played a role for the significantly low core density of the OSB and in essence for the density variation. According to Rathke *et al*. [45], large variations between zones across the thickness could lead to premature failure under mechanical stress in the center part of the panel that includes the core layer as well as the transition zone between the core and the face layer. With regard to this

variation, the difference observed for the control, 40, 50 and 60 % formulations was about 17 %, and it was not statistically significant. In summary, the density profile study showed that the 40, 50 and 60 % formulations created a density profile pattern in OSB panels that was comparable to a commercial control adhesive.

7.4. Conclusions

Hydrolyzed waste animal proteins were used as feedstock to produce wood adhesives for oriented strand boards with mechanical performance that met the CSA requirements. FTIR studies provided a strong evidence of covalent crosslinking between the isocynate functional group of MDI and hydrogen groups of hydrolyzed protein that resulted in urea and urethane linkages. Owing to the hydrophilic nature of some of the functional groups of hydrolyzed proteins, the water resistance of the developed adhesive did not meet the CSA requirement for structural applications. The OSB developed here should therefore be used for indoor, dry condition applications. An ongoing effort that includes capping some of the hydrophilic functional groups of the hydrolyzate, coating and lamination of the OSB panels is currently in progress in the same research group. The technology platform demonstrated here is relevant for the livestock, rendering and OSB adhesive industries. The valorization of hazardous waste protein to industrially relevant safe feedstock and value-added utilization is insightful that can be extended to other agricultural waste proteinaceous biomasses.

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Chapter 8

Conclusions and outlook

Specified risk material is a waste generated by the rendering industry and its utilization as food/feed is banned in most countries that put BSE control in place. On the other hand, the use of renewable sources for drop-in-chemicals and material is receiving much global attention as a result of concerns of fossil fuel depletion, and environmental pollution. The goal of this thesis was to develop valorization technology platform of an otherwise waste SRM into renewable industrial feedstock and to develop industrially relevant bio-based applications such as bioplastics, biocomposites, and adhesives. This research demonstrated that hydrolysis protocols approved by the CFIA and FDA can be used to transform waste animal tissues into safe proteinaceous feedstock for non-food, bio-based applications. The studied hydrolytic protocols cleaved the recovered proteins, improved their water solubility, and reduced molecular size and size distributions. Residues remained after proteinaceous material recovery was mainly ashes and insoluble protein fractions that can be used for organic fertilizer applications, mineral recovery etc.

Control of the molecular size and size distribution of the protein hydrolyzate was also achieved by varying either the hydrolyzing solution concentration or hydrolysis temperature. Besides the molecular size, free and total amino acid quantifications, deamidation and deamination of proteins and organic acid generation were used as quantitative tools of SRM hydrolysis. Although the higher temperature ranges (240 and 260 °C) resulted in severe protein cleavage

and degradation of the amino acids, the molecular weight distribution of the biorefined protein hydrolyzates obtained as such were more uniform. Such uniformity is usually an advantage for functional utilization. The hydrolysis and biorefining techniques reported in this work could be extended to other waste biomasses including blood meal, meat and bone meal, chicken feathers, general food waste, etc.

Chemical modification of the proteinaceous material extracts involved covalent crosslinking with epoxy resin for thermosetting plastic and composite development. The plastics made as such exhibited promising thermal and mechanical properties, good moisture resistance at neutral, acidic and basic pH in all the studied formulation ranges. Among the studied formulations, the use of 20 % protein hydrolyzate by weight of epoxy resin exhibited high tensile strength and glass transition temperature, low temperature curing and better solvent resistance behaviors. Crosslinking techniques developed in this study may be adapted to unhydrolyzed, long molecular chains proteins of animal source (such as blood meal and meat and bone meal) and plant source (such as soy protein, corn zein or canola protein).

These developed proteinaceous plastic was also utilized as matrix of composite materials. The developed composites consist of (i) a control synthetic resin composed of epoxy resin cured with APS and (ii) bio-based resin composed of epoxy resin cured with hydrolyzed proteins. The reinforcing fillers were two types of fiberglass (woven roving and chopped strand mat) and hemp-fiber mat. The study showed that all the reinforcing fibers improved the tensile and flexural

stiffness of the biocomposites made. Thermal stability study also displayed that all composites prepared were stable until a temperature close to 230°C. The study also showed that the mechanical performance of the biocomposites was negatively affected by the water absorption. Generally, the conjuncture of hydrolyzed protein cured epoxy with hemp fiber exhibited comparatively lower water resistance. The influence of water absorption was in such a pattern that water-saturated samples presented poor mechanical properties. Water absorbed hemp based composites specifically exhibited an improvement in tensile strain due to plasticization effect of the water molecules on the cellulose component. These results highlight the importance of interfacing the matrix and reinforcing fiber phases may be needed to improve the mechanical performance and degradation behavior under moisture sorption.

Modifications of hydrolyzed proteins into adhesives were also conducted by crosslinking it with methylenediphenyl diisocyanate. FTIR studies provided a strong evidence of covalent crosslinking between the isocynate functional group of MDI and hydrogen groups of hydrolyzed protein that resulted in urea and urethane linkages. Owing the hydrophilic nature of some of the functional groups of hydrolyzed proteins, the water resistance of the developed adhesive for oriented strand board did not meet the CSA requirement for structural applications. However, the good mechanical strength achieved by the adhesive showed that the developed OSB could be applied for indoor and dry condition structural applications. Overall, moisture resistance was the major challenge of protein based materials with the exception of the epoxy crosslinked proteins.

Thus, further effort that includes capping some of the hydrophilic functional groups of the hydrolyzate, coating and lamination of OSB panels and biocomposites should be further studied.

The conversion technology platforms demonstrated here is relevant not only to SRM, but also to other animal protein residues such as blood and blood meal, meat and bone meal, feather meal, and it can be extended to other undervalued agricultural residues and wastes as a renewable feedstock, providing an alternative to “virgin biomass”. The developed applications in this research could generate an alternative income to the rendering/livestock industry beside their main product line. It also reduces the environmental burden of deploying SRM into the landfill across North America.

Appendices

Appendix A. Other peer reviewed journals published as co-author (second author):

Appendix A1. Nayef El-Thaher, Tizazu Mekonnen, Paolo Mussone, David Bressler, Phillip Choi. Effects of Electrolytes, Water, and Temperature on Cross-Linking of Glutaraldehyde and Hydrolyzed Specified Risk Material. Industrial and Engineering Chemistry Research. 2013, 52, 4987 – 4993.

Appendix A2. Nayef El-Thaher, Tizazu Mekonnen, Paolo Mussone, David Bressler, Phillip Choi. Nonisothermal DSC Study of Epoxy Resins Cured with Hydrolyzed Specified Risk Material. Industrial and Engineering Chemistry Research. 2013, 52, 8189 – 8199.

Appendix B. Literature review paper authored (first author) as part of the PhD program:

Tizazu Mekonnen, Paolo Mussone, Hamdy Khalil and David Bressler. Progress in bio-based plastics and plasticizing modifications: Feature Article. Journal of Materials Chemistry A, 1, 13379-13398.

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Progress in bio-based plastics and plasticizing modifications

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Over the coming few decades bioplastic materials are expected to complement and gradually replace some of the fossil oil based materials. Multidisciplinary research efforts have generated a significant level of technical and commercial success towards these bio-based materials. However, extensive application of these bio-based plastics is still challenged by one or more of their possible inherent limitations, such as poor processability, brittleness, hydrophilicity, poor moisture and gas barrier, inferior compatibility, poor electrical, thermal and physical properties. The incorporation of additives such as plasticizers into the biopolymers is a common practice to improve these inherent limitations. Generally, plasticizers are added to both synthetic and bio-based polymeric materials to impart flexibility, improve toughness, and lower the glass transition temperature. This review introduces the most common bio-based plastics and provides an overview of recent advances in the selection and use of plasticizers, and their effect on the performance of these materials. In addition to plasticizers, we also present a perspective of other emerging techniques of improving the overall performance of bio-based plastics. Although a wide variety of bio-based plastics are under development, this review focuses on plasticizers utilized for the most extensively studied bioplastics including poly(lactic acid), polyhydroxyalkanoates, thermoplastic starch, proteinaceous plastics and cellulose acetates. The ongoing challenge and future potentials of plasticizers for bio-based plastics are also discussed.

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1. Introduction

Plastics are amorphous organic solid polymers covering a wide range of polymerization products suitable for the manufacture of diversified products. Worldwide annual plastics production is estimated to surpass 300 million tons by 2015 (ref. 1) representing trillions of dollars in terms of global economic returns.[2]

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Tizazu Mekonnen earned his BSc degree in Applied Physics (2004) and MSc degree in Chemical Engineering (2009) from Addis Ababa University, Ethiopia. He is currently finalizing his PhD in Bioresource Engineering under the supervision of Professor David Bressler at the Biorefining Conversions and Fermentations Lab at the University of Alberta, Canada. Mr Mekonnen's PhD research work entails hydrolysis and biorefining of hazardous waste protein biomass to fabricate and study novel biopolymers and biopolymer–synthetic polymer hybrid materials for various industrial applications.

Dr Mussone holds a position as Research Associate with the Biorefining Conversions and Fermentations Laboratory at the University of Alberta. Dr Paolo Mussone earned an MSc degree in Process Engineering from the Polytechnic University of Milan in Italy and a PhD in Surface Chemistry and Colloidal Science from the University of Manchester in the United Kingdom. Dr Mussone's general research focus is on the conversion of biomass into value-added chemicals and materials. Of particular interest is the development of renewable polymeric surfactant platforms for heavy petroleum processing and waste water treatment processes.

Plastics are highly valued materials because of their low cost and extraordinary versatility and they constitute the largest petroleum application second only to energy.[3] Among the many applications of plastics, packaging accounts for almost one-third of their use followed by construction and consumer products.[4] The materials science community has been striving for decades to generate bio-based plastics to substitute or complement conventional synthetic plastics based on exclusively petroleum feedstock. According to current estimates, the global production of bioplastics is expected to grow at an annual rate of up to 30% in the coming decade to reach 3.5 million tonnes in 2020.[5]

Bioplastics may also be bio-based (*i.e.* polymer derived from renewable feedstock) and biodegradable (*i.e.* polymer that can return to nature).[6] Biodegradability and compostability depend on the chemical structure rather than the feedstock source. According to the US Department of Agriculture (USDA), bio-based products are defined as commercial or industrial goods (other than feed or food) composed in whole or in significant part of biological products.[7] Thus, synonymous use of the terms bio-based plastic and biodegradable plastic is not correct. Some of the most commonly known bio-based plastics in today's marketplace in terms of production and renewability are poly(lactic acid) (PLA), polyhydroxyalkanoates (PHAs), starch plastics, cellulose esters and protein based plastics (Fig. 1). Other bio-based plastics, such as bio enriched polyurethane manufactured using modified vegetable oils, polyethylene monomers derived from the dehydration of bio-ethanol, polypropylene monomers derived from dehydration of bio-butanol and poly(ethylene terephthalate) monomers produced *via* fermentation, catalytic pyrolysis or gasification of biomass,[8] that have at least partial sourcing from plants constitute emerging technologies expected to make a significant market impact.

Bio-based plastics could overcome the sustainability issues and environmental challenges posed by the production and disposal of synthetic plastics. However, the large scale commercial deployment of bio-based plastics to replace conventional plastic materials remains challenged by several factors. Some of the challenges are attributed to the relatively poor performance, variability of properties of the feedstock associated with location and the time of harvest, high production cost and lack of infrastructure. Recent development in bacteria synthesized plastics (PHAs) and the utilization of nature's own building blocks such as proteins, fats, carbohydrates, lignin, *etc.* obtained from agricultural feedstock and agricultural industry wastes constitute a major progress towards bio-based plastics in the last decade.

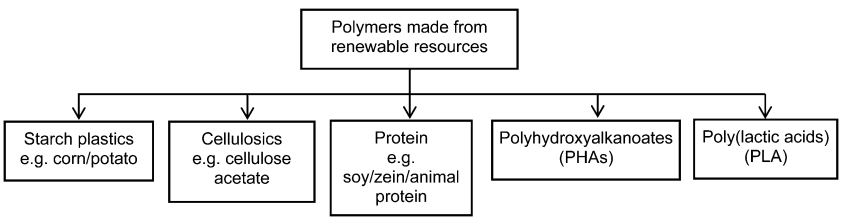

Fig. 1 Major bio-based plastics and their production routes.[6]

Dr Hamdy Khalil is the Senior Global Director for Advanced Technologies and Innovation. He pioneered the introduction of chemicals derived from renewable resources into the manufacturing of automotive parts and the application of Phase Transfer Catalysis in the synthesis of heterocyclic compounds. Dr Khalil is a member of the Board of Directors of several industrial organizations. He chaired and participated in many International Conferences related to Biotechnology as well as Polyurethane Technology. Dr Khalil is a member of the American Chemical Society and the Canadian Institute of Chemistry. He has several patents and publications in the areas of Bio-polyols, Sealants, Latex and Polystyrene.

Dr Bressler is currently a tenured Professor in the Food and Bioresource Technology Division of the Faculty of Agricultural, Life, and Environmental Sciences at the University of Alberta. He is also Founding Director of the Biorefining Conversions Network, an organization focused on facilitating the development of novel, commercially viable biomass conversion technologies, and value-added products within Alberta, Canada. Dr Bressler's general area of research is the industrial application of chemical, thermal, and biological systems for the conversion of conventional agricultural products to platform chemicals and other value-added commodities.

Plasticizers have long been known for their effectiveness in enhancing the flexibility of synthetic plastics such as polyvinyl chloride (PVC) and epoxy resins. New types of plasticizers compatible with bio-based plastics are being developed. For technical and economic reasons, polymer additives are a large and increasingly significant component of the polymer industry.[9] Among the additives, plasticizers constitute about one third of the global additive market,[10] with a worldwide consumption of over 4.6 million metric tonnes in 2003 (ref. 11) and over 6.4 metric tonnes in 2011.[12] Generally, plasticizers are small, relatively non-volatile, organic molecules that are added to polymers to reduce brittleness, impart flexibility, and improve toughness, reducing crystallinity, lowering glass transition and melting temperatures.[13,14] Plasticization reduces the relative number of polymer–polymer contacts thereby decreasing the rigidity of the three-dimensional structure thereby allowing deformation without rupture.[15] Consequently, plasticizers improve processability, flexibility, durability and in some cases reduce the cost of polymers.[16,17]

The use of plasticized polymers in pharmaceutical applications ranging from packaging materials or auxiliary substances in conventional dosage forms to membranes or matrices modifying and controlling the drug release characteristics in therapeutic systems has been reported in the literature.[16,18,19] The processing behaviour, such as film formation and coating dispersion, and properties of polymers in various applications are greatly improved by adequate choice of plasticizer type and quantity.[16,20] Generally, the choice of these plasticizers to be used as modifiers of plastics is limited by the required safety, environmental favorability, chemical and physical property that dictate their miscibility, processing temperature and required flexibility towards the target application.[17]

The risk of leaching out of certain plasticizers, such as phthalates during storage or end-user application, constitutes a major safety risk.[21–24] This coupled with other shortcomings (*e.g.* toxicity, poor compatibility) limits some plasticizers from application in the medical, pharmaceutical and food packaging fields. The ideal plasticizer significantly lowers the glass transition temperature (T_g), is biodegradable, nonvolatile, and nontoxic, and exhibits minimal leaching or migration during use or aging.

Recent advances in bio-based plastics are spurred by factors such as public concern over the depletion of petroleum based raw materials, the desire of manufacturing companies to develop more sustainable raw material sources, the improvement in properties as well as cost competitive relationship of bioplastics.[25,26] As these bio-based plastic industries continuously grow, the demand for new types of plasticizers with new characteristics, performance and other additives that are compatible with the bioplastics also grows in the same direction.[27] In the realm of developing packaging materials from bio-based materials, a high ductility at room temperature is required and thus, there is no tolerance for the polymer film tearing or cracking when subjected to stresses during package manufacturing or use.[28] Moreover, increase in the utilization of plasticized polymers for biomedical and pharmaceutical application,[16] the search for safer plasticizers for commodity plastics such as poly(vinyl chloride)[29–31] and efforts to produce renewable and biodegradable plasticizers[29,30] constitute an additional motive for the recent development of new plasticizers. This review briefly reports recent progress in the development of plasticizers utilized for bio-based plastics, and their influence on the performance of bio-based plastics.

2. Plasticization mechanism

Two types of plasticizers are defined in polymer science: internal and external.[22,23,32] Internal plasticizers are part of the polymer molecules, co-polymerized into the polymer structure, grafted or reacted with the original polymer thereby making the polymer chains more difficult to fit and compact with each other closely.[23] They soften polymers by lowering the glass transition temperature (T_g) and reducing the elastic modulus.[22] External plasticizers, on the other hand, are low volatility molecules added to interact with polymers and produce swelling without chemical reaction. Internal molecular forces between plasticizer molecules and between a plasticizer and a polymer such as dispersion forces, induction forces, dipole–dipole interaction, hydrogen bonds are important in external plasticization.[23]

Several theories have been proposed to explain the mechanism and action of plasticizers on polymers. Among those theories, the following plasticizing mechanisms have been widely accepted to describe the effect of plasticizers on polymeric networks:[33–36] (a) the lubricity theory: this theory is similar to metal parts lubrication by oil. The plasticizer acts as a lubricant to reduce friction and facilitates polymer chain mobility past one another, consequently lowering deformation; (b) the gel theory: this theory extends the lubricity theory and suggests that a plasticizer disrupts and replaces polymer–polymer interactions (hydrogen bonds, van der Waals or ionic forces, *etc.*) that hold polymer chains together resulting in reduction of the polymer gel structure and increased flexibility; and (c) the free volume theory: for any polymeric material the free volume is defined as the internal space available in a polymer for the movement of chains. Free volume is usually described as the difference between the observed volume at absolute zero and the volume measured at a selected temperature. Rigid resins are characterized by limited free volume whereas flexible resins have relatively large amounts of free volume. Plasticizers increase the free volume of resins and also maintain the free volume after the polymer–plasticizer mixture post processing is cooled down. The free volume theory explains the effect of plasticizers in lowering the glass transition temperature.

Although these theories are widely accepted and utilized in the selection of plasticizer for polymers, Shtarkman and Razinskaya[35] stressed the limitation of the current plasticization theories. According to these authors,[35] the plasticization theories are limited and not feasible for plasticizer selection for the following reasons: (1) direct studies of the plasticization mechanism is lacking and (2) the existing plasticization theories have limited predicting capability and are limited to only particular cases. For this purpose, the authors[35] suggested the

necessity of a compatibility–efficiency–property study that takes into account the structure of the polymeric system to select a specific plasticizer rather than relying on the theories.

The aforementioned plasticization theories/mechanisms were developed for synthetic plastics, particularly PVC. Limited attention has been devoted to developing new theories/mechanisms or improving established theories to explain the plasticization mechanism of the newly developed and emerging bio-based plastics. The complex nature of some of the biological feedstock macromolecules makes bio-based plastics radically different from the common repeating monomer based synthetic polymers. Hence, renewed efforts are required to investigate other more explanatory plasticization possibilities and theories.

3. Plasticization of bio-based plastics

3.1. Poly(lactic acid) plastics

Poly(lactic acid) (PLA) is one of the most promising innovative plastics for various end-use applications. This polymer is thermoplastic, renewable, biodegradable and biocompatible, a set of highly attractive attributes for pharmaceutical, biological and medical applications.[37,38] The raw material of PLA, L-lactic acid, can be produced by fermentation of renewable sugar resources such as starch and other polysaccharides.[39,40] Moreover, PLA exhibits a remarkable balance of performance properties comparable to traditional thermoplastics[39] processed using conventional plastic processing techniques. From a physical property standpoint it is often loosely compared to polystyrene.[37] Similar to polystyrene, standard grade PLA has high modulus and strength.[37,41] Moreover, the degradation products of polylactides are nontoxic which enhances practical applications in biomedicine.[42] PLA is currently being commercialized for a wide spectrum of technologically important fields and applications by companies such as Cargill and Dow Chemicals.[38]

PLA belongs to the family of aliphatic polyesters commonly made from lactic acid (2-hydroxypropionic acid) building block shown in Fig. 3. The synthesis of lactic acid into high-molecular weight PLA can follow two different routes of polymerization,[40,43] as depicted in Fig. 4. The monomer lactic acid is condensation polymerized to yield a low-molecular weight, brittle, glassy polymer in the first route, which, for the most part, is unusable unless external coupling agents are used to increase the molecular weight of the polymer.[40] The second route of producing PLA is to collect, purify, and ring-open and polymerize lactide to yield high molecular weight (average M_w > 100 000) PLA.[40,43,44]

The combination of the chiral lactic acid monomers (Fig. 2) or the depolymerization of low molecular weight PLA (Fig. 3) could give rise to distinct forms of polylactides. These polylactides are poly(L-lactide) (or LL-lactide), poly(D-lactide) (or DD-lactide), poly(LD-lactide) (or *meso*-lactide) as shown in Fig. 4 or a mixture of L-and D-lactides, called racemic lactide (*rac*-lactide).[38,46,47] While the D- and L- lactides are optically active, *meso*- is not (Fig. 4).[46] Highly crystalline PLA can be obtained with low D content (<2%), fully amorphous PLA on the other hand can be obtained with high D content (>20%).[48] Semi-crystalline PLA is obtained with 2 to 20% of D content.[2] The amount and stereosequence of these lactides in the polymer backbone give rise to a wide range of molecular weights. These changes as a result impact the melt behavior, thermal, mechanical, optical properties, barrier properties and biological properties of PLA.[49,50]

PLA is brittle, with relatively poor impact strength and low thermal degradation temperature limiting its applicability.[39,40] Relatively poor strength, coupled with its hydrophobicity, semi-crystalline properties, limited thermal processability, lack of reactive functional groups along the polymer backbone and high cost constitute the majority of its limitation in wide industrial and medical applications.[39,45] Accordingly, to compete with the low-cost and flexible commodity polymers and upgrade the PLA performance, considerable research effort is being carried out. These attempts include modifying PLA with plasticizers, blending with other polymers,[51] copolymerization, and incorporation of fillers.[37,45,52]

3.1.1. Plasticizers for poly(lactic acid). Low molecular weight compounds such as oligomeric lactic acid, glycerol, triacetin, and low molecular weight citrates,[17] partial fatty acid esters[53,54] are common plasticizers of PLA. A large number of investigations have also been reported on blending PLA with various polymers as plasticizers, for example, thermoplastic starch (TPS),[51] poly(ethylene oxide),[55,56] poly(ethylene glycol)(PEG),[55] poly(ε-caprolactone),[55–57] poly(vinyl acetate),[58] poly(hydroxy butyrate),[59] cellulose acetate,[60] poly(butylene succinate),[61] and poly(hexamethylene succinate),[61] to improve its flexibility. Most of the resulting plasticized PLA materials exhibited better impact resistance, increased deformation at break and improved resilience. Table 1 reports common monomeric and polymeric plasticizers and their plasticization effects on the glass transition temperature and mechanical property of PLA.

The results in Table 1 show that all citrates at 20% concentration reduced the glass transition temperature and improved the flexibility while reducing the tensile strength of the PLA control. A significant improvement of elongation at break was achieved at the expense of tensile strength. Ljungberg and Wesslen[63] also investigated the use of triacetin, tributyl citrate, triethyl citrate, acetyl tributyl citrate, acetyl triethyl citrate as potential plasticizers of PLA and reported a drastic lowering of the glass transition temperature of PLA at concentrations as low as 15% resulting in a homogeneous and flexible film. However, it was reported that the migration of citrates onto the film surfaces during aging, especially the low molecular weight citrates, was a major challenge.[17] This issue could be addressed

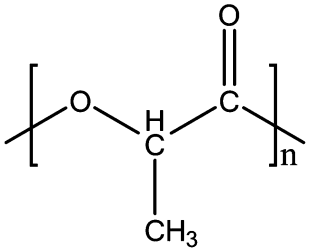

Fig. 2 Basic structure of PLA.

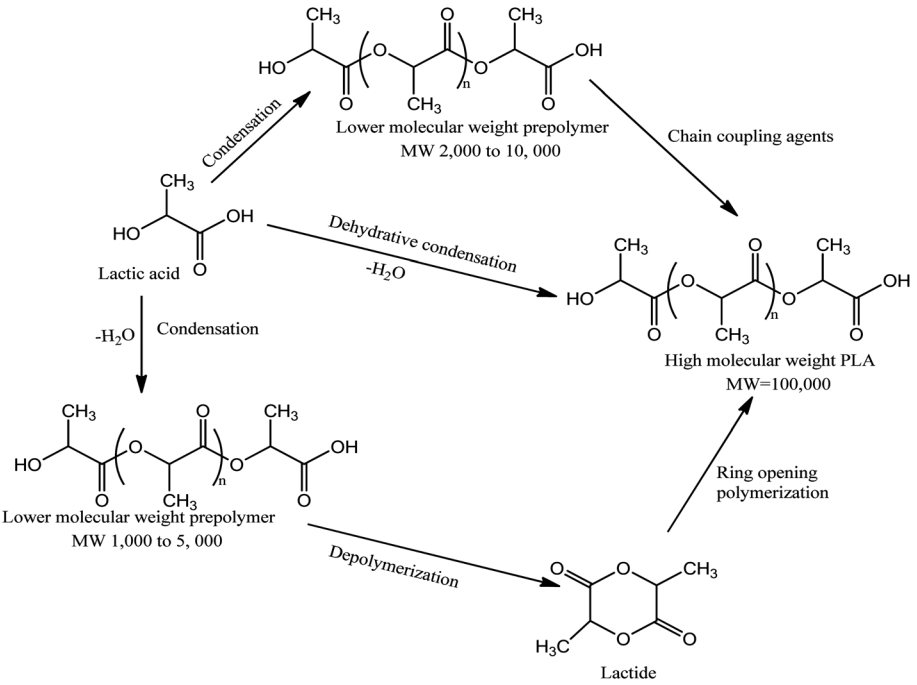

Fig. 3 Synthesis methods for high molecular weight PLA.[44,45]

by increasing the molecular weight. For instance, Ljungberg and Wesslen[17] transesterified tributyl citrate (M_W 360 g mol^{-1}) with diethyl glycol that resulted in two oligomeric plasticizers with higher molecular weights (M_W 4500 g mol^{-1} and 63 600 g mol^{-1}). The investigation of the effects of these oligomers on thermo-mechanical and aging properties of PLA shows that both oligomers did not lower the T_g as greatly as monomeric citrates. Among the two oligomeric plasticizers, a relatively larger reduction in T_g was achieved by the oligomer with the lower molecular weight.

Similarly, molecular weight variation, concentration and the presence of polar amide groups of plasticizers can positively interact with PLA chains, affecting the compatibility between PLA and the plasticizer and controlling elongation and morphological stability that result in leaching during aging or use.[17,63] The plasticizer with lower molecular weight that resulted in lower T_g of PLA may also facilitate the migration of the plasticizer from the bulk of the material compared to the higher molecular weight plasticizer.[17] The effect of triacetin (0–30%) and tributyl citrate (0–25%) loading on PLA was studied[63] and an almost linear decrease in T_g with the increase of plasticizer content was observed.

3.1.2. Other methods of improving PLA performance. New PLA toughening strategies using citrate family (tributyl *O*-acetylcitrate and tributyl citrate) to improve the PLA ductility were reported by Hassouna *et al.*[64] This strategy involves grafting of tributyl citrate onto neat and maleic anhydride modified PLA with tributyl *O*-acetylcitrate.[62] The maleation of PLA was carried out by reactive extrusion with the aim of incorporating hydroxyl functional groups into the PLA. The neat PLA and hydroxyl functionalized PLA were then copolymerized with tributyl citrate that already contains a hydroxyl functional group. Such toughening was shown to drastically decrease the T_g of PLA. However, the grafting reaction of tributyl citrate into anhydride-grafted-PLA revealed a shift of PLA T_g toward higher values compared to neat PLA grafted with tributyl citrate. After six months of aging, no phase separation was observed and no major leaching phenomenon was noticed in both cases. These observations indicated that the mobility restriction as a result of hydrogen bonding occurring between PLA and tributyl citrate as well as the grafting reaction of tributyl citrate into anhydride-grafted-PLA diminished the leaching phenomena.

Maglio *et al.*[55] studied the copolymerization of PLA with poly(ε-caprolactone) (PCL) and poly(oxyethylene) (PEO) to

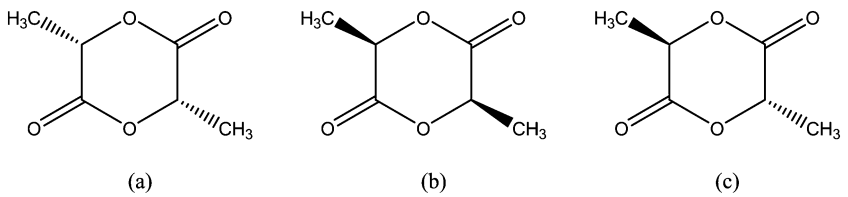

Fig. 4 Chemical structures of dimeric (a) D-lactide, (b) L-lactide and (c) *meso*-lactide.

Table 1 Mechanical and thermal performance of PLA plasticized with different modifiers

Modifiers	M_W (g mol^{-1})	Conc. (wt%)	T_g (°C)	E^a (MPa)	ε^a (%)	σ^a (MPa)
PLA[54,55]	137 000	100	59	1720	7	51.7
Triethyl citrate[54]	276	20	32.6		382	12.6
Tributyl citrate[54]	360	20	17.6		350	7.1
Acetyl triethyl citrate[54]	318	20	30		320	9.6
Acetyl tributyl citrate[54]	402	20	17		420	9.2
Poly(oxyethylene)[55]	10 000	21	31	320	7	49
Poly(ε-caprolactone)[55]	10 000	20	35	961	25	19
Glycerol[51]	92.09	20	53		—	—
PEG monolaurate[51]	400	20	21		142	—
Plasticized TPS[51]	—	25	—		2.9	30.2
PEG[62]	1500	10	34.3	1750	150	15.1
PEG[62]	1500	20	23.2	1460	150	14.6

[a] Tensile modulus (E), tensile stress at yield (σ), and elongation at break (ε).

improve the brittleness and reduce T_g. The copolymers obtained as a result exhibited high elongations at break and as a result much lower tensile moduli than the PLA pure polymer (Table 1). The T_g of the copolymers was also much lower than that of the original PLA. A recent study by Hassouna *et al.*[62] investigated grafting of poly(ethylene glycol) (PEG) onto PLA through reactive extrusion to develop plasticized PLA. It was shown that the T_g and modulus were invariably reduced. In all cases, both T_g and elastic modulus were dependent on the content of PEG grafted onto the PLA. The *in situ* reactive grafting of PEG onto PLA exhibited a marked T_g reduction than the blending option. Other plasticizers of PLA reported in the literature include epoxidized soybean oil,[65] ionic liquids,[66] mixed plasticizers,[67] *etc.*

In summary, it can be pointed out that several studies have demonstrated that plasticizers play a significant role in determining the performance properties of PLA plastics. Plasticizers can solve most of the problems that occur during processing or in final use. New characteristics of PLA observed during plasticization may also pave the way to novel applications. The limitations of the currently studied PLA plasticizers include leaching during use, lack of thermal stability, need for offering more ductility and more performance, biocompatibility issues, cost, need for high percentage loading to lower price of PLA, need for a bio-based plasticizer that reduces the overall carbon footprint, *etc.*

3.2. Polyhydroxyalkanoates (PHAs)

Microbial-produced PHAs are fully biodegradable bio-polyesters produced by a wide variety of microorganisms for internal carbon and energy storage as part of their survival mechanism.[68,69] PHAs, also known as poly(4-alkan-2-oxelanones) according to IUPAC naming, have attracted much attention recently as alternative polymeric materials that can be produced from renewable and biowaste resources. PHAs, with the general structure shown in Fig. 5, vary widely in their structure and properties (flexibility, crystallinity, melting temperature, *etc.*), depending on the producing microorganisms, the conditions of biosynthesis and the type of carbon source.[70] PHAs are piezoelectric, perfectly isotactic/optically active and biocompatible thermoplastic polyesters amenable to melt-processing into various final forms.[71–74] High molecular weight PHAs have attracted considerable attention as potential replacements for non-degradable commodity plastics (*e.g.* polyethylene and polypropylene), as well as biodegradable and biocompatible biomaterials for implant purposes.[72,75]

Poly(3-hydroxybutyrate) (PHB), R = methyl, being the first among the isolated PHAs, is the most extensively studied PHA produced in nature in the presence of excess carbon by bacteria as storage granules providing food, energy and reducing power.[76,77] This polymer and its copolymer with polyhydroxyvalerate to make poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) are at present the only known PHAs relevant for practical applications.[78] PHB and PHBV are completely biodegradable in the environment and can be extruded, moulded and spun on conventional plastic processing equipment.[71,79] These features make these polymers an ideal candidate for the production of biodegradable packaging materials and other disposable articles.[79] However, the industrial scale production of PHB/PHBV is hindered by roadblocks. Thermal processing is challenging because of their relative low decomposition temperatures near their melting points, pronounced brittleness, very low deformability and susceptibility to a rapid thermal degradation.[78,80] Furthermore, the current cost of production of PHB is high compared to other high-volume synthetic plastics.[70,78] Because of its limited thermal stability, the melt flow index changes rapidly with time

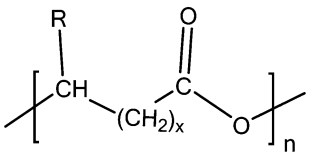

Fig. 5 General structure of PHAs. n = 1 (R = hydrogen, poly(3-hydroxypropionate); R = methyl, poly(3-hydroxybutyrate); R = ethyl, poly(3-hydroxyvalerate); R = propyl, poly(3-hydroxyhexanoate); R = pentyl, poly(3-hydroxyoctanoate); R = nonyl, poly(3-hydroxydodecanoate)), n = 2 (R = hydrogen, poly(4-hydroxybutyrate)), and n = 3 (R = hydrogen, poly(5-hydroxyvalerate)).

and its volatile decomposition products need to be handled safely. PHB's slow crystallization rates also lead to tacky products (*e.g.* fibres, films, *etc.*).[70] Its copolymer with valerate (PHBV) has overall better properties, especially regarding improved toughness with an acceptable loss of strength and modulus.[73] However, the present large-scale production cost of PHBV remains higher than that of PHB.[78]

The toughness and processability of PHB can be improved by incorporation of the hydroxyvalerate (HV) monomers in the bacterial fermentation process.[81] While PHBV with a high HV content has high flexibility, low crystallinity, and low crystallization rate, it compromises the yield strength and Young's modulus of PHB, which can result in rubbery materials, meanwhile, it increases the cost of materials.[81] Various approaches, such as use of nucleating agents, plasticizers and agents that promote crystallization of the polymer, modification of the polymer structure, blending, *etc.*, have been carried out to overcome the processing and product difficulties and other shortcomings.[70,73,78,82]

3.2.1. Plasticizers for PHAs. The use of monomeric and polymeric plasticizers of PHA lowers the glass transition temperature and the melting points, allowing processing at lower temperature and avoiding thermal degradation.[79,82] In addition, plasticizers improve both toughness and softness of the polymer by decreasing its crystallinity, weakening the intramacromolecular bonding and facilitating conformational changes.[83] The plasticization of PHBV using soybean oil, epoxidized soybean oil, epoxidized linseed oil, dibutyl phthalate, polyester plasticizer (Lapol 108), triethyl citrate, acetyl tributyl citrate and polyethylene glycol has been described in the literature[82–88] as shown in Table 2.

It is observed from Table 2 that the plasticizers induced depression of glass transition temperature and improvement in the elongation at break with all the plasticizing additives used, with the exception of triglyceride soy oil. From Park and Choi's study,[83] triethyl citrate was the most effective plasticizer in terms of reduction of the glass transition temperature as well as in terms of improvement of the impact strength and elongation. The difference in the effectiveness of these plasticizers can be attributed to the variation in the combined effect of chemical structure, molecular weight compatibility or solubility of the plasticizer with the polymer.[83] On the other hand, studies of impact strength and elongation properties by Seydibeyoglu *et al.*[88] showed that functionalized oils such as epoxy soyate are much more effective than triglycerides of epoxidized soybean or linseed oil. This might be due to the better reactivity of epoxy soyate than the counterpart triglycerides owing to its lower molecular size and simple molecular structure.

The use of low molecular weight, biodegradable and non-toxic compounds as plasticizing additives such as dibutyl sebacate (DBS), dioctyl sebacate (DOS), polyethylene glycol (PEG), Lapro1503 (L503), Lapro15003 (L5003), and a nonpolar polymer polyisobutylene (PIB) with concentration up to 50 wt% was investigated to improve the deformative characteristics of PHB.[89] These plasticizers were completely compatible with the polymer and formed a monophase system in mixtures of up to 15–20 wt%. Conversely, when the concentration was beyond 20 wt% the system becomes considerably weak, because of overloading. The majority of the plasticizers examined by the author cause a considerable decrease in crystallization temperature and improvement of mechanical properties. Other plasticizers reported in the literature include dodecanol, lauric acid, tributyrin, and trilaurin.[90]

3.2.2. Other methods of toughening. Blending of PHB or PHBV-based materials with polymers such as poly(butylene succinate),[81] poly(ethylene succinate),[81,91] polyethylene,[92] polypropylene,[92] poly(ε-caprolactone),[93] poly(lactic acid),[93,94] *etc.* has been extensively examined, and improvements of mechanical and thermal properties were reported. However, in most of these studies poor interfacial adhesion and phase separation of the PHB (V) and the other polymer blends was reported. These limitations could be improved through the use of a compatibilizing agent, functionalization, chain extension, controlled chemical crosslinking and optimizing the process conditions.

Ma *et al.*[81] used a free radical initiator (dicumyl peroxide) to induce compatibilization and partial crosslinking between PHB and PBS. The resulting compatibilized blends were shown to have a smaller particle size, improved interfacial adhesion and consequently resulted in improved tensile strength, impact toughness and elongation at break. Sadi *et al.*[95] evaluated the compatibilization efficiency of polypropylene/PHB blends with copolymers such as poly(propylene-*g*-maleic anhydride), poly(ethylene-*co*-methyl acrylate), poly(ethylene-*co*-glycidyl methacrylate), and poly(ethylene-*co*-methyl-acrylate-*co*-glycidyl methacrylate). Their study showed that poly(propylene-*g*-maleic anhydride), having the strongest adhesion between the phases, was the most efficient in terms of improving the mechanical performance of the blend.

Table 2 Effect of plasticizing modifiers on the glass transition temperature and mechanical properties of PHBV

Sample	Modifier concentration (wt%)	M_W (g mol^{-1})	T_g (°C)	ε (%)	TS (MPa)
PHBV[83]	100	680 000	−6.6	6	43.1
Soy oil[83]	20	814.3	−3.4	3	33.7
Epoxidized soy oil[83]	20	874.2	−19.0	7.2	22.1
Dibutyl phthalate[83]	20	278.2	−28.5	10	11.7
Triethyl citrate[83]	20	276.1	−30.0	10	10.9
Epoxy soyate[88]	20	—	—	7.51	13.8
Soy oil[88]	10	—	—	5.09	18.7
Epoxidized linseed oil[88]	10	—	—	7.46	19.6

Optimization of the processing conditions, taking the relationship among structure, composition, and polymer properties into account, is of particular importance as well.[96] High shearing rates during process operations such as extrusion (10–100 000 S^{-1}) and injection molding (1000–100 000 S^{-1})[97] in addition to the heating applied during processing are expected to change the molecular weight and as a result its performance. For instance, Yamaguchi *et al.*[98] reported 25–30% molecular weight reduction of PHB through shearing of PHB at 180 °C, at a shear rate of 6.3 s^{-1} within 5 min interval in addition to an order-of-magnitude decrease in shear viscosity.

3.3. Thermoplastic starch (TPS)

Starch, a polysaccharide of granular structure, is one of the most attractive feedstock for the development of biodegradable polymers because it is relatively inexpensive, abundant and renewable. Starch plays an important role both in the development of the commercialized bio-based plastics[99] and in the bioethanol industry. The role of starch in the development of bio-based plastics could be in the development of (a) thermoplastic starch (TPS) plastics where TPS is obtained through direct modification of starch, (b) poly(lactic acid) where its feedstock (lactic acid) is originated from starch derived sugars fermentation and (c) PHAs where starch derived sugars are used as a carbon source for the microorganisms producing PHAs.

Starch is composed of two homopolymers of D-glucose: the linear (1,4)-linked α-D-glucan amylase, typically constituting about 30% of starch depending on the source of starch, and a highly branched (1,6)-linked α-D-glucan amylopectin (Fig. 6). Commonly, amylopectin takes part in the formation of a crystalline structure and amylose does not.[100] Virgin starch is brittle and difficult to process into articles due to its relatively high glass transition and melting temperatures. The T_g of virgin dried starch is estimated to be approximately 240 °C,[101] which is above the starting point of its thermal degradation (about 220 °C).[102] High T_g and brittleness of starch are mainly caused by the presence of strong inter- and intra-molecular hydrogen bonds between the starch macromolecules.[101] Furthermore, TPS polymers based solely on starch are extremely water sensitive[103] and can suffer from significant molecular weight change during processing (extrusion or injection molding).[104] These drawbacks limit the possible shapes that can be imparted to the materials into films with adequate mechanical properties[105] and thus of limited practical value. Therefore, starch must be modified to breakdown the crystalline granules, decrease the T_g and melting temperature (T_m) either by incorporating plasticizers,[106] blending with other polymers,[51,52] chemical modification or combinations before they can be processed into plastics.[107]

3.3.1. Plasticizers for thermoplastic starch. During the thermoplastic processing of starch, typically between 70 and 90 °C in the presence of a plasticizer (*e.g.* water), a semicrystalline granule of starch is transformed into a homogeneous material with hydrogen bond cleavage between starch molecules. This process, called gelatinization, leads to loss of crystallinity[106] and is associated with the loss of double helices as well as with the loss of lamellar and long range crystalline structure.[108] Plasticizers penetrate starch granules and destroy the inner hydrogen bonds of starch, and eliminate starch–starch interactions because they are replaced by starch–plasticizer interactions. The plasticized moldable thermoplastic material, called TPS, is fit for injection molding, extrusion or blow molding similar to other synthetic thermoplastic polymers.[109]

There are several substances used as plasticizers for the preparation of thermoplastic starch. Some of the most studied and reported TPS plasticizers in the literature include polyols such as glycerol, glycol, sorbitol, xylitol, maltitol, ethylene glycol, propylene glycol, butanediol;[107,110,111] sucrose, fructose, mannose,[112] fatty acids (such as myristate or palmitate),[113] *etc.* It is also necessary to note that water is a good plasticizer of starch. However, the use of water alone as a plasticizer is not preferable because the resulting product will be brittle when

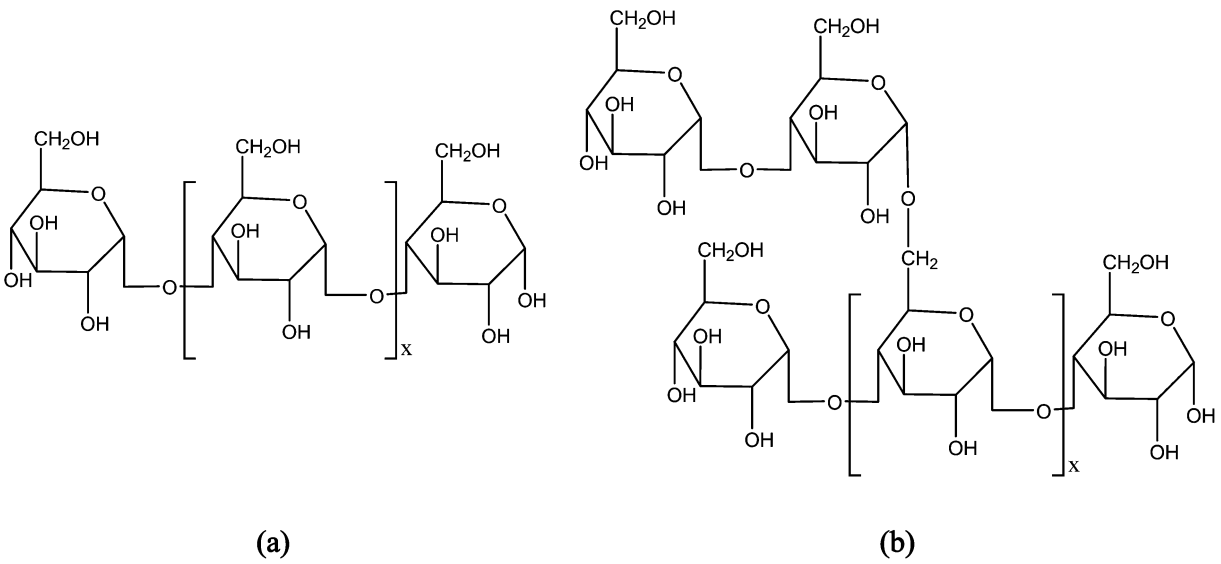

Fig. 6 Structure of starch polymers (a) amylose and (b) amylopectin.

equilibrated with ambient humidity[110] and due to volatilization of water. Glycerol, a classical plasticizer of starch, is perhaps the most widely studied and used polyol plasticizer of TPS. This is because of its low cost, nontoxicity (for food and biomedical application) and high boiling point (292 °C). Moreover, the hydrolysis and/or transesterification of lipids (triglycerides) into fatty acids for the biodiesel industry produce glycerol as a by-product. Utilizing such by-products provides glycerol with an additional market driver in addition to the opportunity of improving the economics of both the biodiesel and the bio-plastic industries. Nonetheless, glycerol is known to leach out during aging and humidity exposure, a major limitation for large scale applications.

The properties of plasticized starch can be tuned by changing the temperature of processing, water content and the properties and amount of plasticizers. For instance, Yu *et al.*[101] reported that the elongation at break of the thermoplastic starch is significantly improved by plasticization with glycol, glycerol and hexylene glycol. In addition, the thermal properties of plasticized starch are a function of water and plasticizer content.[110,114] The source of starch is also important for the property of TPS. This is because starches from various sources have different amylose/amylopectin ratios, molecular weights, molecular weight distributions and granular size crystallinity (Fig. 7). This as a result influences the gelatinization and glass transition temperatures[115,116] that are directly correlated with the thermoplasticity of the TPS. The effect of various plasticizers at different concentrations on the gelatinization temperatures, thermal stability, and glass transition temperature has been studied and reported in the literature. Some of the plasticizers and their effects are reviewed and shown in Table 3.

Abdorreza *et al.*[117] showed that the type and concentration of plasticizers govern the heat sealability as well as the seal strength of sago starch based films. The same authors showed that sorbitol-plasticized films exhibited significantly better heat sealability than did the glycerol type. However, the highest seal strength was obtained with a combination of sorbitol and glycerol. The effect of starch gelatinization in the presence of high molecular weight polyol plasticizers and water was also studied under static and dynamic conditions by Taghizadeh and Favis.[108] Their investigation showed that glycerol and sorbitol exhibited similar gelatinization temperatures, while an ascending T_g was observed from glycerol to diglycerol and polyglycerol attributed to the viscosity and molecular weight increase and hydroxyl bond density diminution of the latter two plasticizers.

Other plasticizers such as urea, formamide, combinations of urea and formaldehyde,[125] used with thermoplastic corn starch at different concentrations were also reported. Property evaluation by Ma *et al.*[125] showed that mixtures of urea (20 wt%) and formamide (10 wt%) plasticized TPS exhibited better thermal stability, water resistance and better mechanical properties than conventional glycerol plasticized TPS. According to Ma *et al.*,[125] the reasons behind such property improvement with the urea–formamide mix plasticizer could be due to the formation of more stable and stronger hydrogen bonds with the hydroxyl groups of starch molecules than with glycerol. Yang *et al.*[126,127] reported ethylenebisformamide, synthesized from methyl formate and ethylenediamine, as a novel and effective plasticizer of corn starch and potato starch. Ethylenebisformamide was shown to be effective in destroying the crystalline morphology of the native starch granule and conversion into a homogeneous phase TPS through plasticization and extrusion under shear and pressure. The morphology of the native crystalline starch and the homogeneous plasticized starch at 25% and 30% ethylenebisformamide loading was studied by scanning electron microscopy (SEM)[126,127] and shown in Fig. 7.

The SEM study (Fig. 7) clearly showed that the action of ethylenebisformamide and temperature processing (in this case extrusion) resulted in destruction of the crystalline native starch granules (Fig. 7a and d) morphology to form a continuous phase of TPS having a different crystallinity as further confirmed by X-ray diffraction crystallography.[126,127] The effect of plasticizer

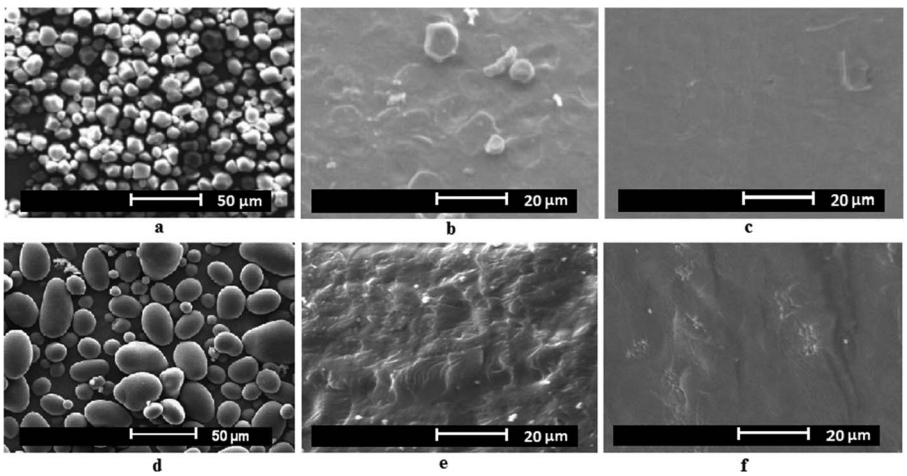

Fig. 7 Scanning electron microscopy micrograph of (a) native corn starch granules, (b) ethylenebisformamide (25%) plasticized TPS, (c) ethylenebisformamide (30%) plasticized TPS (adapted from: ref. 126 John Wiley & Sons, copyright © 2006) and (d) native potato starch.

Table 3 Common plasticizers for some of the common starch sources

Starch source	Plasticizer	Plasticizer concentration (wt%)	Gelatinization onset (°C)	Gelatinization peak/ conclusion (°C)/T_g
Wheat starch in the presence of water[108]	Glycerol	65	74.7	91.5[a]
	Sorbitol	65	73	92[a]
	Diglycerol	65	90	115[a]
Sago starch[117]	Starch (control)	0	123.7	157.2[b]
	Glycerol	30, 40, 50	149, 152, 141	169, 175, 164[b]
	Sorbitol	30, 40, 50	124, 126, 122	158, 151, 155[b]
	Sorbitol : glycerol (1 : 1)	30, 40, 50	120, 118, 142	150, 147, 176[b]
Corn starch[118,119]	Glycerol,[118,119] PLA,[120] poly(butylene adipate-*co*-terephthalate)[120]	—	—	—
Potato starch[111,121]	Glycerol–xylitol	40	—	−66.4[c]
	Glycerol–sorbitol	40	—	−69.3[c]
	Xylitol–sorbitol	40	—	−44.1[c]
Rice starch[122–124]	Glycerol	20, 25, 30, 35		
	Poly(ethylene glycol)	3, 6, 9		
	Sorbitol	30, 35, 40, 45		
	Others(formamide,[123] urea,[124] propylene and triethylene glycol[124])	10–30		

[a] Gelatinization conclusion. [b] Gelatinization peak. [c] T_g.

loading had also an effect on the continuity of the plasticized TPS phase. Higher concentrations resulted in more uniform phases for the studied loading range. Possible hydrogen bond formation between ethylenebisformamide and starch[126] during plasticization is shown in Fig. 8 below. The hydrogen bonds formed can be stronger than the intra and intermolecular bonds in starch, and as a result corn and potato starch were effectively plasticized with ethylenebisformamide.[126,127] In general, besides the plasticizer type, the quantity of plasticizer used and the processing method applied also affect the physical, thermal and mechanical properties of the resulting starch based bioplastics. For example, Flores *et al.*[128] studied and reported the effect of different gelatinization and drying techniques on the performance of glycerol plasticized starch films. The authors[128] finding shows that gelatinization and drying techniques used to obtain TPS films affected network characteristics that as a result determines the changes in physical properties potentially affecting the film performance as well.

In recent studies, the use of novel multifunctional ionic liquid plasticizers such as 1-allyl-3-methylimidazolium chloride[109,129] and 1-butyl-3-methyl imidazolium chloride as a plasticizer[130] and a compatibilizing agent[131] of starch has been reported. Ionic liquids, organic salts that are liquid at ambient temperature, are gaining interest because of their unique properties including non-volatility, non-flammability, low viscosity, chemical and electrochemical stability.[132] These liquids (examples of structures are shown in Fig. 9) have strong hydrogen bond forming abilities with starch owing to their high concentration of chloride ions. TPS plasticized using 1-butyl-3-methylimidazolium chloride shows less hygroscopicity and a much higher elongation at break in the rubbery state than the control glycerol-plasticized TPS samples.[130] The potential application of ionic liquids plasticized starch as solid biopolymer electrolytes was also reported by Wang *et al.*[129] This paves the way for a wide variety of potential applications of TPS bioplastics such as antistatic plastics, electronic shielding, biosensor, and environmentally sensitive membranes.

3.3.2. Other methods of improving performance of TPS. Chemical modification can also be an effective method of improving the processability and product performance of TPS. For example, hydroxylation,[133,134] acylation,[135] oxidation[136] and acetylation[136,137] of starch by substituting the ester or ether groups for the hydroxyl were reported to improve the processing behavior, hydrophobicity and mechanical properties. The synthesis of thermoplastic starch acetate with a high degree of substitution through acetylation of starch is one of the common

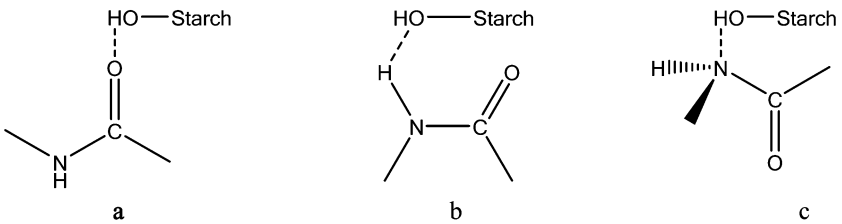

Fig. 8 Possible hydrogen bonds between ethylenebisformamide and starch.[126]

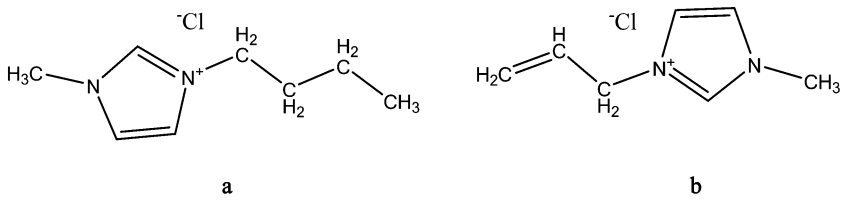

Fig. 9 Chemical structure of ionic liquids 1-allyl-3-methylimidazolium chloride and 1-butyl-3-methyl imidazolium chloride.

chemical modifications of starch.[137,138] The starch acetates were shown to have higher thermal stability and hydrophobicity due to the reduction of the hydroxyl group with the acetylation. Some of the chemical modifications may reduce biodegradability, biocompatibility and generate some toxic chemical by-product during synthesis.[139] On the other hand, some of the chemically modified starch (*e.g.* starch acetate) could be biocompatible and safe enough to be used in tissue engineering and other medical applications.[138]

Other toughening modifications recently reported in the literature to improve performance and overall economics of TPS based polymers include blending of TPS with protein,[131] PVA,[140] polycaprolactone, polyhydroxybutyrate, polymethacrylate, polystyrene mostly in the presence of urea and polyol plasticizers.[103,120] Surface modifications such as polymeric surface coating,[109] chemical and photo crosslinking of TPS and blends were also shown to reduce surface hydrophilic characteristics and improve water resistance, increase the tensile strength and Young's modulus while decreasing the elongation at break.[140–142] In summary, starch based plastics have grown to represent a major portion of the biodegradable polymer market. The commercial success of TPS polymers is hugely affected by the source, safety, quality, cost and functionality of plasticizers. Selective plasticization/toughening methods of TPS provide an attractive base for developing starch polymers that can be used as biodegradable and renewable packaging materials, environmentally sensitive membranes, and in biomedical and pharmaceutical applications such as drug and protein carriers, tissue engineering applications, *etc.*

3.4. Protein based plastics

Proteins are renewable, biodegradable and optically active natural[143] polymers produced by animals, plants and bacteria. Until recently proteins have been utilized exclusively in the food industries. Recent studies on non-food uses of agricultural feedstock initiated an interest in protein based plastics as well. Due to the continuous and cohesive matrix forming ability of proteins, various proteins of both plant and animal origin have received attention for the production of biodegradable plastics, edible films and sheets.[144,145] Furthermore, microencapsulating agents and active compounds in pharmaceutical applications,[146,147] adhesives, blend and composite materials,[142,148] wound dressing[149] and bionanocomposites[142,150] can be produced. Protein based biomaterials can also promote tissue regeneration, such as new bone growth,[149] integrate into blood clots and stimulate collagen deposition, and stimulate cells to produce new tissue, with no need for expensive growth factors.[151] Plant proteins that can be used for bio-based plastics include soy protein,[150] corn zein,[33] wheat protein,[152,153] cottonseed protein,[154] sunflower protein,[155] *etc.* Animal proteins such as blood meal,[156] gelatine and collagen,[157] keratin and feather quill,[158] egg protein,[159] whey protein,[155] meat and bone meal[160,161] can also be used as a feedstock of such bio-based plastics (Table 4).

Proteins are interesting biomaterials based on 20 amino acids which confers a wide range of functional and film-forming properties as a function of various extrinsic or intrinsic conditions such as plasticizer type and concentration.[162] The major drawback of protein-based plastics, with the notable exception of keratin, is their sensitivity towards relative humidity.[163] For example, Zheng *et al.*[163] reported that soy protein sheets submerged in water for 20 h absorbed up to 180% water. In addition, protein films and coatings are often quite stiff and brittle due to extensive intermolecular interactions between protein chains through hydrogen bonding, electrostatic forces, hydrophobic bonding and disulfide cross-linking.[34] Thus, thermoplastic processing of proteins into bio-based plastics is usually accompanied by plasticization and/or other form of modification for the successful development of useful proteinaceous biopolymers. Plasticizers can reduce the aforementioned chain-to-chain interaction and induce flexibility, moisture resistance and ease of processability.

Thermosetting protein plastics processing, on the other hand, occurs through chemical crosslinking that involves the formation of covalent bond bridges between protein chains by using a crosslinking agent. The crosslinkers chiefly target the reaction between themselves and protein functional groups – such as primary amines, carboxyl, hydroxyl, and sulfhydryls – of amino acid residues to provide mechanical strength and moisture resistance.[160,161] Protein-polymer grafting is another method of producing a protein based biomaterial usually with complementary advantages of each component. Thermoplastic processing, which involves melting a polymer followed by shaping and cooling, is the most widely adopted method for the production of protein-based bioplastics.

3.4.1. Plasticizers of protein based plastics. Plasticizers of proteins are generally added to the protein matrix during thermoplastic processing such as extrusion or injection molding to improve processability, reduce brittleness and modify the properties of the final structure.[164] Plasticizers added to the protein resin or compound usually consist of low molecular weight, low volatility substances, which mainly compete for hydrogen bonding and electrostatic interactions

Table 4 Examples of plasticizers, animal and plant derived protein biomass, and the mechanical properties of the biopolymers made

Plasticizer	Protein studied	TS (MPa)	*E* (MPa)	ε (%)	Ref.
Urea (20%)	Blood meal	12.3	608	—	156
	Wheat gluten	—	—	—	165
Diethyl tartrate (30%)	Feather quill	19.2	1267.9	1.6	158
	Chicken fibers	19.0	907.9	3.3	166
Dibutyl tartrate (30%)	Corn zein	20	1325	—	167
Glycerol (30%)	Corn zein	19.3	620	—	167
	Wheat gluten	6.7	51	118	168
	Soy protein	13.8	250.5	177.5	169
	Feather quill	15.2	380.5	13.6	158
	Chicken feathers	15.7	332.3	8.5	166
	Peanut proteins	8.0	147.0	63.0	168
	Sunflower protein	8.5	—	140	170
Ethylene glycol (30%)	Chicken feathers	17.8	354.0	43.8	166
	Sunflower protein	8.7	—	23	170
	Feather quill	16.8	321	64.9	158
Propylene glycol (30%)	Chicken feathers	22.3	811.2	7.6	166
	Sunflower protein	7.2	—	63	170
	Feather quill	20.5	529.5	11.2	158
	Soy protein	4.5	108.4	8.5	169
Oleic acid (20%)	Gelatin	54	2500	2.9	171
Sorbitol (20%)	Gelatin	52	1997	4.4	171
Mannitol (20%)	Gelatin	57	2250	4.5	171

with the protein chains to produce swelling.[34] Table 4 below summarizes common plasticizers for different protein biomass sources that have been used in the thermoplastic processing of proteins in the literature.

Similar to most other bio-based plastics, the composition, size, and shape of plasticizers influence the mechanical, physical, thermal, moisture permeability and aging behavior of proteinaceous plastics.[158,166,170,171] Orliac *et al.*[170] demonstrated that sunflower protein isolate films plasticized with different polyalcohols, such as glycerol, ethylene glycol, propylene glycol, polyethylene glycols, and polypropylene glycols, exhibited high mechanical properties, and good moisture impermeability to the level that it can be used for agricultural mulching. Cao *et al.*[171] compared the plasticizing effect of polyethylene glycol (PEG) with different molecular weights (300, 400, 600, 800, 1500, 4000, 10 000, 20 000) on gelatin films. The result showed that PEG with lower molecular weight gave better plasticizing effect (higher elongation), lower water vapor permeability and better visual effect. An increase in molecular weight of PEG in contrast induced an increase in the tensile strength, elastic modulus and a decrease in the elongation of gelatin films. Polar groups (–OH) along plasticizer chains are believed to develop polymer–plasticizer hydrogen bonds replacing the polymer–polymer interactions in biopolymer films.[172] Thus, hydrogen bonding ability of PEGs was affected by factors such as the number of hydroxyl groups per mole, molecular size, solubility and polarity that will explain the observed variation. Recent studies by Ullah *et al.*[158,166] also demonstrated that the variation in hydrogen bonding interactions between plasticizers (glycerol, diethyl tartrate, propylene glycol and diethyl tartrate) and keratin from poultry feather quills and poultry feather fiber could be responsible for the variation in plasticization efficacy. The best mechanical properties, transparency, flowability, and processability were observed in the case of ethylene glycol plasticized keratin quill and keratin feather, conceivably because of the formation of strong hydrogen bonding between the ethylene glycol and quill keratin.

Proteins are hydrophilic materials and as such they need to be coupled with adequate plasticizers to reduce the water absorbance of the corresponding plastics. Therefore, extensive attempts to improve moisture barrier properties are being conducted.[173–175] The introduction of hydrophobic materials such as lipids, long chain fatty acids and waxes incorporated into protein films has shown promising results.[173,176,177] For instance, Sohail *et al.*[173] studied and reported the moisture barrier property improvement of protein biopolymers (casein films), as a result of wax incorporation in the film formation and surface wax coating. The wax application on moisture barrier properties was more efficient in wax-coated casein films than wax incorporated biopolymers. While both wax-coating and incorporation improved the flexibility of the films at the expense of tensile strength reduction, the wax incorporated polymers exhibited better flexibility than the coated ones. Pommet *et al.*[176] likewise reported an improvement in the water vapor permeability of gluten protein films with the use of saturated fatty acids with an even number of carbons from 6 to 18 (C6 : 0 : hexanoic acid, C8 : 0 : octanoic acid, C10 : 0 : decanoic acid, C12 : 0 : lauric acid, C14 : 0 : myristic acid, C16 : 0 : palmitic acid, C18 : 0 : stearic acid).

Shellhammer and Krochta[178] studied the effect of lipid type and amount on the plasticization of whey protein biopolymer using beeswax, candelilla wax, carnauba wax and a high melting fraction of anhydrous milk fat. According to the authors, an increase in lipid level decreased the strength of the biopolymers.

Among the studied lipids, candelilla wax incorporation provided the weakest films, followed by beeswax, milk fat, and carnauba wax. Furthermore, a positive correlation between water vapor permeability of the lipids and the lipid plasticized protein plastics explains the increment in water vapor permeability of some of the biopolymers. Fabra *et al.*[177] reported the formation of bilayer structures by saturated fatty acids in sodium caseinate film forming solution that led to water vapor permeability improvement. The self-association of saturated fatty acid molecules occurs to form bilayers of different sizes in the film forming dispersions, and these laminar structures grow and persist in the dried film. The crystal formations as a result greatly limit water vapor permeability and yield rigid nonflexible films that show opacity and low gloss. According to the same authors,[177] unsaturated fatty acids such as oleic acid did not form laminar structures due to the double bond while it provokes a synergic plasticizing effect with water that seriously increased the water vapor permeability and film flexibility at intermediate relative humidity levels.[179]

The synergetic effects of using mixed glycerol (polar) and oleic acid (amphiphilic) plasticizers on sodium caseinate[179] and zein protein biopolymers[180] were also reported recently. According to Ibragimo *et al.*,[181] the plasticization obtained by glycerol is structural (inter-packet) and that of oleic acid is molecular (intra-packet). These two different molecules with different plasticization mechanisms provide the possibility for their interaction during film formation. The combination of these two plasticizers in zein films exhibited synergy and as a result a change in tensile strength (highest at 3 : 1 ratio of oleic acid to glycerol), decrease in glass transition temperature and change in microscopic molecular structure were observed.[180]

Tummala *et al.*[182] reported the use of glycerol, sorbitol and their blend to plasticize and compatibilize soy-protein and polyester amide, and compared their influence on the performance of the resulting biopolymers. While sorbitol plasticized soy-polyester amide plastics were more rigid, with a higher tensile modulus and tensile strength and thermal stability, glycerol plasticized soy-polyester amide plastics had the highest impact strength. The blend of the two on the other hand provided an intermediate tensile strength and modulus. Other types of protein biopolymer modifications reported in the literature include blending of gelling agents such as agar, agargel, phytagel,[183] incorporation of nanoclays,[184] *etc.* A recent study by Kim and Netravali[183] demonstrated that the blending of gelling agents with soy protein significantly improved the mechanical, thermal stability resistance of the protein biopolymers. This is because of the possible formation of interpenetrating network (IPN) structures between the gelling agents and the protein with a high degree of intermolecular interactions.[183]

3.4.2. Other methods of improving performance of protein based plastics. The most commonly utilized techniques for polymer modifications besides plasticization are blending, grafting, crosslinking, and composite formation, which are all multicomponent systems.[185,186] While blending is the physical mixing of multiple polymers to obtain the requisite properties, grafting and crosslinking are among the major irreversible methods of chemically modifying polymer properties. Grafting is a method by which a monomer or a polymer is covalently attached onto another polymer chain. Crosslinking on the other hand is the joining of two or more molecules or molecular chains through a covalent bond by another monomer or polymer called crosslinking agent. Grafting of polymers to protein chains results in a new class of proteinaceous biomaterials comprising natural and synthetic building blocks that are important in diverse fields of application including drug delivery, biotechnology, nanotechnology and nanobiotechnology.[187,188] Materials produced as a result of covalent attachment of synthetic polymers to proteins have the potential to synergistically merge the advantages of proteins and synthetic polymers. The hybrid materials may keep the chemical structure, diverse functionalities, stability, solubility, biocompatibility and biocompostability of proteins while keeping the stability and processability of synthetic polymers.[189,190]

The graft polymerization of styrene on soy protein isolate,[191] 2-hydroxyethyl methacrylate on soy protein,[192] poly(ethylene glycol) on soy protein,[193] polycaprolactone on zein,[194] waterborne polyurethane on soy protein,[195] poly(ethylene oxide) diglycidyl ether on wheat protein,[196] methyl methacrylate, ethyl methacrylate and butyl methacrylate on camelina meal[197] has been widely reported. Wu *et al.*[194] reported that the grafting of polycaprolactone onto zein protein resulted in a dramatic flexibility improvement, while the strength remained constant. Moreover, the glass transition temperature and melting temperature were also shown to decrease due to the plasticizing effect of polycaprolactone on the protein. Kurniawan *et al.*[196] also showed that the chemical modification of wheat protein based biopolymers with poly(ethylene oxide) diglycidyl ether resulted in the formation of a different network structure of the biopolymer with an improved flexibility, and improved mechanical performance.

Chemical crosslinking modification of protein with various agents to improve the mechanical, thermal and moisture resistance of the resulting biopolymers is another technique that has been widely studied.[160,161,196,198,199] Chemical crosslinking of proteins usually depends on the availability of particular chemicals that are capable of reacting with the specific kinds of functional groups that exist in proteins. The most extensively used chemical crosslinking agents of proteins include aldehydes (formaldehyde, glutaraldehyde, glyoxal, benzaldehyde),[161,200,201] carbodiimide, maleic anhydride, hydroxysuccinimide, *etc.*[202–204] Most of the studies show that crosslinking improved the tensile strength, tensile modulus, and moisture and solvent resistance, while the flexibility is reduced.[160,161] In summary, protein-based plastics can be easily modified through plasticization; grafting or crosslinking due to the presence of several functional groups provides protein-based plastics great promise in a wide range of applications. Further research into plasticizer/modification technique selection that combines the characteristics of the different protein feedstock with performance is necessary if protein based plastics are to achieve their full commercial potential.

3.5. Cellulose acetate

Cellulose is an abundant, renewable, and biodegradable natural polymer that constitutes the skeletal part of plants. It is a

homogeneous polysaccharide formed by repeating connection of D-glucose building blocks (Fig. 10), with an average degree of polymerization of 1500 to 3000 depending on the source.[205] Industrial materials are being developed from cellulose and its derivative over a broad range of application because of its abundance, environmental and biocompatibility benefits, relatively low cost and ease of modification. Cellulose by itself is poorly soluble in common solvents and is not melt processable as it decomposes before it undergoes melt flow.[206] Nonetheless, the chemistry of cellulose opens the way to various chemical modifications, such as esterification and etherification give entry into a broad variety of products including coatings, base for photographic films, filters, pharmaceutics, fragrances, polymer additives, membranes and building materials.[207–209] The most industrially relevant and oldest biodegradable cellulose ester derivative is cellulose acetate.[208] Cellulose acetate and mixed cellulose esters, such as cellulose diacetate, cellulose triacetate, cellulose acetate propionate, and cellulose acetate butyrate, are all commercially available materials.[208] These thermoplastic materials are usually synthesized through esterification of cellulose,[210] where other substituent groups replace the hydroxyl groups of cellulose (Fig. 10). For instance, cellulose acetate is the product of esterification reaction between cellulose and acetic anhydride in the presence of sulfuric acid catalyst to form fully acetylated cellulose triacetate, followed by partial hydrolysis to remove acid catalyst and produce a degree of substitution in the polymer that yields the desired working properties.[211] Other methods of cellulose ester synthesis, such as transesterification of cellulose with vinyl esters under catalysis, are reported in the literature recently.[212]

Raw materials such as cotton, recycled paper, wood cellulose, and sugarcane are used in making cellulose ester biopolymers in powder form.[210] Cellulose ester powders combined with plasticizers and additives are extruded to produce various grades of commercial cellulosic plastics in pelletized form. Of great interest as potential biodegradable plastics are also long chain aliphatic acid esters of cellulose.[206,213] These cellulose esters are characterized by stiffness, moderate heat resistance, high moisture vapor transmission, grease resistance, clarity and appearance, and moderate impact resistance.[206] The presence of polar functional groups in the cellulose acetate chain offers an additional advantage of affinity to solvents including plasticizers and lithium ions for the development of polymer electrolytes.[214] Nevertheless, owing to the high viscosity and elevated glass transition temperature, cellulose acetate derivatives themselves are not processable as a thermoplastic.[209] In an effort to modify its properties and facilitate processing, cellulose acetate is modified through plasticization by various aliphatic and aromatic esters,[215,216] chemically modified through grafting onto the polysaccharide backbone, and modification by forming polymer blends.[208,216,217]

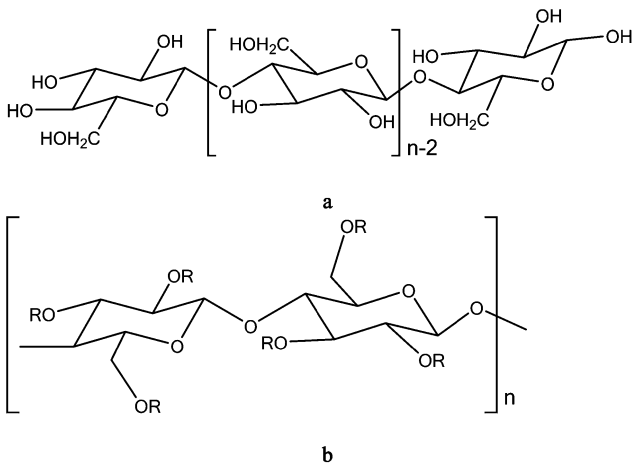

Fig. 10 Schematic representation of the molecular structure of (a) cellulose (*n*-degree of polymerization) and (b) cellulose ester (R-functional group for each type of cellulose ester).

3.5.1. Plasticizers for cellulose acetate. The thermal and rheological properties of polymers are crucial factors for thermoplastic processability of polymers. A broad processing window without thermal degradation of polymer is necessary to adjust the required rheological behavior of the polymers. Cellulose acetate is characterized by high glass transition and melting temperatures. As such the addition of processing aids such as plasticizers is required to improve its rheological behavior or thermal processability in the polymer melt. The most common plasticizers for cellulose acetate plastics reported in the literature include diethyl phthalate, dimethyl phthalate, triphenyl phosphate, ethylhexyl adipate, flexol, triacetin, glycerol triacetate, triethylene, glycol dipropionate and a wide variety of other plasticizers.[218–220]

Zepnik *et al.*[221] have recently studied the effect of plasticizer type and concentration on cellulose acetates using benzoate, acetates, phosphate and citrates based plasticizers. An increase in plasticizer concentration resulted in significant broadening of the thermoplastic processing window due to a strong decrease in glass transition temperature. It was thus possible to tune the rheology, melt strength and thermoplastic processing cellulose acetate by changing the plasticizer concentration. On the other hand molecular size, chemical structure, and solubility variation of plasticizers were shown to influence its compatibility, and ultimately the efficacy. It is generally agreed that plasticizers that have higher thermodynamic compatibility with the base polymer cause better plasticization than those with limited compatibility. The selection of an efficient plasticizer for cellulose esters was suggested by Fridman and Sorokina[219] who developed a set of criteria for efficient plasticization of cellulose acetate. An efficient plasticizer should take into account the compatibility of components, temperature durability and mechanical properties during processing and service time of the final polymer.

The efficiency of a plasticizer depends also on the loading concentration. Fig. 11 and 12 show the effect of one of the common cellulose acetate plasticizer (diethyl phthalate) concentration on the thermal and mechanical properties of cellulose acetate (drawn from tabulated data reported by Fridman and Sorokina[219]). As the plasticizer concentration increases, a reduction in glass transition temperature was observed and hence a significantly lower processing temperature is needed, while a substantial thermal stability drop

resulted in the cellulose ester plastic (Fig. 11). On the other hand, an increase in plasticizer concentration resulted in an increment of impact strength (Fig. 12) and elongation at break accompanied by a drop in tensile strength. Similar trend of cellulose acetate stiffness and toughness properties was observed by Mohanty *et al.*[210] upon increasing triethyl citrate plasticizer concentration. In summary, the type of the plasticizer and optimum plasticizer concentration are key parameters to reduce the processing temperature without compromising the stability and other performances of the plastic.

While cellulose acetate or its degradation products are safe, some of its common plasticizers such as phthalates, triacetin, glycerin, polyethylene glycol are associated with high toxicity, relatively high diffusion and water solubility.[222,223] For example, deterioration of cellulose acetate as a result of migration or evaporation of plasticizers, reaction of plasticizers with other chemicals in their surroundings to form other products has been documented in the literature.[218] As a result of such deterioration, not only unsafe plasticizers and plasticizer reaction products are released to the environment, but materials developed from cellulose ester became prone to cracking, warping, discoloration, exudation, shrinkage and powdering as they age.[224]

To mitigate these safety issues in addition to awareness of green technology and government legislations, several mitigation efforts are being conducted. These efforts include the development of safe, more stable and more compatible, bio-based, and functional plasticizers. Sugar based plasticizers, such as sorbitan,[216,225] polyoxyethylene sorbitan monopalmitate,[225] polyoxyethylene sorbitan monostearate,[216] are also reported. The use of an ionic liquid plasticizer synthesized from choline chloride and urea, such as deep eutectic solvent (DES), has been recently reported as a safe and novel plasticizer of cellulose acetate.[226] These plasticizers have the high solvating potential of crystalline cellulose acetate, and are less expensive, non-toxic and biodegradable in addition to their large electronegativity and delocalization of charge that enables them to positively influence ionic conductivity of cellulose ester.[226,227] Other ionic solvent cellulose ester plasticizers reported in the literature include 1-allyl-3-methylimidazolium chloride,[228] and other ionic liquids based on methylimidazolium and methylpyridinium cores with allyl-, ethyl-, or butyl-side chains.[229]

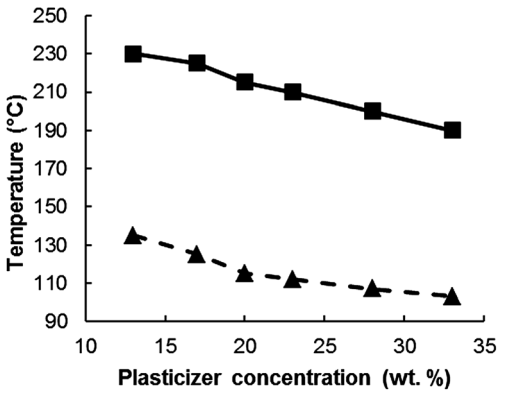

Fig. 11 Influence of diethyl phthalate concentration on T_g (▲) and processing temp (■) based on the data from Fridman and Sorokina.[219]

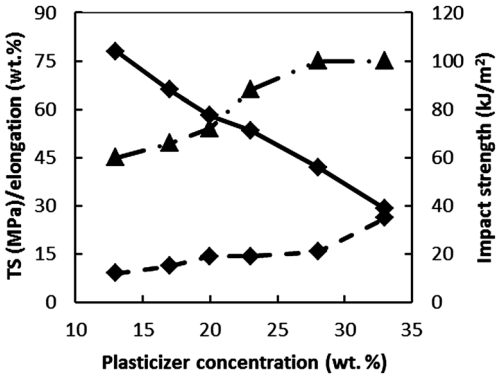

Fig. 12 Influence of diethyl phthalate concentration on impact strength (▲), elongation (■) and tensile strength (TS) (◆) based on the data from Fridman and Sorokina.[212]

3.5.2. Other techniques of improving performance of cellulose acetate. Chemical modification of cellulose acetate through grafting and crosslinking with other polymers is the other widely reported strategy for improving the performance of cellulose acetate.[208,217,230] Graft copolymerization of polymers generally offers an attractive and versatile means of imparting a variety of functional groups to a polymer.[185] Cellulose acetate grafting is a process aimed at the introduction of branches of synthetic polymers along the main polysaccharide chain to confer specific additional properties to the former without modifying its intrinsic characteristics.[231] Grafting offers the opportunity to combine the best properties of two or more polymers in one physical unit. Moreover, by varying parameters such as the degree of polymerization, polydispersities of the main chain and the side chains, graft density, and the distribution of the grafts, a more polymeric material with more valuable properties may result.[217] Grafting of cellulose acetate with other polymers such as poly(lactic acid),[232,233] poly(methyl methacrylate),[231,234] poly(hydroxybutyrate-*co*-valerate),[235] caprolactone,[32] polystyrene,[236] poly(ethylene glycol) and poly(hydroxybutyrate)[237] with new and improved performance features including better conductivity, thermal stability, and other marked advantages with better control and property tuning capability has been reported in the literature.

4. Concluding remarks

The rapid technological development of bio-based plastics, such as PLA, polyhydroxyalkanoates, (PHA), bio-based epoxy resin and bio-based PE, has yet to be translated into significant market impact, primarily due to high production cost and performance limitations. Plasticizers are important additives and performance enhancers of polymers. As such they can augment the processability of most of these bio-based plastics, and constitute a significant opportunity and at the same time barrier in the applications of bio-based plastics in various fields of applications. Moreover, the performance, safety, biodegradability, economics and functional utilization of bio-based plastics are strongly dependent on the performance of the incorporated plasticizers. This review highlights that selective

use of a plasticizer at an optimum concentration allows controlling the balance between the processability, strength, modulus, toughness and other important mechanical properties of plastics. Such a selection is usually conducted based on reasons for application of plasticizer, mechanism of plasticizer action, interaction and effect of the plasticizer on other additives besides the biopolymer, concentration range depending on the application.

In addition to the sanction of some common plasticizers (*e.g.* phthalates) for various applications, due to environmental and health concern as a result of migration and leaching during aging or use of plastics, newer concerns are emerging with regard to the effect of plasticizers in maintaining the renewability and biodegradability of bio-based plastics. The development and utilization of bio-based plasticizers such as polyols, fatty acids and fatty acid derivatives, epoxidized soy oil, ester amides, citrates and ionic liquids, such as methylimidazolium chloride and deep eutectic solvent are widely reported to tackle such issues. These new forms of plasticizers offer new dimensions of plasticizer selection that provide additional functionality (*e.g.* electric conductivity by ionic liquids) to the bio-based plastics and some of the others provide high safety to be used even in edible food packaging applications. While most of the fundamental mechanism, physico–chemical interaction or rule of thumb in the selection of a suitable plasticizer were established for the synthetic plastics (mainly for PVC), there are hardly no newer theories/mechanisms for the relatively new bio-based plastics. As a result, most of the current plasticization investigations are conducted under the assumption that the mechanisms developed for PVC would also be valid for the bio-based plastics. A fundamental understanding of the plasticization mechanism of bio-based materials is essential, along with their similarity and difference with PVC, if they are to reach their full potential and success.

Besides the usual purpose of improving flexibility and processability, research is progressing in areas such as the search and modification of plasticizers that impart additional functions of flame retardancy, optical quality, electric conductivity or insulation, thermoxidative stability, chemical and temperature (high and low) resistance in demanding environments; reactive plasticizers that provide chemical integrity, gas and moisture impermeability improvement, provide or improve biodegradability and biocompatibility to the polymers are under investigation. The migration of some of the current bio-based plasticizers through either volatilization or mass transfer to a liquid or solid in contact poses another challenge. These and the other challenges led to the search for newer types of plasticizers and alternative methods of improving the processability and overall performance. Some of the alternative methods to plasticization include molecular orientation, physical and reactive blending, chemical cross-linking and grafting of the bio-based polymers with other polymers to tailor the ultimate product properties.

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