Transglutaminase catalyzed amination of food protein and peptides with biogenic amines: studies on functionality and antioxidant capacity

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

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Abstract

There is a growing demand for innovative and affordable technologies in the food industry to modify ingredient functionality and to ensure product safety. Proteins are essential components in many food systems, and their structure can be modified in a variety of ways to control and improve functional characteristics of a food system. The enzymatic modification of proteins with transglutaminase is one such novel technology that has a broad range of applications, and was the focus of this research.

In the first study of this research, hydrolyzed pea protein were modified by microbial transglutaminase (MTGase) from the *Streptomyces mobaraensis* species. Biologically active (biogenic) amines histamine and tyramine were used as amine donor substrates in this enzyme-catalyzed reaction. Conjugation of biogenic amines and pea protein hydrolysates was achieved in the presence of MTGase after 6 h of incubation at 37°C. Conjugation progress was monitored by high performance liquid chromatography and fluorescence spectroscopy. Seventy six percent of histamine was covalently incorporated to protein hydrolysates by MTGase, thereby reduced the concentration of undesirable biogenic amine. Also, the MTGase-catalyzed introduction of tyramine into pea protein hydrolysates substantially improved the antioxidant potential of the newly formed conjugates.

In the second study, porcine skin gelatin was modified by MTGase-induced amination with tyramine in an aqueous media at 50°C. Gelation and melting

temperatures, viscoelastic behaviour, as well as gelation rate and gel strength of tyramine-gelatin conjugates and MTGase-cross-linked gelatin were determined. MTGase-catalyzed cross-linking increased the gelation and melting temperatures of the gel. Incorporation of tyramine via MTGase did not change the gelling and melting temperatures of the gelatin. Tyramine incorporation negatively affected the gelling behaviour of the gelatin and was likely due to covalent tyramine-gelatin bonds that interfered with the normal formation of triple helix networks.

Overall, this research demonstrated biogenic amines, as potential substrates for MTGase, were able to introduce into food proteins and peptides. A combination of glutamine-containing peptides together with MTGase has the potential to decontaminate fermented foods and beverages for biogenic amines.

Acknowledgements

I would like to express deep gratitude to my supervisor, Dr. Mirko Betti, for his constant guidance and full support throughout my study and research, without which this work would have been a frustrating and overwhelming pursuit.

I would like to thank Dr. Zeb Pietrasik for giving advice and guidance in the course of completing the experiments. His constructive inputs in order to improvise the thesis was valued greatly. In addition, I express my appreciation to the external examiner Dr. Hoon Sunwoo for willing to spend his precious time in evaluating this thesis.

My special thanks go to Dr. Yuliya Hrynets, who provided assistance and comments to help me.

I am also grateful to my colleagues and numerous friends for giving helpful advices throughout this academic exploration.

Finally, my immense gratitude goes to my family for the support they provided me through my entire life. Without their unconditional love and encouragement, I would not have been able to complete this thesis.

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List of Abbreviations

%RSA, Percentage of relative scavenging activity

A, Absorbance

ANOVA, Analysis of variance

Asp, Aspartic acid

BAs, Biogenic amines

BHT, Butylated hydroxytoluene

BVPC, Bitter vetch protein concentrate

Cys, Cysteine

DDGSs, Dried distillers grains with solubles

DH, Degree of hydrolysis

DNA, Deoxyribonucleic acid

DPPH, 2,2-diphenyl-1-picrylhydrazyl

FMP, Fish myofibrillar protein

G-L bonds, ε-(γ-glutamyl) lysine bonds

G', Storage modulus

G'', Loss modulus

GF, Gluten-free

Gln, Glutamine

Gly, Glycine

G_N, Gel strength

HHP, High hydrostatic pressure

HIS, Histamine

HPLC, High performance liquid chromatography

HyPro, Hydroxyproline

K_{gel}, Gelation rate

LVR, Linear viscoelastic region

Lys, Lysine

MTGase, Microbial transglutaminase

MW, Molecular weight

MWCO, Molecular weight cut-off

PPH, Pea protein hydrolysates

Pro, Proline

PSG, Porcine skin gelatin

RNA, Ribonucleic acid

TG2, Transglutaminase 2

TGase, Transglutaminase

T_{gel}, Gelation temperature

T_m, Melting temperature

TYR, Tyramine

Z-Gln-Gly, N-Benzyloxycarbonyl-L-Glutaminylglycine

Chapter 1: Literature review: Microbial transglutaminase and its applications in food processing

1.1 An overview on the transglutaminase enzyme

In recent decades, multi-disciplinary efforts have searched for unique products and processing methodologies that can alter the functional properties of food matrices. Proteins represent major components of food, and apart from nutritional value, provide various functionalities that affect food quality and consumer perception. Proteins can be tailored in chemical and enzymatic ways (Singh, 1991; Gerrard, 2002). Modified proteins with improved functional properties usually have a wider scope of use to satisfy new food trends and consumer expectations. Proteins with distinct properties can be created by chemically modifying specific site amino acid residues of original protein structures. These modifications include acylation, methylation, phosphorylation, succinylation, esterification and glycosylation (Lundblad, 2014; Boutureira and Bernardes, 2015). The utilization of chemical modification can be limited and unfavorable due to harmful by-products that may form during processing; indeed many of the chemical reagents used are toxic. Thus, a milder approach to produce protein cross linkages is certainly desirable. Accordingly, different enzymatic modifications have been proposed as alternatives. There are several procedures that employ enzymes such as protein partial hydrolysis, incorporation of functional groups to the side residues of the proteins, and the formation of covalently linked protein networks with versatile properties. These modifications

can be achieved under biological conditions by peptidases (EC 3.4.x), transglutaminases (EC 2.3.2.13), tyrosinases (EC 1.14.18.1), laccases (EC 1.10.3.2), and peroxidases (EC 1.11.1.x) (Buchert et al., 2010; Heck et al., 2013). The investigation of how enzymes can be used to modify the functional properties of food proteins is still growing. Currently there are two major enzymatic methods for the conjugation of proteins with a variety of natural polymers using the cross-linking activity of the enzymes tyrosinases and transglutaminases (Mariniello et al., 2014). Tyrosinases oxidize phenolic residues of protein tyrosines to quinones, which can further cross-link with other side chain residues of a different protein (Selinheimo et al., 2008). However, the application of tyrosinases in food applications is limited due to the color of oxidation products, limited accessibility of phenolic residues in certain globular protein structures and the chemical nature of catalyzed covalent bonds that are not yet fully characterized (Gerrard 2002; Mariniello et al., 2014). Until now the only commercially available food-grade protein crosslinking enzymes are transglutaminases.

Transglutaminase (TGase, protein-glutamine: amine-γ-glutamyltransferase; EC 2.3.2.13) belongs to the acyltransferases group, which catalyzes acyl-transfer reactions between a γ-carboxyamide group of peptide- or protein-bound glutamine residues (Gln, acyl donors) and a variety of primary amines or the ε-amino group of lysine residues (Lys, acyl acceptors) in proteins. This results in the formation of isopeptide bonds, like ε-(γ-glutamyl) lysine bonds (G-L bonds). One molecule of ammonia is generated for each cross-linking reaction. When

lysine residues or primary amines are not available from the reaction system, TGase can catalyze the deamidation of glutamine residues using water as an acyl acceptor. TGase possess glutamine substrate specificity, but is compatible with a wide range of acyl acceptor substrates (Buchert et al., 2010). Moreover, bonds formed by TGases are highly resistant to proteolytic degradation (Griffin et al., 2002). Transglutaminase-catalyzed reactions are shown in Figure 1.1. The reaction promoted by TGase has been reported with a variety of food proteins as substrates to improve their solubility, water-holding capacity, emulsifying, foaming, and gelling capacity in food matrices. Their commercial availability, reaction efficiency and versatility result in the increased use of TGases in meat, fish, dairy and cereal industries.

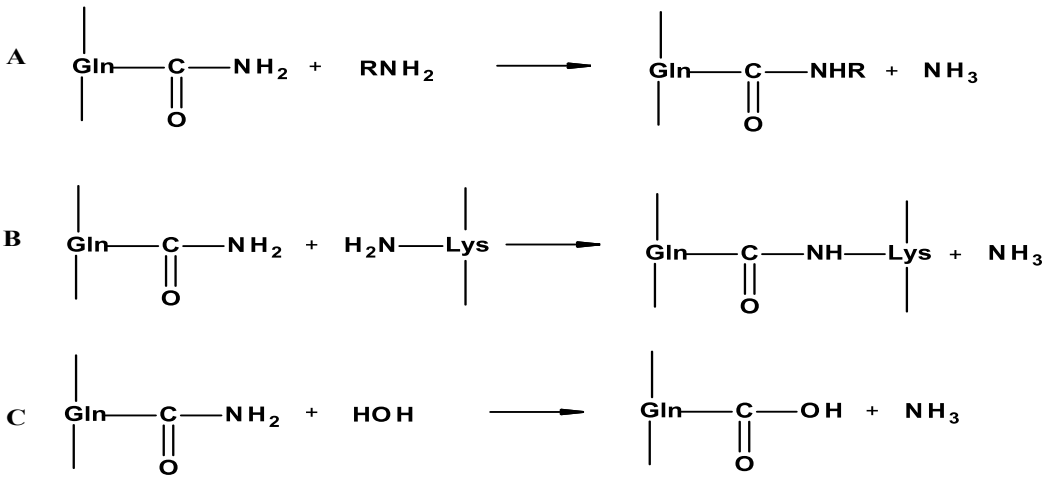


Figure 1.1: Transglutaminase-catalyzed reactions: (A) Incorporation of a variety of primary amines into proteins; (B) Cross-linking of proteins and (C) Deamidation. Adapted from *Applied Microbiology and Biotechnology,* "Properties and applications of microbial transglutaminase", 64(4), 2004, p.448, Yokoyama, K., Nio, N., and Kikuchi, Y, 2004. Copyright 2004 by Springer-Veriag. Adapted with permission.

1.2 Insight into transglutaminase of microbial origin

1.2.1 Different origins of TGase

TGases are widespread in most animal tissues and bodily fluids. Eight TGases have been identified so far; they are Factor XIII (fibrin stabilizing factor), tissue transglutaminase, prostate transglutaminase, epidermal transglutaminase, keratinocyte transglutaminase, TGM X, TGM Y, and TGM Z (Griffin et al., 2002). These mammalian enzymes can be found in almost all cell compartments such as the cytoplasm, mitochondria, recycling endosomes, and nucleus (Kanchan et al., 2015). They are characterized with several physiological and pathological roles including maintaining the extracellular matrix (ECM) integrity, regulating cell adhesion, modulating signal transduction and serving as protein scaffolds (Eckert et al., 2014). The first TGase now designated as transglutaminase 2 (TG2), was identified in guinea pig liver more than 50 years ago and was used to incorporate amines to proteins (Sarkar et al., 1957). Guinea pig liver was the sole source of commercial TGase for food industries until the end of the 1980s (Connellan et al., 1971; Brookhart et al., 1983). The rare source, extremely high cost, complicated separation and purification procedures for manufacturing tissue TGases prompted the search for new sources of this enzyme. Since then, TGases were identified in various mammalian tissues (Yasueda et al., 1994), fish (Ha and Iuchi, 2003), and in soy fodder beet and orchard apple plant tissues (Falcone et al., 1993). The separation and purification of this enzyme from these newer sources are still in their infancy and yet are applied in food processing on an

industrial scale (Zhu et al., 1995). A microbial transglutaminase (MTGase) was first isolated from the culture broth of *Streptomyces* S-8112 in 1989, later identified as a variant of *Streptomyces mobaraensis* (Motoki et al., 1989; Ando et al., 1989). Then efforts scaled up the production of MTGase for commercial applications. Ajinomoto Co. Inc produces *Streptomyces* TGase at an industrial scale and is the only transglutaminase product used for commercial applications so far (Macedo et al., 2011). MTGase is generally recognized as a safe (GRAS) for human ingestion (Kuraishi et al., 2001) by the U.S. Food and Drug Administration (FDA) since 1998. According to Directive 2000/13 EC of the European Parliament, MTGase is considered an adjuvant technology and does not need to be identified in the end product (Ajinomoto, 2013). Since this enzyme is not antigenic and is safe for food use its popularity as a useful tool in food processing is increasing.

1.2.2 Production of MTGase

The main approach to obtain industrially useful MTGases is using techniques of isolation and screening to identify TGase-producing strains from thousands of different microorganisms. Fermentation technology is used to manufacture the enzyme as purified, well-characterized preparations with high activity. *Streptomyces* MTGase is currently the only one commercially produced at a reasonable scale (Kirk et al., 2002). Naturally synthesized *Streptomyces* TGase is expressed as intracellular insoluble inclusion bodies or as inactive pro-MTG; these avoid uncontrolled cross-linking of cellular proteins, which are then

activated by several exogenous proteases, such as bovine trypsin, intestinal chymotrypsin through the removal of the *N*-terminal pro-peptide (Pasternack et al., 1998; Salis et al., 2015). In order to improve the production of *Streptomyces* TGase, researches have focused on optimizing the cultivation conditions and the nature of culture medium. Numerous studies have examined the expression of *Streptomyces* TGase using bacterial host microorganisms, such as *Escherichia coli* (Yokoyama et al., 2000) or *Corynebacterium glutamicum* (Kikuchi et al, 2003). Research regarding the expression of MTGase is systematically advancing; especially with *Escherichia coli* due to its ease of culture, short doubling time, and being the most suitable screening platform for directed evolution (Chen et al., 2013). Recent attempts to increase production efficiency have been made by synthesizing the active-form MTGase using *Streptomyces lividans* as a host (Noda et al., 2013). The composition of fermentation media is critical, especially the carbon and nitrogen sources as the two main components, since it influences the production, yield, and volumetric productivity of *Streptomyces* TGase (Guerra-Rodríguez and Vázquez, 2014). In the industrial biotechnology processes it is not economical to use culture media since the large amount of nutrients are required such as yeast extract and peptone, which are prohibitively expensive (Kieliszek and Misiewicz, 2014). Numerous publications have discussed using agricultural waste materials as medium sources for MTGase production. Rodríguez-Castillejos et al. (2014) studied the feasibility of the production of TGase by a selected *Streptomyces mobaraensis* strain grown in a medium based on enzymatic sorghum grain hydrolysates and dried distillers grains with solubles (DDGSs).

This crude medium was able to cultivate organisms that could produce a reasonable MTGase activity (0.66 U/mL). The cost of sorghum is relatively low since it is a livestock feed. DDGSs are generated as waste in the production of ethanol from corn. Although not as economical as sorghum and DDGSs, the greatest yield of MTGase production (3.2 U/mL) was obtained with a medium formulated using skim milk, potato, and glycerol (Guerra-Rodríguez and Vázquez, 2014). This formulation did not require the addition of supplemental nutrients such as yeast extract, peptone and sodium caseinate. The search for both productive and inexpensive alternatives for MTGase production media is a key to its future commercial viability.

1.2.3 Characteristics of MTGase

Some of the physico-chemical properties of purified MTGase, such as molecular weight, molecular structure, and enzymatic properties, have been documented. TGase from microbial origin has a molecular weight of approximately 38 kDa, which is much lower than that isolated from animal tissues (Ando et al., 1989). The primary structure of MTGase is a single polypeptide comprised of 331 amino acid residues, with only a single cysteine (Cys^{64}) residue in its overall sequence (Kanaji et al., 1993). Its secondary structure forms a compact internal domain with a deep cleft, containing a central 8-stranded β-sheet surrounded by 11 α-helices (Figure 1.2) (Kashiwagi et al., 2002). MTGase contains a Cys-His-Asp catalytic triad similar to human TG2 (Strop, 2014). Its nucleophilic attack on the substrate begins with deprotonation of the active site

Cys64 thiol group by nearby His274 side chain; these two residues are principally involved in the acyl-transfer mechanism reaction, where Asp255 plays a critical catalytic role in maintaining the structure orientation (Martins et al., 2014). The substrate specificity of MTGase is likely determined by the combination of primary sequence, secondary structure, its flexibility and accessibility to a specific glutamine (Strop, 2014). In contrast to mammalian TGase, MTGase's independence of Ca^{2+} makes it very attractive to the food industry, since many food proteins such as milk caseins, soybean globulins and myosins are susceptible to Ca^{2+} precipitation (Motoki and Seguro, 1998). Furthermore, its activation requires no special cofactors such as Cu^{2+}, Zn^{2+} and Pb^{2+}, which tend to bind the thiol group of the Cys residue. This explains why the enzyme has a broader substrate specificity and lower deamidation activity (Yokoyama et al., 2004). MTGase is tolerant to a broad range of reaction conditions. Its isoelectric point is approximately 8.9, and it acts in a wide range of pHs, from 5.0 to 8.0, with pH 6.0 to 7.0 being the most favourable for its catalytic activity (Ando et al. 1989). MTGase is active at temperatures between 0 and 70°C with the optimum temperature of 55°C, capable of maintaining its activity at 70°C temperature for 10 min. The enzyme is very susceptible to heat and rapidly loses activity within a few minutes above 70°C. At near-freezing temperatures MTGase maintains total enzyme activity (Yokoyama et al., 2004).

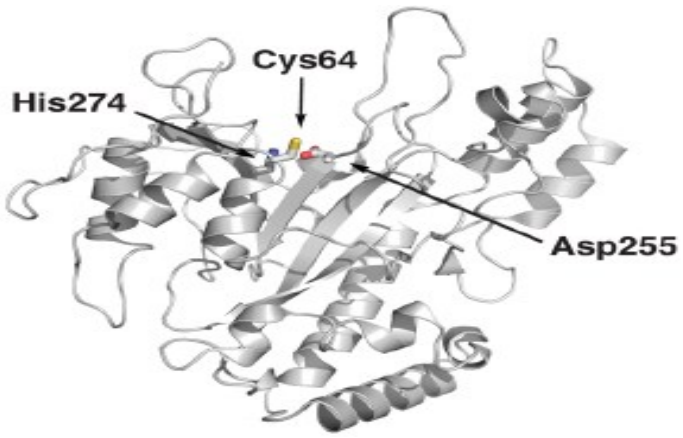


Figure 1.2: Mricrobial transglutaminase three-dimensional structure with the predicted catalytic triad (Cys64, His274, Asp255). Reprinted with permission from Strop P. (2014) "Versatility of microbial transglutaminase", *Bioconjugate Chemistry,* 25 (5), p. 856. Copyright 2014 by American chemical society.

MTGase achieves a site-specific protein modification at the level of one or a few Gln and Lys residues in protein substrates, resulting in the covalent incorporation of amino acids or peptides (Strop, 2014). This reaction could enhance the nutritional value of food proteins, since these additional amino acids or peptides would behave like endogenous amino acids and be liberated by the action of digestive enzymes in the normal mammalian gastrointestinal tract (Yokoyama et al., 2004). In food matrices, the formation of an isopeptide G-L bond stabilizes the protein network; it has a great resistance to mechanical stress and proteolytic degradation (DeJong and Koppelman, 2002). The MTGase-mediated formation of cross-linkages through amines of different lengths can result in high molecular weight polymers, causing extensive protein conformational changes (Serafini-Fracassini et al., 2009; Carvajal et al., 2011).

The rapidly increasing practical applications of MTGase give the food industry the ability to modify various functional properties of food products (Figure 1.3).

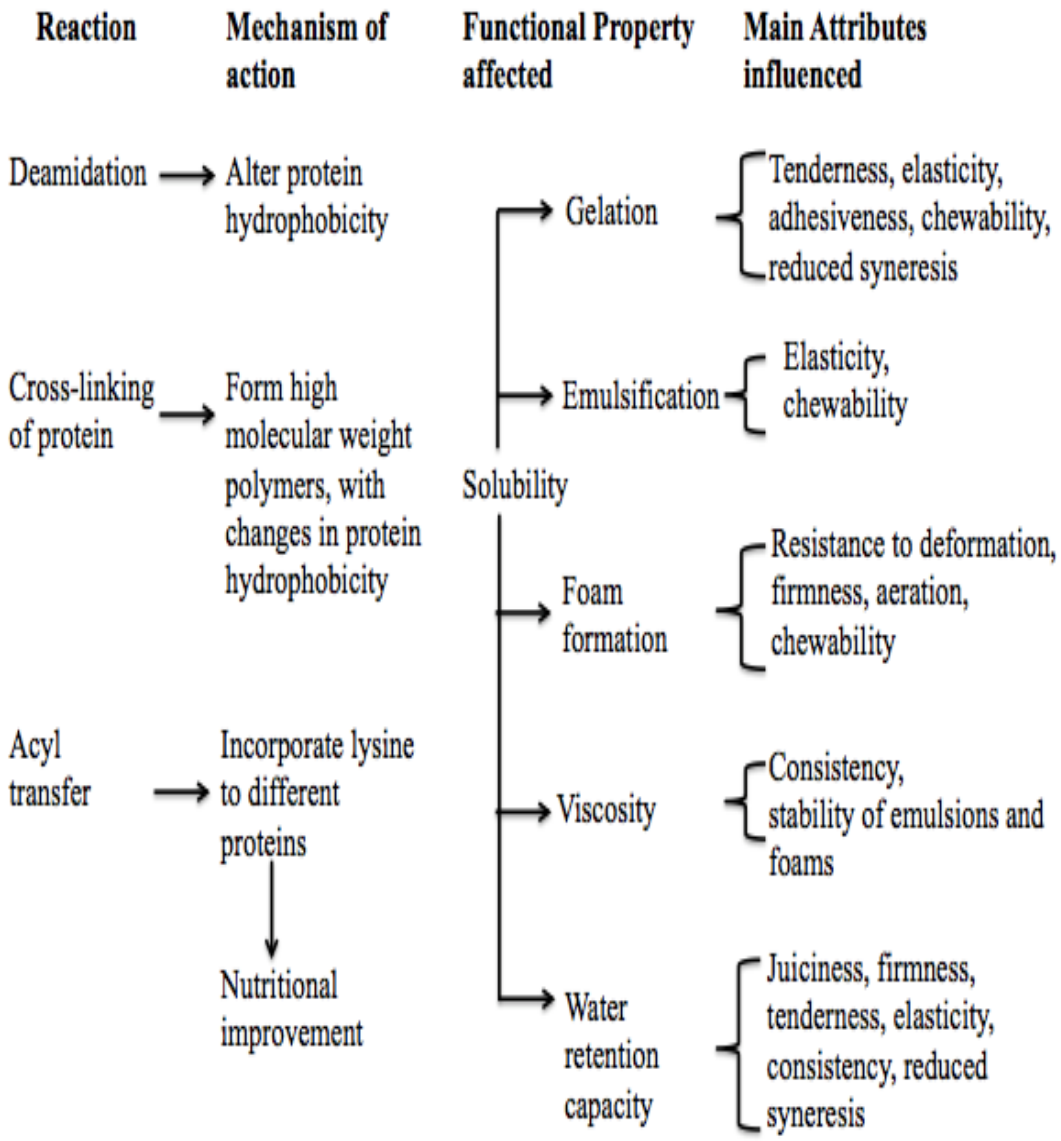


Figure 1.3: Practical use of protein cross-links induced via the action of transglutaminase. Adapted from *Food chemistry,* 171, Gaspar, A. L. C., and de Góes-Favoni, S. P., "Action of microbial transglutaminase (MTGase) in the modification of food proteins: A review", p. 318, 2015. Adapted with permission. Copyright 2014 by Elsevier Ltd.

1.3 Application of MTGase in food processing industry

1.3.1 Meat and fish products

The demand for high quality but low-priced meat production has motivated the industry to develop novel processing methodologies to utilize carcasses and low-value meat cuts of poorer quality. Restructured meats and various sausages make it possible to exhibit consumer-desirable texture and appearance, with the goal of increased retail product value (Maróstica and Pastore, 2010). The MTGase-aided manufacture of restructured meat is one of the main technologies used here, since the process acts as a binding agent. This maximizes the efficiency of carcass utilization by creating palatable meat pieces resembling intact muscle (Gerrard, 2002). Apart from a positive impact on the texture and consumer acceptance of the final products, the use of MTGase does not require the addition of salt, phosphates or thermal treatment, all traditionally used in restructured meats. This means MTGase can be used to satisfy consumers demand for healthier low sodium products (Motoki and Kumazawa, 2000). de Ávila et al. (2012) investigated the effect of using MTGase on restructuring deboned dry ham. The authors reported the formation of a three-dimensional protein network in the pork cubes catalyzed by cross-linking reactions, and the bonds were stabilized further upon drying of MTGase-treated cured boneless pork shank. Canto et al. (2014) concluded that hardness, springiness and resistance increased in low sodium restructured caiman steaks containing MTGase without compromising sensory qualities.

Myofibrillar proteins are the key functional muscle proteins, and primarily contribute the main functional properties of muscle foods, including gelation, emulsification and water-binding capacity (Tornberg, 2005). Hong and Xiong (2012) demonstrated that MTGase increased solubility of pork myofibrillar proteins under various pH conditions, and obtained maximum solubility at pH of 3.0. The authors also noted an improved emulsion capacity and viscosity by increasing the pH due to a network formation via enzymatic action. A partial reaction of MTGase with myosin heavy chains alters structure of myosin by creating protein-protein cross-links; the newly formed protein mesh has fewer α-helices but an increased number of β-sheets (Ahhmed et al., 2009a). These modifications contribute to the formation of a strong gel network and play an important role in texture properties such as stiffness, elasticity, cohesion and adhesiveness. Ahhmed et al. (2009b) confirmed that the gel strength in both beef and chicken improved through the reaction of myosin with MTGase, and was significantly greater in beef.

Surimi is the concentrated myofibrillar protein obtained from mechanically deboned fish flesh. Industries strive to produce high quality surimi products with improved gel strength and whiteness, since the raw fish materials have a large amount of dark muscle containing significant levels of lipids and myoglobin (Chanarat and Benjakul, 2013). In an evaluation of the gelation properties of surimi from different fishes modified with MTGase (0.2-0.6 U/g), Chanarat and Benjakul (2012) observed an increase in the gel strength of threadfin bream fish. Here the natural actomyosin extracted from this type of fish had the greatest

amount of ε-amino group content and provided the reactive groups necessary for cross-linking.

Combining non-meat ingredients with meat products using MTGase can improve the product's functional properties to create new food products. Kudre and Benjakul (2013) concluded that the gel strength of sardube surimi was greatly improved by the addition of MTGase (0.6 U/g) in combination with bambara groundnut proteins (4.5 or 6% (w/w)). Here enzyme-catalyzed G-L bonds of this mixed gel were formed between muscle and groundnut proteins possessing an increased breaking force. Similar increases in gel strength were reported with heat-treated myofibrillar protein isolate/pea protein isolate mixtures (3:1) with inclusion of appropriate amounts of MTGase (Sun and Arntfield, 2012). Zhang et al. (2013) conducted experiments involving the addition of different starches from corn, potato and tapioca to MTGase-treated surimi-beef gel. They concluded that addition of potato starch yielded the greatest gel strength. Authors also observed that a mixed gel with MTGase as a binder had the least cooking loss.

1.3.2 Dairy products

Research has also been published exploiting the use of MTGase with milk proteins. Casein, the major protein in milk, is an excellent substrate for TGase due to its open chain structure making it accessible to the enzyme (Bönisch et al., 2006). Among the caseins in milk, κ-casein was the most susceptible to enzyme reaction in unheated milk, followed by β-casein and α-casein (Sharma et al., 2001). The greatest susceptibility in κ-casein is due to its content of Gln and Lys

residues and their location on the micelle surface making it more accessible to the enzyme (Traore and Meunier 1991). Globular whey proteins are much more difficult to undergo this cross-link reaction since they are stabilized by disulfide bridges (Bönisch et al., 2007). Dithiothreitol promotes partial denaturation of whey proteins by cleaving disulfide bonds, liberating Gln and Lys residues as substrates, making them more available to the enzyme (Lee et al, 2002). MTGase also can contribute to the quality of yogurt, which is a major product of the dairy industry. The appearance, sensory quality and consumer acceptability of yogurt are strongly determined by its physicochemical properties, particularly acidity, syneresis and viscosity. In a set-style yogurt, the basic idea behind in using MTGase is to improve its texture. Instead of adding extra dry protein powder or stabilizer, cross-linking milk proteins with MTGase may positively influence the functional properties of the final yogurt product, such as increasing the gel strength/firmness, preventing syneresis and contributing to a dry, stable, smooth surface. Jooyandeh et al. (2015) investigated the effects of MTGase treatment on the selected properties of reduced fat milk-based set-style yogurt. Compared to a control samples, pre-incubation with MTGase prior to fermentation decreased the extent of syneresis and improved the rheological properties of this low fat yogurt. MTGase treatment led to a considerable decrease in the serum separation on the yogurt's gel surface. This effect is supported by the results published by Lorenzen et al. (2002), who found serum separation decreased up to 20% with MTGase enzymatic addition. The excellent G-L cross-linking properties of casein via the action of MTGase gives rise to a strong and stable gel, which directly decreases

the syneresis phenomena (Anema et al., 2005). Furthermore, this increased gel strength influences other functional properties such as water-holding capacity and viscosity, due to the formation of the homogeneous microstructures with small pores (Han et al., 2009). According to Jooyandeh et al. (2015) and Ozer et al. (2007), MTGase treatment was still effective in the development of acidity in yogurt. Though enzymatic cross-linking led to a minor imbalance of the associative growth of the yogurt starter culture and delayed bacterial multiplication.

1.3.2.1 Effect of MTGase on quality characteristics of cheeses

The introduction of additional covalent cross-links by MTGase for low fat cheese production shows promise. Various studies report that the reduction of the fat content has problems. It often leads to various defects such as increased moisture content, decreased cheese yield, rise in curd acidity, poor cheese ripening indices and lower total volatile fatty acids compared with full-fat cheese (Fenelon and Guinee, 2000; Shehata et al., 2004; Sahan et al., 2008). An investigation carried out by Ahmed et al. (2015) to improve the textural profile and organoleptic properties of low fat Gouda cheese employed either a protein-based fat replacer or MTGase. They demonstrated an increased protein recovery and cheese yield after MTGase treatment. The investigation by Sayadi et al. (2013) on the manufacture of MTGase-treated low fat Iranian white cheese also supported the former results. The enzyme-catalyzed cross-linking of casein micelles limits the movement and the rearrangement of protein chains to form casein gels that entrap free proteins present in whey and decrease wheying-off.

This results in a denser network structure that ultimately yields a firmer cheese even though the moisture content of the cheese was greater (Lucey et al., 2003; Aaltonen et al., 2014). The organoleptic properties of MTGase-treated cheeses had improved body and texture and an increased water holding capacity (Ahmed et al., 2015).

1.3.2.2 Effect of MTGase-induced cross-linking on ice cream

Ice cream is a complex colloidal system mainly comprised of ice crystals, air bubbles, partially coalesced fat globules and aggregates (Goff, 2002). From a structural perspective, milk proteins emulsify the fat and contribute to the partial coalescence and fat structure formation, leading to enhanced aeration and foam stability (Vega and Goff, 2005). The formation of ice cream structure is hindered when the fat content is reduced, since it is a critical component conferring consistency on the product by affecting ice crystallization, hardness and melting rate (EI-Nagar et al., 2002). Appropriate amounts of MTGase can be applied efficiently in the production of ice creams as a partial replacement for fat. According to Rossa et al. (2012), MTGase-induced cross-links occur in higher molecular weight whey and casein proteins. With a decreased fat ice cream product, the enzyme-catalyzed polymerization can stabilize air bubbles and increase the product volume. The formation a more cohesive protein network led to both better melting resistance and improved pseudoplastic properties of partially fat replaced ice cream.

1.3.3 Cereal products

Gluten is a key ingredient found in grains such as wheat, barley and rye. Individuals with celiac disease must adhere to what is commonly referred to as a gluten-free (GF) diet due to their inappropriate immune response to gluten proteins. Foods sources allowed in a GF diet include rice, corn, sorghum quinoa, buckwheat, amaranth flours and by-products from these raw materials (Schuppan et al., 2005). However, the quality of a baking product is largely dependent on the superior viscoelastic properties of gluten in the dough. During dough mixing, gluten is an essential protein that can form covalent bonds (e.g., disulfide bonds) and non-covalent bonds (e.g., hydrophobic interactions). The viscoelastic network has a critical impact on volume, texture and flavor of the dough (Delcour et al., 2012). Flours from GF cereals result in a batter rather than dough, since their proteins lack adequate viscoelastic properties and the necessary gas holding capacity during the baking process (Cauvain, 2015). In this respect, seeking better alternatives of gluten is still a technological challenge for the food industry. The TGase enzymatic technique can be applied as replacement to mimic gluten functionality. The first research on this hypothesis was provided by Gujral and Rosell (2004) on rice flour modified with 1% (w/w) MTGase. They showed progressive enhancement on the rheological properties of rice dough with an increased specific volume (2.75 mL/g) and a softer crumb quality. Renzetti et al. (2008) evaluated the use of MTGase in flours from six different GF cereals (brown rice, buckwheat, corn, oat, sorghum and teff) during bread-making. They concluded that brown rice and buckwheat flours were the optimal substrates for

the MTGase application. The rheology of batters and the resulting breads from buckwheat and rice flour improved due to the formation of large protein complexes catalyzed by the enzyme. This study indicated that addition of MTGase improves the elastic-like behavior of corn batters, but was not effective to obtain breads from oat, sorghum or teff. MTGase was also applied to improve the quality of GF-cookies using buckwheat flour formulated with rice and corn (Altındağ et al. 2015). Their study showed significant changes in the moisture level of cookies prepared with buckwheat (100%) and a buckwheat-corn (50%-50%) flour mix when MTGase was added. The moisture level was enhanced due to enzyme-catalyzed deamidation, resulting in increase in the glutamic acid residues content. Subsequently, the negative charges of these groups increased the water binding ability of the proteins. Buckwheat-rice flour with added MTGase had the greatest spread ratio, a characteristic desirable for better cookies quality. Addition of MTGase further led to improved textural properties and gave softer but more brittle cookies, measured by decreased hardness and increased fracturability values.

1.3.4 Edible protein films

Packaging is used to coat fresh and processed food products to reduce secondary contamination, maintain food quality and extend shelf life after processing and during storage. Hydrocolloidal biopolymers, such as proteins and polysaccharides, have attracted extensive interest to form edible films. They are relatively abundant and can form excellent films that are biodegradable and

renewable (Vargas et al., 2008; Pires et al., 2011). The unique abilities of MTGase make its applications in the creation of edible films feasible. Inherent problems of films that must be addressed include a low resistance to tension, susceptibility to mechanical damage, poor elasticity, and poor resilience of traditional natural polymers. Protein films can be used as a vehicle for the delivery of antimicrobial agents, antioxidants or functional ingredients such as probiotics, minerals and vitamins (Marquez et al., 2014; Chambi and Grosso, 2006). New protein materials represent a natural source for biofilms that has been explored and characterized in recent years, and were formerly regarded as waste products (Di Pierro et al., 2011). Myofibrillar and sarcoplasmic proteins from fish muscle by-products have been widely used to produce biofilms. Rostamzad et al. (2016) developed MTGase modified fish myofibrillar protein (FMP) film with a nanoclay. They concluded the biofilm had improved physical and mechanical properties when MTGase was added. The tensile strength of FMP films increased by about 62.3% by incorporating 3 wt% of MTGase. This was due to cross-linking in the polymer matrices which improved the rigidity of polymer molecules by creating a dense protein network with an increased molecular weight. The presence of 3 wt% MTGase resulted in significant decrease of water vapor permeability in these FMP films as compared to the untreated control. The new intra- and intermolecular covalent bonds catalyzed by MTGase likely created a continuous matrix with greater cross-linking density, resulting in decreased water solubility and increased water vapor barrier properties. A grain legume modified with MTGase was also used to produce edible films. Bitter vetch cultivated for

forage and seed is an inexpensive protein source found today in many countries around the world (Sadeghi et al., 2009). The possibility of using MTGase in the manufacture of bitter vetch protein concentrate (BVPC) edible films was investigated. Here the impact of enzyme treatment on morphological and functional properties of BVPC-based films was studied (Porta et al., 2015). MTGase containing BVPC films had a more compact microstructure when cross-links were formed among the molecules. This improved both the resistance and stiffness of the film. This film exhibited a 50-fold lower CO_2 permeability and 700-fold O_2 permeability compared to the non-MTGase treated film because the enzyme increased the molecular weight and cross-linking among the protein structure. Improved film mechanical features and gas barrier properties benefit the maintenance of food quality to maximize food shelf life and consumer appeal.

1.4 Introduction of biogenic amines

Biogenic amines (BAs) are basic nitrogenous compound formed by amination and transamination of aldehydes and ketones or by decarboxylation of amino acids (Askar and Treptow, 1986; Maijala et al., 1993). They are a group of organic bases with a low molecular weight. BAs are generally formed and degraded by the normal metabolism of plants and living organisms, but large amount of them are also produced in foods by microbial decarboxylation of amino acids (Ten Brink et al., 1990). The chemical natures of BAs include aliphatic (cadaverine, putresince, spermine, spermidine), aromatic (tyramine, phenylethylamine) and heterocyclic (histamine, tryptamine) structures (Figure 1.4). BAs formation results from the decarboxylation of free amino acids as

precursors by enzymes of bacterial origin. The prerequisites for BAs formation by microorganisms include the availability of corresponding free amino acids (Marklinder and Lönner, 1992), the presence of decarboxylase-positive microorganisms (Tiecco et al., 1986; Ten Brink et al., 1990; Hose et al., 1990), conditions that allow bacterial growth, and decarboxylase synthesis and decarboxylase activity (Brink et al., 1990). When all of these prerequisites are met, amino acid decarboxylation is able to take place and removes a carboxyl group to produce the corresponding amine. For example, lysine can be decarboxylased to cadaverine, while histidine can be converted by bacterial reaction into histamine. Spermine and spermidine are both formed by arginine, and tyramine, tryptamine, serotonin, and phenylethylamine arise by the same manner from tyrosine, tryptophane, hydroxytryptophan and phenylalanine, respectively. BAs are naturally present in wide variety of foods including vegetables, fruits, meat products, fish, dairy, beer, wine and their products. Furthermore, BAs can be expected in all foods that contain proteins or free amino acids, which are subjected to conditions enabling microbial activity, especially in fermented food products.

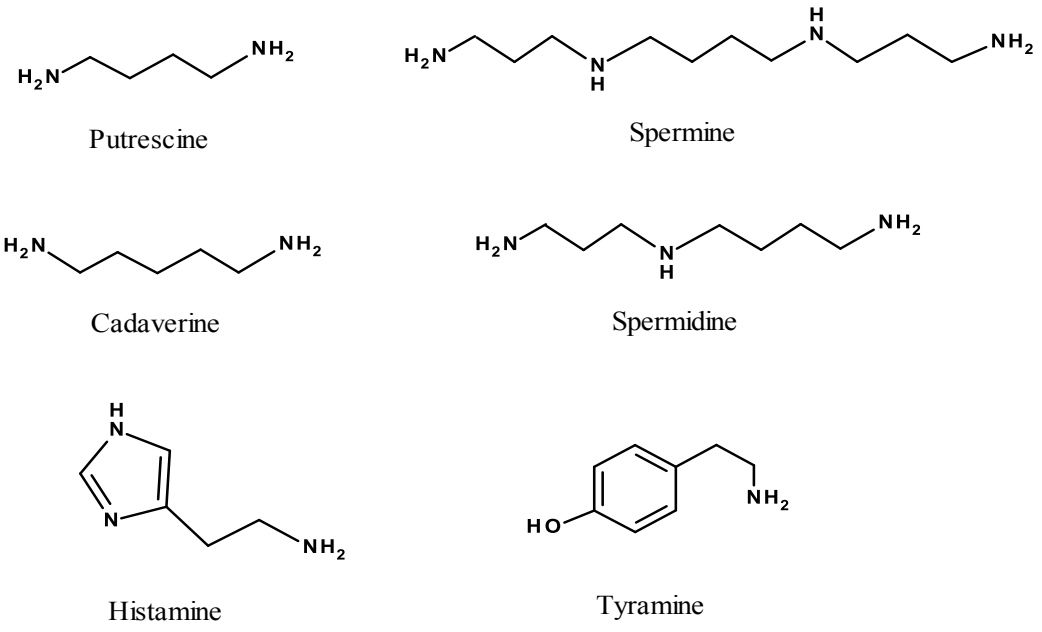


Figure 1.4: Biogenic amines structures.

BAs also play important roles in many human and animal physiological functions, such as the regulation of body temperature, the intake of nutritional factors, and blood pressure control (Ten Brink et al., 1990, Greif et al., 1999). Polyamines (putrescine, spermidine and spermine) are indispensable components of all living cells and are involved in signal transduction, and DNA, RNA, and protein synthesis. They are essential for maintaining high metabolic activity of the normal functioning and immunological system of the gut (Bardócz et al., 1993, Santos, 1996). The presence of some amines (catecholamine, indolamine and histamine) is related to vital metabolic functions in humans, especially to the nervous system and the control of blood pressure (Halász et al., 1994). Histamine serves as a primary mediator of the acute symptoms of an allergic response (Taylor, 1986; Stratton et al., 1991). BAs such as putrescine cadaverine and

spermindine have been reported to be free radical scavengers (Yen and Kao, 1993). Tyramine possesses remarkable antioxidant activity due to the presence of amino and hydroxyl groups (Halász et al., 1994).

BAs in low concentrations are essential for many physiological functions, whereas in greater amounts can have toxicological effects. Several symptoms can be nausea, sweating, headache, hypo or hypertension, cardiac palpitations, and renal intoxication (Maijala, 1994; Halász et al., 1994). Certain classes of amines, tyramine, tryptamine, and phenylethylamine cause vasoconstriction; in contrast, others (histamine and serotonin) possess a vasodilatador effect (Önal, 2007). Some BAs (agmatine, spermine and spermidine) are potential precursors for the formation of carcinogenic N-nitroso compounds by reacting with nitrites and produce a range of labile N-nitroso products (Kim et al., 2009). Histamine is connected to a worldwide problem known to be an inducer of so called "scombroid fish poisoning", characterized by difficulty in breathing, itching, rash, vomiting, fever, and hypotension (Naila et al., 2010). A large dietary intake of tyramine can initiate migraines and hypertensive crises (McCabe-Sellers et al., 2006). Low levels of BAs in food are not considered as a serious risk because they are normally metabolized by the natural detoxification system present in the gastro-intestinal tract of mammalians to physiologically less active degradation products (Bodmer et al., 1999). However, upon ingesting greater levels of BAs, the detoxification system is unable to digest these sufficiently and hence they can be toxic. The determination of the exact toxicity threshold of BAs in humans is

difficult because the toxic dose strongly dependents on the efficiency of the particular detoxification status of each individual (Halász et al., 1994).

1.5 Production of gelatin

Gelatin is a product obtained from the acid, alkaline, or enzymatic hydrolysis of collagen, and is the chief protein component of animal skin, bones, and connective tissue, including fish and poultry (Bogue, 1922). Collagen is mainly found in connective tissues and bones of vertebrate animals (Guzelian et al., 1980). Pure, dry commercial-grade gelatin is a brittle solid, faintly yellow in color and nearly tasteless and odourless. It is soluble in highly polar, hydrogen-bonding, organic solvents such as glycerol and propylene glycol, but is insoluble in less polar organic solvents such as benzene and acetone (Ward and Courts, 1977). Gelatin has a high molecular weight, and as a functional polymer is an important hydrocolloid. It is widely used in a range of products mainly due to its superior gelling and thickening properties (Mariod and Adam, 2013). From a nutritional perspective, unlike other popular hydrocolloids such as polysaccharides, gelatin is a digestible protein containing all essential amino acids except tryptophan (Yadav et al., 2015).

Native collagen molecules are composed of three α-chains intertwined in a collagen triple-helix. Glycine (Gly), proline (Pro) and hydroxyproline (HyPro) exist in large levels in collagen. The regular repetition of a Gly-X-Y unit forces the formation of a triple helix by intra- and inter-chain hydrogen bonding, where X is mostly Pro and Y is mostly HyPro (Asghar and Henrickson, 1982). Gly occupies the crowded center of the triple helix since it has a small side chain,

whereas Pro and Hypro point outwards and impart rigidity to the triple helix. Four to eight collagen molecules in cross-section constitute the basic unit of collagen fibrils, held together by covalent bonds. Cross-linking between collagen molecules reinforces and stabilizes the fibrils, and confers a structure typical of the strong rigid nature of skins, tendons and bones (Gómez-Guillén et al., 2011).

Gelatin can be made from many different sources of collagen, including cattle bones, hides, pigskins and fish, all which are principal commercial sources (Mariod and Adam, 2013). Pigskin is the most abundant source (Karim and Bhat, 2008); Figure 1.5 shows a representative production process for porcine skin gelatin (Wang and Shi, 2011). The insoluble native collagen must be pre-treated before converting it into a form suitable for extraction (Gómez-Guillén et al., 2011). A chemical pre-treatment disorganizes the protein structure to produce adequate swelling and solubilisation of gelatin achieved by breaking non-covalent bonds (Stainsby, 1987). Subsequent heat treatment destabilizes the triple-helix structure by cleaving the hydrogen and covalent bonds, giving rise to the helix-to-coil transition to solubilize the gelatin (Gómez-Guillén et al., 2002). Hence gelatin is a water-soluble substance, which involves the destruction of the tertiary, secondary, and to some extent the primary protein structures of collagens (Fernandez-Dıaz et al., 2001). The yield and quality of gelatin are determined not only by the species or tissue from which it is extracted, but also by the extraction process, as a function of pH, temperature and extraction time (Montero and Gómez- Guillén, 2000).

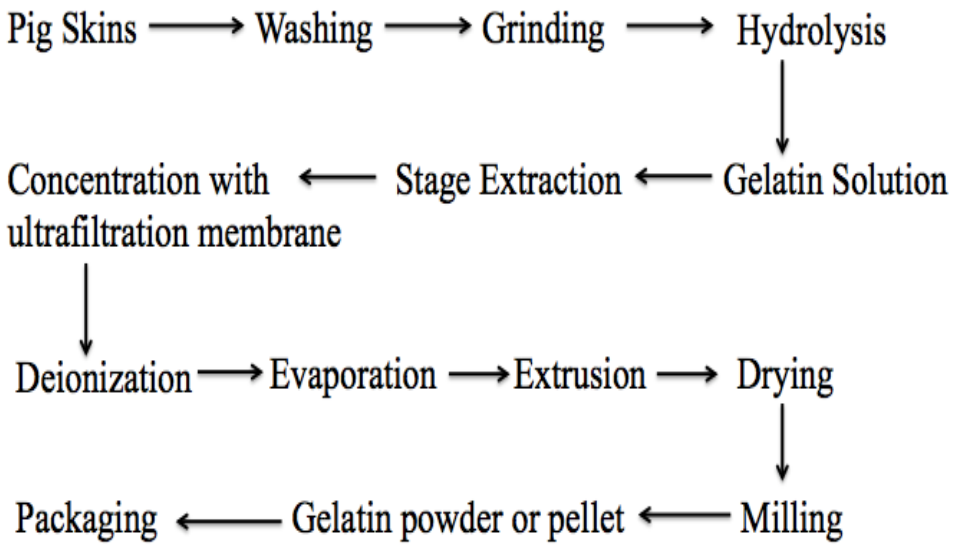


Figure 1.5: Porcine skin gelatin production. Adapted from *Food and Bioproducts Processing,* 89(3), Wang Y. and Shi, B., "Concentration of gelatin solution with polyethersulfone ultrafiltration membranes", p. 164. Adapted with permission. Copyright 2010 by the institution of Chemical Engineers.

Two types of gelatins are known commercially as type-A and type-B gelatin obtained under acidic and alkaline pre-treatment conditions, respectively. Type A gelatin has an isoelectric range of 7 to 9; here the acid processing results in the limited hydrolysis of the asparagine and glutamine amino acid side chains (Cole 2000). Type B gelatin comes from collagen subjected to a caustic soda or lengthy lime process prior to extraction, and has an isoelectric point of 4.7 to 5.4 (Keenan, 1994). Gelatin in solution is amphoteric; in acidic solutions it is mainly positively charged, whereas in alkaline solutions gelatin it is mainly negatively charged. The quality of good food-grade gelatin is determined largely by its functional properties such as gel strength, viscosity and thermal stability (gelation and melting temperatures). Gel strength and viscosity are the main properties used for

grading any gelatin determined by performing a well-defined protocol at a given gelatin concentration (6.67%) under careful standardized conditions (Simon et al., 2003). Gel strength and thermal stability are mainly dependent on the amino acid composition and the molecular weight distribution of gelatin. The proportion of the proline and hydroxyproline particularly correlates with its melting and gellation temperature (Gómez-Guillén et al., 2002). Gelatin has been used as a gelling agent in various food applications and its ability to form thermoreversible gels has been the central focus of investigations. Gelatin forms gels similar to those of polysaccharide hydrocolloids by forming micro-structural network. During cooling, gelation occurs by physical cross-linking which leads to the formation of a three-dimensional branched network (Gilsenan and Ross-Murphy, 2000). Hydrophobic, hydrogen, and electrostatic bonds in the binding of gelatin molecules can be disrupted upon heating and hence converts the gel to a solution as the temperature rises to about 30°C to 40°C. This imparts its special property of melting in the mouth (Morimura et al., 2002). Gelatin has excellent foaming and emulsification properties due to the amphiphilic nature of its molecules (Zandi, 2008). In addition, gelatin contributes to the viscosity of the continuous phase of an emulsion and is able to delay the flocculation and coalescence (Djagny et al., 2001). All these unique functional properties of gelatin make it one of the most widely applied additives in the food industry, especially in desserts, candies, bakery, meat and dairy products.

1.6 Transglutaminase and gelatin

Introducing a more extensive covalent cross-link network within or between gelatin chains is an interesting field of research. This can be expected to create substantial changes in the functional properties of gelatin. One possibility that is acceptable in food area is using transglutaminase to catalyze covalent bonds. Babin and Dickinson (2001) evaluated the affect of MTGase on acid-processed gelatin gels and concluded that enzyme treatment could result in either a negative or positive effect on the thermoreversility of gelatins. The resultant effect depended on whether or not the 'chemical' covalent cross-linking was predominant over the 'physical' triple helix network of gelatin. The study found that a cold-set gelatin gel remained un-melted on heating to 40°C for very high gelatin and enzyme concentrations due to the predominant hindrance effect of the covalent crosslinks within and between the chains. Norziah et al. (2009) reported that the treatment with MTGase could be a practical way to modify the gel properties of extracted fish gelatin, since the gel strength increased by MTGase. Cross-linking by TGase of gelatins and other food proteins, such as soy protein isolate, has also been studied. Zhang et al. (2013) evaluated the functional properties of such modified product and showed that TGase-treated products had improved rheological properties due to the cross-linking formed. However, these products exhibited an impaired emulsifying activity since crosslinking greatly increased the molecular weights of the modified protein structures.

1.7 Research hypotheses and objectives

A review of the literature regarding TGase enzyme indicated a potential application to conjugate BAs with food proteins or peptides. Large amounts of

BAs have been identified in various foods and their toxicological effect on humans has been documented. However, using an MTGase-aided reaction to reduce BAs has not been explored thoroughly. Heinrich Waelsch discovered animal TGase in 1957 together with collaborators Nirmal Sarkar and Donald Clarke. They speculated that TGase functioned possibly in histamine fixation. The first study which indicated such reaction indeed could occur and was performed with live mice by Ginsburg in 1963 (Ginsburg et al., 1963).

The covalent attachment of hisamine (HIS) and tyramine (TYR) into *N*-benzyloxycarbonyl-L-glutaminylglycine (Z-Gln-Gly) peptide via the action of MTGase was investigated previously (Tams et al., 2011). This study indicated that these BAs are good acyl acceptor substrates. Furthermore, the antioxidant activity of aromatic amine TYR to quench free radicals has been well documented (Yen and Hsieh, 1997). Therefore, it is proposed to target BAs by binding them covalently to food proteins or peptides by an enzyme-catalyzed reaction. There is limited information available regarding the preparation, identification and characterization of these amine-protein or amine-peptide conjugates. This research produced and identified MTGase-mediated protein/peptide-amine conjugates and assessed their effects on the functionality and antioxidant activity of these modified proteins and peptides.

The following hypotheses were tested:

- The conjugation of proteins or peptides with BAs in the presence of MTGase can reduce the BA content and thus decrease their toxicity;
- MTGase-catalyzed amination of protein/peptide with TYR increases the antioxidant activity by introducing phenol structures into the food system;
- MTGase-catalyzed amination improves the specific protein functional properties due to the hydrophobic features of BAs.

To prove the hypotheses the following specific objectives were addressed:

- To incorporate HIS or TYR into alcalase-hydrolyzed pea protein isolate (PPH) with the aid of MTGase;
- To confirm the formation of PPH-HIS and PPH-TYR conjugates;
- To assess the effect of conjugation of TYR on the antioxidant activity of modified protein hydrolysates;
- To investigate the effect of MTGase-catalyzed TYR-gelatin conjugation on viscoelastic properties of modified porcine skin gelatin.

[1]Chapter 2 (Study 1): Transglutaminase-catalyzed amination of pea protein peptides using biogenic amines and its effect on antioxidant activity [1]

2.1 Introduction

Biogenic amines (BAs), also known as biogenically active amines, are a group of low molecular weight organic bases that contain at least one primary amine group. They are mainly formed and degraded as a result of regular metabolic activity in animals, plants and microorganisms, and are usually produced through the decarboxylation of amino acids by corresponding amino acid decarboxylases (Shukla et al., 2011) or by amination and transamination of aldehydes and ketones (Santos, 1996). BAs can be found in a wide variety of foods, including fish, meat, cheese, vegetables, beers and wines. The most common types of BAs in food include heterocyclic histamine and tryptamine, aromatic tyramine and 2-phenylethylamine, and aliphatic cadaverine, spermine, spermidine, putrescine and tryptamine. Low levels of BAs in food are not considered a serious risk due to a gastro-intestinal detoxification system which is capable of metabolizing the normal dietary intake of BAs to physiologically less active degradation products (Stevanato et al., 2011). However, excessive amounts of BAs in food, especially in fermented food products, can cause health risks due to their pharmacological and toxicological effects. For instance, histamine (HIS) can induce so-called “scombroid fish poisoning”, the allergy-like food borne disease (Bulushi et al., 2009), while tyramine (TYR) is responsible for a "cheese

[1] *A version of this chapter has been submitted for publication in the Journal of the Science of Food and Agriculture.*

reaction", associated with a hypertensive crisis (McCabe-Sellers, 2006). Polyamines like putrescine and cadaverine, are considered potential precursors of carcinogens such as *N*-nitrosamines (Kim et al., 2009). To mitigate the negative impact of BAs and polyamines, technologies such as high temperature processing, high hydrostatic pressure (HHP), irradiation treatment, amine-negative starter cultures and the use of certain additives have been explored (Osborne and Bremer, 2000). However, the majority of these techniques proved not fully efficient. High levels of HIS were still found in thermally processed fish paste due to the heat stability of histamine. HHP reduced TYR, putrescine and cadaverine, but substantially increased the amount of spermidine in fermented sausages (Ruiz-Capillas et al., 2007). The use of additives including sodium sorbate, D-sorbitol and sodium nitrite can cause adverse effects due to lack of consumer acceptance (Naila et al., 2010). Thus, it is important to develop an inexpensive, food-compatible method that can reduce BAs.

Transglutaminases (TGase, EC 2.3.2.13) are a group of enzymes are widely used for cross-linking proteins in the food industry. TGase catalyzes the acyl-transfer between an acyl donor, like the γ-carboxamide group of peptide- or protein bound glutamine residues, and an acyl acceptor, including a variety of primary amines (Motoki and Seguro, 1998). This reaction modifies proteins either by forming intra- and intermolecular isopeptide bonds via ε-(γ-glutamyl-lysine) links or by the covalent attachment of amines, such as polyamine or putrescine (Colas et al. 1993). Microbial transglutaminase (MTGase) from *Streptomyces mobaraensis* is a Ca^{2+}-independent enzyme used in various food applications due

to its availability, ease of handling and stability over a wide range of pHs and temperatures (Zhu and Tramper, 2008). MTGsae has been exploited previously to covalently incorporate putrescine, spermine, and spermidine into glutaminyl residues in polypeptides (Williams-Ashman and Canellakis, 1979). Research has demonstrated that MTGase can fortify a protein source that is limited in essential amino acids (Ikura, 1985, Bercovici et al., 1987). Taking advantage of the non-specificity of the acyl acceptor, Gundersen et al. (2014) demonstrated that MTGase could be used to incorporate small amines (i.e. cadaverine) into glycine and the esterified threonine, serine, cysteine, and tryptophan. Furthermore, Tams et al. (2011) demonstrated that HIS and TYR could be covalently bound to a Z-Gln-Gly peptide using MTGase. Therefore, it is possible that MTGase could be used to covalently attach BAs to food proteins or peptides and thus mitigate the negative toxicological effects of exogenous BAs. BAs-protein and peptides conjugates could be strategically created possessing custom functionalities and bioactivities. For instance, the glutamine-containing peptides could be conjugated with TYR, since the TYR possesses radical scavenging activity due to its phenol structure (Yen and Hsieh, 1997). To the best of our knowledge, no study has reported an enzyme-aided reaction to control BA production. More specifically MTGase has not been used to conjugate BAs to a food protein hydrolysate, nor has any study evaluated the antioxidant capacities of the resulting amine-peptide conjugates.

In our study pea protein isolate was used since it contains 16.5% of glutamine and glutamic acid (Pownall et al., 2010). Pea protein isolate is a good

candidate to use as a substrate for MTGase to detoxify food from BAs. Heterocyclic HIS and aromatic TYR were chosen as amine donors candidates because they are the most common BAs occurring in food as well as for their different structural features. The aim of this work was: (1) to study the possibility of the covalent incorporation of HIS or TYR into hydrolyzed pea protein isolates with the aid of MTGase, and (2) to evaluate the antioxidant power of the subsequent amine-peptide conjugates. The covalent attachment of BAs to PPH was evaluated by liquid chromatography, and antioxidant capacities were tested against DPPH radical and by the ability to inhibit lipid oxidation in a linoleic acid model system.

2.2 Materials and Methods

2.2.1 Materials

Pea protein isolate was obtained from Leduc Food Processing Development Centre (Edmonton, AB, Canada). Alcalase (protease from *Bacillus lichenifromis*, activity 2.4 units/g of protein), Flavourzyme (protease from *Aspergilluus oryzae,* activity 500 units/g of protein) were obtained from Novo Nordisk (Bagsvaerd, Denmark). Trypsin from porcine pancrease (EC 3.4.21.4), histamine dihydrochloride (HIS, ≥ 98.0%), tyramine hydrochloride (TYR, ≥ 98.0%), dansyl chloride (5-(dimethylamino) naphtalene-1-sulfonyl chloride, ≥ 99.0%), ammonia (30%), DPPH (2,2-diphenyl-1-picrylhydrazyl), ammonium acetate (≥ 98.0%), linoleic acid, ammonium thiocyanate, ferrous chloride, butylated hydroxytoluene (BHT), acetonitrile and acetone were purchased from Sigma-Aldrich (St. Louis,

MO, USA). MTGase (Activa-TI, with an activity of 100 U/g, 1% MTGase blended in 99% maltodextrin) was purchased from Ajinomoto Food Ingredients (Eddyville, IA, USA). The crude enzyme's activity was tested by a transglutaminase assay kit (Sigma-Aldrich) and used without further purification. All other reagents were of analytical grade from Sigma-Aldrich or Fisher Scientific (Fair Lawn, NJ). Ultrapure water was obtained through a Millipore Milli-Q system (Milford, MA, USA).

2.2.2 Experimental design

Enzymatic hydrolysis of pea protein isolate was achieved with three different enzymes: alcalase, trypsin and Flavourzyme. Three independent batches were conducted for each enzyme and assays were performed in duplicate for each batch. Following this exploratory test, pea protein hydrolysate (PPH) produced using alcalase was selected for the preparation of PPH-BAs conjugates using MTGase. The hydrolysate was incubated with HIS in the presence of MTGase at 37°C for 6 h. Liquid chromatography with pre-column derivatization using dansyl chloride was performed to quantify the HIS left in the system after the enzymatic reaction, thus providing an indirect evaluation of the conjugation efficiency between PPH and HIS. Due to peptide interference during the chromatographic separation of PPH-TYR, the MTGase-induced conjugation of PPH with TYR was assessed measuring the changes in fluorescence emission. For this purpose, the PPH was incubated for 6 h with MTGase in the presence (+) or absence (-) of TYR (PPH -TYR (+) MTGase and PPH + MTGase, respectively) and the

fluorescence emission was then measured. Both untreated PPH and untreated TYR were included as controls. For fluorescence emission and antioxidant assays three independent trials were conducted, where in total 24 tubes were prepared (2 tubes × 4 treatments (samples + controls)) on three different occasions, resulting in six replicates for each treatment. All trials were prepared on different days and tubes were randomized within the incubator.

2.2.3 Preparation of pea protein hydrolysates (PPH)

Pea protein hydrolysates were prepared according to Humishi et al. (2007) and Khiari et al. (2014) with (A) alcalase, pH 8.5, 50°C; (B) Flavourzyme, pH 7.0, 50°C and (C) trypsin, pH 7.0, 37°C. The pH and temperature values selected corresponded to optimal conditions for the enzymes. The hydrolysis reactions were performed using a pH-stat titration system (Metrohm Titrando 842; Software Tiamo 1.3, Herisau, Switzerland) equipped with a bath circulator (Haake S7, Fisher Scientific, Newington, NH, USA). Five percent (w/v) pea protein isolate solutions were dispersed in deionized water, adjusted to pH 8.0 with 1 M NaOH, and stirred for 15 min at 50°C. The enzymatic digestion was initiated by the addition of enzymes to attain the enzyme to substrate ratio of 1:50 (w/w) for each enzyme tested. The pH was maintained at 8.0 during the hydrolysis period using the pH-stat titrator. The aliquots were collected at 3, 6, 12, 18 and 21 h intervals and heated at 80°C for 10 min in a water bath (Isotemp 2320, Fisher Scientific, Marietta, OH) to inactivate the enzymes. Subsequently, the aliquots were cooled to 25 ± 2°C and centrifuged (Avanti, Beckman Coulter Inc., Palo Alto, CA, USA)

at 10,000 × *g* for 20 min at 4°C. The hydrolysates were then filtered using Whatman No. 1 filter paper (Sigma-Aldrich). The clear permeate solution was collected and passed through an ultrafiltration cartridge of 10 kDa molecular weight (MW) cut-off membrane (Amicon Ultra, Millipore, Cork, Ireland). The ultrafiltration was performed using a peristaltic pump (Masterflex, Model 7518-00, Thermo Fisher Scientific, Martham, MA, USA) in a closed loop with silicone tubing (Masterflex, Cle-Parmer Instrument Co.). The resulting retentate containing peptides with MW < 10 kDa was collected, lyophilized and stored at -20°C for further analysis.

2.2.4 Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, was calculated from the amount of base (NaOH) necessary to add to keep the pH constant during hydrolysis (Adler-Nissen,1986):

$$DH\ \% = 100 \times B \times N_B \times (1/\alpha) \times (1/MP) \times (1/h_{tot})$$

where B is the base consumption (mL); N_B is the molarity of the base; α is the average degree of dissociation of α-NH_2 groups released during hydrolysis and expressed as $\alpha = 10^{pH\text{-}pK}(1+10^{pH\text{-}pK})$, where pH and p$K$ are the values at which proteolysis was conducted; M_p the mass of protein being hydrolyzed (g) and h_{tot} the total number of peptide bonds in the pea protein substrate (8.0 meq/g of protein).

2.2.5 MTGase amination of PPH with histamine (HIS)

Histamine (0.04 g) was dispersed in 100 mL of deionized water. PPH and

HIS in ratios of 1:1 (w/w) were mixed and pH was adjusted to 7.0. The samples were incubated (Innova 44 incubator shaker, New Brunswick Scientific, USA) at 37°C for 6 h with MTGase (10 unit/g PPH). At the end of incubation, samples were heated at 80°C for 10 min to inactivate MTGase's activity. Proof of PPH-HIS conjugation was evaluated using derivatization with dansyl chloride, followed by liquid chromatography-ultraviolet detection.

2.2.6 MTGase amination of PPH with tyramine (TYR)

PPH (0.4 g) were added to TYR at a weight ratio of 4:1 (w/w). Each of the weighed powders was dissolved in 40 mL of 0.1 M sodium phosphate buffer (pH 7.0). The aliquots obtained were incubated at 37°C for 6 h with MTGase. PPH treated with MTGase upon 6 h incubation, untreated PPH and untreated TYR were also prepared as controls to ensure that changes in peptide properties are due to the incorporation of TYR. After incubation each sample solution was passed through the ultrafiltration system, using membrane filter cartridge with a molecular weight cut-off (MWCO) of 10 kDa (Amicon Ultra centrifugal filters, Millipore, Cork, Ireland) to remove MTGase, followed ultrafiltration with a MWCO membrane of 650 Da to remove unreacted TYR. At the end of the ultrafiltration system, the retentate of each sample was collected, lyophilized and stored at -20°C for further analysis. Proof of PPH-TYR conjugation was evaluated determining the changes in fluorescence emission spectrum.

2.2.7 Proof MTGase-catalyzed conjugation of PPH with HIS or TYR

To evaluate the extent of MTGase-catalyzed amination of PPH, HIS concentrations were evaluated before and after reaction using liquid chromatography in sections 2.2.7.1- 2.2.7.3. For the amination of PPH with TYR, fluorescence emission spectra profiles were evaluated as reported in the section 2.2.7.4.

2.2.7.1 Derivatization procedure

One milliliter of a sample solution (5-fold dilution) was mixed in a glass vial with 200 μL of 2 M NaOH and 300 μL of saturated sodium carbonate (Gong, 2014, Alberto et al., 2012). To finish the reaction, 2 mL of freshly prepared dansyl chloride solution (10 mg/mL in acetone) was added and the mixture was incubated at 40°C for 45 min in darkness. In order to remove residues of dansyl chloride, 100 μL of ammonia was then added and the mixture was left to stand at 25°C for 30 min. Subsequently, the volume of the mixture was made up to 5 mL with acetonitrile and filtered through 0.22 μm filters (Millipore Millex, Billerica, MA, USA) prior to HPLC injections.

2.2.7.2 High-performance liquid chromatography (HPLC) unit and operative conditions

Identification and quantification of PPH-HIS conjugates produced via MTGase reaction were carried out using HPLC Agilent 1100 system (Agilent Technologies, Inc., Santa Clara, CA, USA) consisting of a G-1312 binary pump, a G-1328A injector, a G-1322A degasser, and a G-1315A photodiode array detector

(PDA), equipped with an Ascentis Express ES-C18 column (150 × 4.6 mm, 2.7 μm particle size; Sigma-Aldrich, St. Louis, MO, USA). The column temperature and flow rate were maintained at 40°C ± 0.5 and 0.5 mL/min, respectively. The gradient elution system consisted of 0.1 M ammonium acetate (phase A) and acetonitrile (phase B) started at 20% B, increased via linear gradient to 75% B at 80 min, then returned to the initial composition within 10 min. The injection volume was 10 μL and PDA data were recorded with a 200-600 nm range at a detection wavelength of 254 nm.

Histamine was identified by comparing its retention time and UV spectrum with that from a standard solution of an authentic sample, and quantified using a calibration curve obtained as reported below.

2.2.7.3 Quantitative analysis

A calibration curve for HIS was constructed by plotting peak area (y) vs. concentration for six concentration (x) levels. The standard stock solution was prepared by dissolving mixtures of PPH and HIS in ratios of 1:1 (w/w) to a 50 mL in 0.1 M HCl. The stock solution was diluted with a suitable amounts 0.1 M HCl to obtain final concentrations of 5, 10, 20, 40, 80, 160 μg/mL. Each diluted solution was subjected to a derivatization procedure as described in section 2.7.1 prior to HPLC analyses. A calibration curve was constructed by analyzing the resulting standard solutions by HPLC-UV at 254 nm. The correlation coefficient was $R^2 \geq 0.999$. The average limit of detection was calculated as 5.72 ± 0.19 μg/mL based on a signal-to-noise ratio of 3:1. The average limit of quantification was determined as 17.36 ± 0.59 μg/mL by assuming an S/N ratio of 10:1.

2.2.7.4 Evaluation of MTGase-amination of PPH with TYR by fluorescence spectroscopy

Fluorescence analyses were performed using a Spectramax M5 multimode microplate reader (Molecular Devices, Sunnyvale, CA, USA). Lyophilized samples were dissolved in 0.1 M phosphate buffer (pH 7.0) to a final concentration of 1.5 mg/mL. The solutions were excited at 280 nm (slit width 10 nm) and the emission intensity was recorded from 300 to 550 nm using 1 cm path-length quartz cuvette (Suprasil, Fisher Scientific, Pittsburg, PA) at a 500 nm/min. A buffer blank was subtracted from all spectra automatically by software.

2.2.8 Evaluation of antioxidant activity by DPPH radical scavenging activity assay

DPPH test was used to assess the ability of PPH-TYR conjugates to transfer labile H atoms to DPPH radical, a likely mechanism of antioxidant protection (Goupy, 2003). The experimental conditions used were adapted from Braca et al. (2002): DPPH was dissolved in methanol to a final concentration of 200 μM. The lyophilized sample (100 μL) was thoroughly mixed with 100 μL of the DPPH solution in the 96-well plate to a final assay concentration of 10 mg/mL. Each mixture was incubated at 25°C in the dark for 30 min and the absorbance at 517 nm was measured using a Spectramax M5 microplate reader. DPPH test for untreated PPH and untreated TYR solutions (from 0.1 to 20 mg/mL) with increasing concentrations was also conducted and the plot of absorbance vs.

concentration was established. The radical scavenging activity was expressed as a percent of relative activity (% RSA) and calculated with the following equation:

$\% \text{ RSA} = ((A_{control} - A_{test}) / A_{control}) \times 100$,

where $A_{control}$: absorbance of the control (DPPH only). A_{test}: absorbance of the test subject (test sample + DPPH).

2.2.9 Inhibition of linoleic acid oxidation

The antioxidant activity of PPH-TYR conjugates was determined by measuring the percent inhibition of peroxidation in linoleic acid system (He et al., 2013; Hong et al., 2014). A 2.0 mL aliquot of the lyophilized sample (at final concentration of 1 mg/mL) was dissolved in 1.5 mL of 0.1 M phosphate buffer (pH 7.0) and the mixture was added to 1.5 mL of 50 mM linoleic acid that was dissolved in 99.5% ethanol. For the blank assay, 2.0 mL of buffer was added to the ethanolic linoleic acid solution. BHT (2 mM) was used to replace sample and referred to a positive control. The tubes with reaction mixtures were sealed tightly with silicone rubber caps and kept at 60°C in the dark for 7 d. The degree of oxidation was measured at every 24 h interval by the ferric thiocyanate method (Osawa and Namiki, 1981). A 100 µl sample of the assay solution was removed from the test tube and mixed with 100 µl of ammonium thiocyanate (30%, w/v), 100 µl of 200 mM ferrous chloride dissolved in 1 M HCl and 4.7 mL of 75% aqueous ethanol. After the mixture has been stirred for 5 min, 200 µl aliquot was transferred to a clear bottom 96-well plate. The degree of color development was measured as increase in absorbance at 500 nm using a Spectramax M5 multimode

microplate reader. The capacity to inhibit the peroxide formation in linoleic acid was calculated as (%) inhibition = $((A_{blank}-A_{test}) / A_{blank}) \times 100$

where A_{blank} was the absorbance of the blank reaction and A_{test} was the absorbance of sample or BHT.

2.2.10 Statistical analyses

Data are reported as mean ± standard deviation of the three independent experiments. The effect of hydrolysis period and type of enzyme on the DH was analyzed by two-way analysis of variance (ANOVA) using the PROC MIXED procedure of SAS (v. 9.4, SAS Institute Inc., Cary, NC, USA). The model, in this case, tested the hydrolysis approach (Alcalase, Trypsin, Flavourzyme) and hydrolysis period (3, 6, 12, 18, 21h) were considered fixed effects. Hydrolysis data (DH) were fitted to a non-linear curve using GraphPad Prism 4 (GraphPad Software, Inc. San Diego, CA) with an R^2 from 0.981 to 0.987. Spectroscopic profiles obtained from the fluorescence analyses from three independent trials were averaged and reported as graphs. Data obtained from DPPH and linoleic acid oxidation activity were analyzed by one-way ANOVA. For both models (1- and 2-way ANOVA), post-hoc analysis for significance was obtained using Tukey's HSD ($p < 0.05$).

2.3 Results and Discussions

2.3.1 Extent of enzymatic modifications of pea protein hydrolysates

The degree of hydrolysis (DH) is generally used as a proteolysis monitoring parameter and is the most common indicator when comparing hydrolysis

efficiency and progress (Gíménez et al., 2009). For this study, Flavourzyme was chosen as being industrial peptidase and due to production of antioxidative or radical scavenging peptides from rapeseed (Xue et al., 2009) or tilapia proteins (Raghavan et al., 2008). Hydrolysis with Alcalase was also reported to produce zein hydrolysate with antioxidant potential (Zhu et al., 2008), while Trypsin hydrolysates inhibited lipid peroxidation in fish (Nagash and Nazeer, 2013). The effects of enzymes and treatments periods on DH of pea protein isolate are shown in Figure 2.1. Pea protein was hydrolyzed using three commercial enzymes at their optimal temperature and pH and the process of hydrolysis was monitored for 21 h using pH-stat. A typical hydrolysis curve obtained under experimental conditions showed that an increase in hydrolysis time resulted in a greater ($p < 0.05$) DH for all enzymes tested (Figure 2.1). The use of Flavourzyme and Trypsin led to the production of PPH with no more than 10.7% of DH by 21 h, while the greater ($p < 0.05$) DH of 24.8% was obtained when hydrolyzed with Alcalase. The results agree with an earlier study on enzymatic hydrolysis of different protein substrates, including fish (Dong et al., 2008; Ovissipour et al., 2012), whey (Mutilangi et al., 1995) and wheat gluten (Kong et al., 2007). This could be because Alcalase and Flavourzyme hydrolyze proteins in different manner. Alcalase is an *endo*-peptidase, therefore cleaves peptide bonds at the interior of the polypeptide chain and produces mainly small to medium-size oligopeptides/polypeptides (Adler-Nisen, 1986), while Flavourzyme is a mixture of *endo*- and *exo*-peptidase. It was also established that alkaline proteases, including alcalase, exhibit higher activities than do acid or neural proteases such

as Flavourzyme (Rebeca et al., 1991). DH significantly increased for Alcalase hydrolysis at 18 h reaching a value of 24.2 %. Prolonged hydrolysis time over 12 h did not significantly increase DH, possibly due to substrate limitation, enzyme inhibition by the end product or a decline in peptide bonds available for hydrolysis (Phengnuam et al., 2013). Such a behaviour is considered typical for enzymatic reactions and has been demonstrated for commercial proteases, including Alcalase and Flavourzyme, acting on protein derived from different legumes (Kim et al. 1990; Betancur-Acona et al. 2009). Therefore, the hydrolysis of pea protein isolate with Alcalase for 18 h was found the most suitable and used for further analyses.

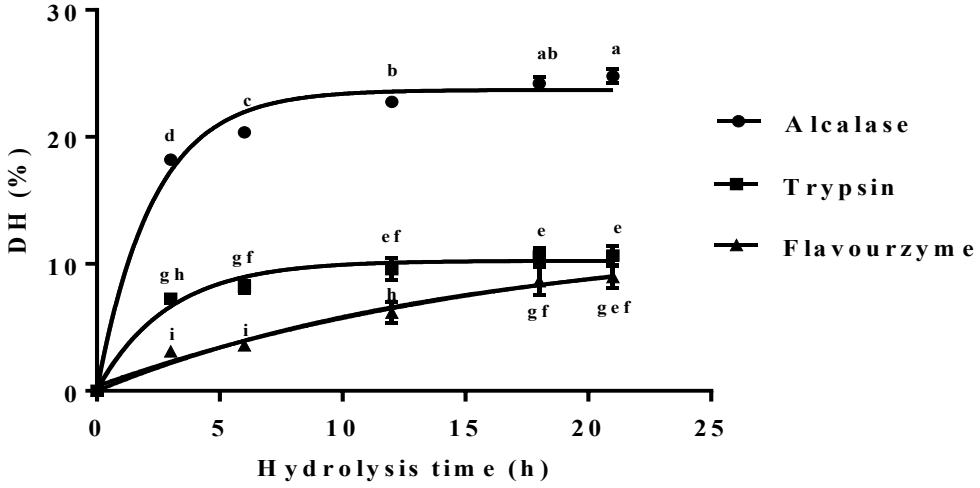


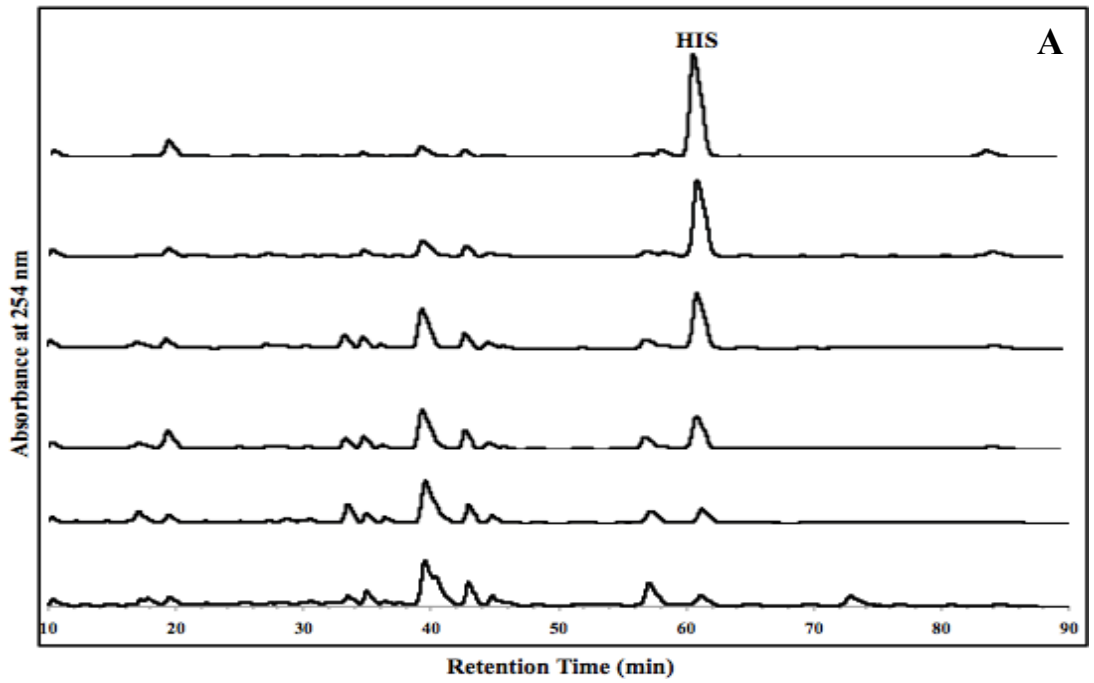
Figure 2.1: Time course of proteolytic degradation of pea protein isolate obtained by three different enzymes: Alcalase, Trypsin and Flavourzyme at 1:50 (w/w) enzyme/substrate ratio. Protein concentration was 5% (w/v). Each data point is the mean of 2 determinations from three independent batches. Vertical bars represent standard deviations (n = 6). Different letters represent significant differences ($p < 0.05$). The data were fitted on a curve with a non-linear model using GraphPad Prism graph-plotting program.

2.3.2 Evidence of PPH-HIS conjugation by liquid chromatography

To demonstrate that the conjugation of HIS to PPH was possible, HIS quantification was performed before and after MTGase-catalyzed reaction. Various methods for accurate and efficient determination of BAs have been developed, and HPLC is by far the most frequently used, owning to its superior sensitivity. Derivatization of the amino group is commonly performed to reduce the polarity of amines and also to provide a chromophore UV or fluorescent detection (Mayr and Schieberle, 2011), with dansyl chloride or benzoyl chloride being the most common derivatizing agents for HPLC analyses.

During a preliminary experiment, similar retention times were observed for dansylated derivatives of TYR and peptides in PPH; this made their identification not accurate. On the contrary, when HIS was dansylated no interferences from the peptides were observed. Since close elution or co-elution may result in lack of resolution for a peak of interest or interfere with compound quantification, proof of PPH-BAs conjugation via MTGase was tested using HIS. Figure 2.2A shows the RP-HPLC chromatograms of six standard solutions of HIS. It clearly indicated that all tested standards were completely separated by the applied elution method. Dansyl chloride - derivatized HIS eluted at the retention time of 61 min and was assigned as “HIS”. The presence of other peaks during the separation was noticed in the chromatograms; however they did not interfere with the identification of HIS derivatives. The results indicate that the proposed derivatization and HPLC analytical method can be applied for quantification of HIS. Figure 2.2B shows the representative HPLC chromatograms of the treatment PPH-HIS (+) MTGase and

the controls (PPH-HIS, PPH (+) MTGase, and PPH only). The chromatogram showed there was no HIS peak detected at the retention time of 61 min in both PPH (+) MTGase and PPH samples. The HIS peak area decreased after the MTGase treatment. As shown in Figure 2.2C, the concentration of HIS decreased from 80.0 to 19.0 μg/mL (76.3% reduction), indicating the formation of peptides-HIS conjugates (amination). These results confirm that MTGase can be used conjunction with glutamine containing peptides as a tool to reduce the concentration of BAs in food. It was of interest to understand if the BA-peptide conjugation has an effect on the bioactivity of peptides, like for instance, the antioxidant activity.


A
HIS
Absorbance at 254 nm
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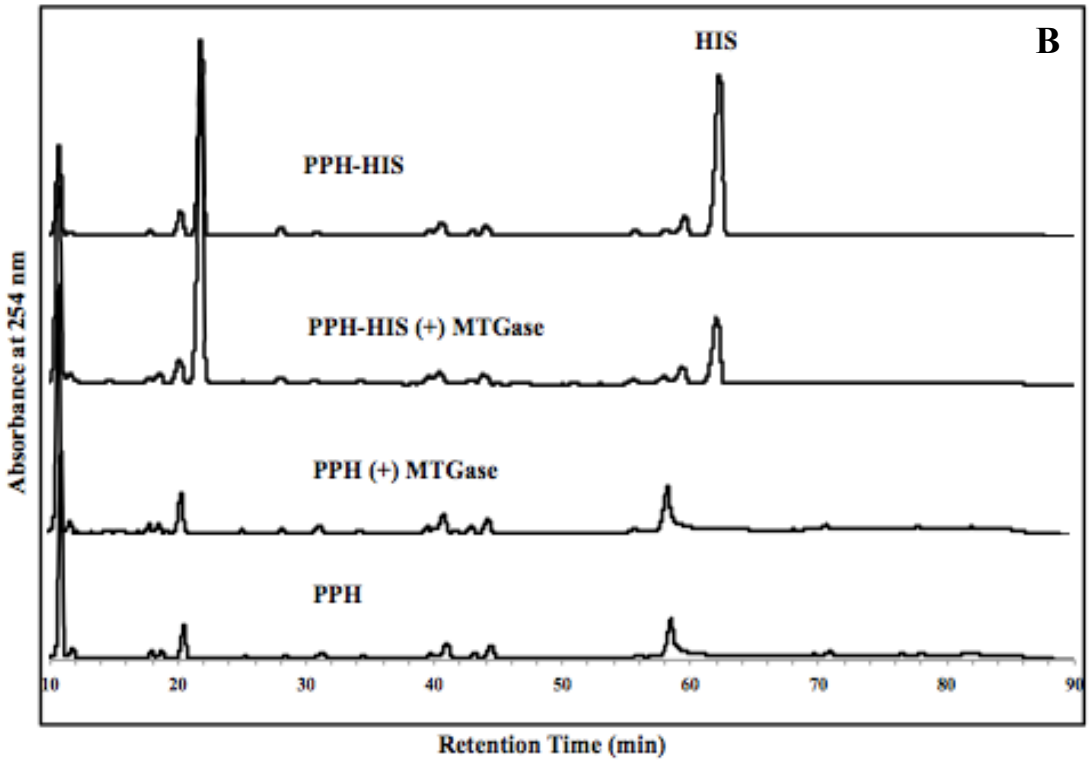
20
30
40
50
60
70
80
90
Retention Time (min)


B
HIS
PPH-HIS
PPH-HIS (+) MTGase
PPH (+) MTGase
PPH
Absorbance at 254 nm
10
20
30
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50
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70
80
90
Retention Time (min)

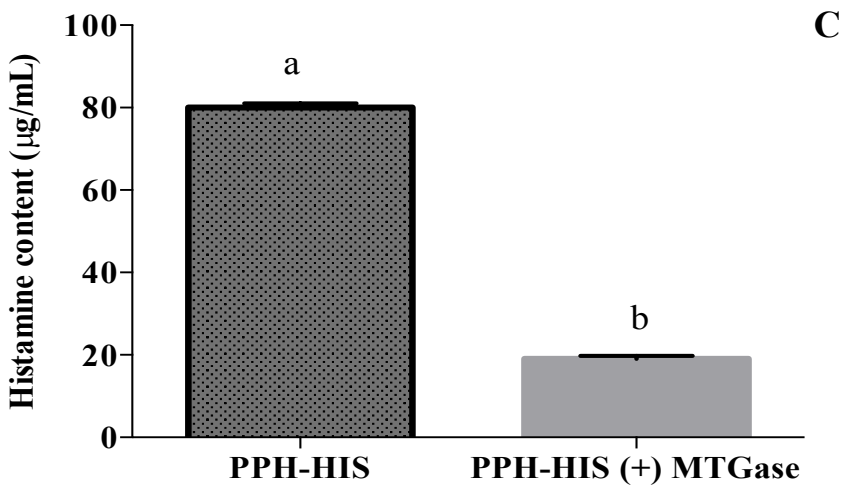

Figure 2.2: Chromatograms of dansylated derivatives of HIS detected at 254 nm. (A) Standard solutions of HIS at 5 (1); 10 (2); 20 (3); 40 (4); 80 (5); 160 (6) μg/mL and (B) PPH, PPH-HIS, PPH (+) MTGase and PPH-HIS (+) MTGase incubated at 37°C for 6 h. Peak designated as 'HIS' refers to a dansylated histamine.

2.3.3 Evidence of PPH-TYR conjugation by fluorescence spectroscopy

As previously reported, proof of PPH amination through MTGase-catalyzed reaction was demonstrated to be possible by determining unreacted HIS concentration using liquid chromatography; applying the same approach using TYR as a primary amine was excluded due to interferences from peptides. Another way to indirectly evaluate the formation of PPH-TYR conjugates would be by using a spectroscopic technique, like fluorescence emission. TYR is a fluorescent and aromatic molecule; therefore its conjugation to a protein or peptides should influence their fluorescence emission pattern. For instance, the intrinsic fluorescence spectrum of a protein is mainly determined on the level of exposure of aromatic amino acids to the reaction solvent, therefore its structural

conformation can be effectively monitored using changes in the intensity and maximum wavelength of fluorescence signal of tryptophan (Trp) and tyrosine (Tyr) (Valeur and Berberan-Santos, 2012). To confirm the formation of PPH-TYR conjugates via the action of MTGase, the intrinsic fluorescence was thus monitored and the results presented in Figure 2.3. PPH exhibited a broad peak with a maximum intensity ranging from 340-360 nm, a typical fluorescence profile for Trp when exposed to an aqueous solution (Lakowicz, 2013). When MTGase was added to PPH, a slight decrease in FI in treatments upon 6 h incubation was observed. This indicates that some of the Trp chromophores were exposed to a more polar environment at 6 h reaction in the presence of MTGase. This seems to be in contrast with results for whey (Agyare and Damodaran, 2010) or soybean protein isolate (Jiang and Zhao, 2010), where higher fluorescence intensities (FI) were observed for MTGase treatments and was attributed to a decrease in the exposure of buried aromatic groups to the solvent as a consequence of the cross-linking reaction between proteins. Sample PPH-TYR (+) MTGase resulted in a hypsochromic shift of the λ_{max} and display at 310 nm and the highest emission. This infers that the incorporation of TYR into PPH causing pronounced effects on the structural rearrangement of the hydrolysates. This demonstrates that amination of PPH may induce a partial folding of peptides to bury previously exposed hydrophobic molecules (i.e. TYR), consequently leading to an increase in the relative fluorescence intensity (Tang et al., 2005). To exclude the interference of the unreacted TYR on the results observed, a solution containing the same amount of TYR was subjected to ultrafiltration system as

reported in section 2.6 and the fluorescence profile of this treatment (TYR) was presented in Figure 2.3 and exhibited a λ_{max} at 310 nm. A very low FI of the TYR control (500 FI) indicates its effective removal by ultrafiltration and thus validating that the observed spectra changes were likely due to amination.

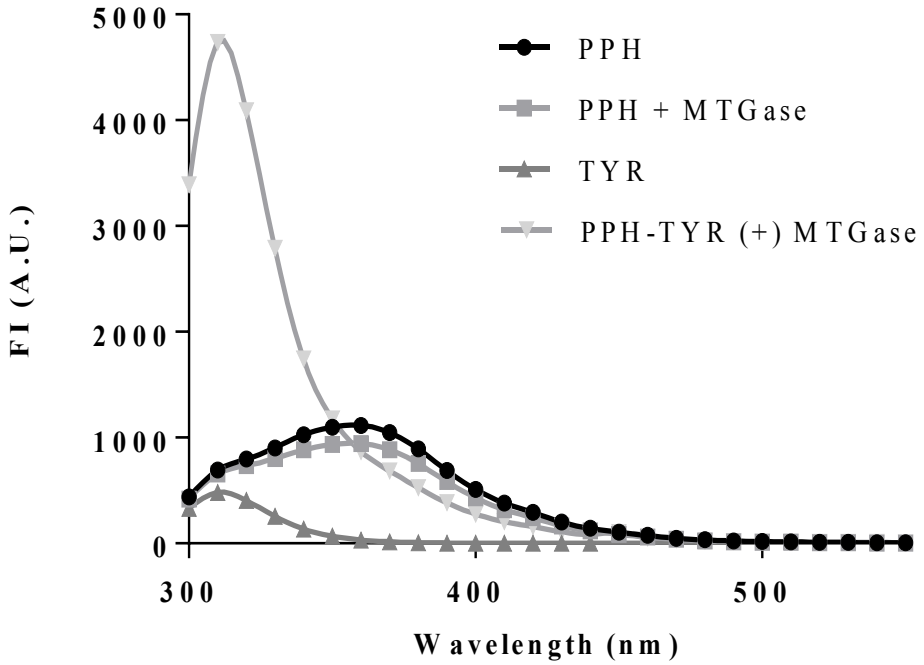

Figure 2.3: Fluorescence spectra profiles of PPH-TYR conjugates obtained in the presence of MTGase and control treatments (PPH, PPH + MTGase, TYR). Data are presented as mean ± SD (n = 6) and expressed in arbitrary units (A.U.). FI refers to fluorescence intensity.

2.3.4 DPPH radical scavenging activity

DPPH is a relatively stable radical that is widely used to evaluate the antioxidant activity of different compounds (Bougatef et al., 2009). The

antioxidant compound could scavenge the radical by donating H and convert it to a stable diamagnetic molecule DPPH-H, followed by the reduction in absorbance (Prior et al., 2005). Figure 2.4A illustrates that ultrafiltrated PPH exhibited 41.1% RSA. PPH showed a concentration-dependent scavenging activity against DPPH radicals with the scavenging capacity increasing with increasing concentration (Figure 2.4B). This is consistent with a study testing canola hydrolysates (Cumby et al., 2008). Furthermore, in the present study, the scavenging activity of 1 mg/mL of PPH was greater (Figure 2.4B) as compared to a previous report that also used alcalase proteases on pea protein isolate (Humiski and Aluko, 2007). These authors have also shown that PPH showed a stronger activity (11%) after hydrolysis by Flavourzyme. A decrease (34.1 %) in scavenging activity was observed in MTGase-treated hydrolysates without TYR when compared to that of untreated PPH. A number of studies have demonstrated that the antioxidant activity of protein hydrolysates depends on their molecular weight distribution (Peña-Ramos et al., 2004; Li et al., 2008; Su et al., 2011). Here peptide fractions with smaller molecular weight were associated with higher antioxidant activity. Our results indicate that MTGase–mediated cross-linking reaction can decrease the proportion of low molecular weight peptides, thus reduce their radical scavenging ability.

Like most synthetic and natural antioxidants, the structural feature responsible for the radical scavenging activity of TYR is the phenol ring. BAs such as dopamine and TYR function as strong free radical inhibitors (Yen and Hsieh, 1997). As expected, the presence of TYR provided a scavenging activity of

44.6 and 56.1% at 2.5 and 5 mg/mL, respectively (Figure 2.4B). However, as reported in the previous section, ultrafiltration was able to remove the majority of the unreacted TYR. Furthermore, a control solution of TYR reduced the DPPH value to 7.6% after ultrafiltration. This is equivalent to a TYR concentration of 0.16 mg/mL, corresponding to a 94% reduction from the original concentration. Among all treatments, the greatest (52.3%) scavenging activity was found in PPH -TYR (+) MTGase treatment, suggesting that the incorporation of TYR into PPH through MTGase increased the radical scavenging capacity of the PPH. Hence, conjugation of BAs to PPH not only has the potential to reduce their toxicity, but also represents the creation of novel peptides with an improved antioxidant activity.

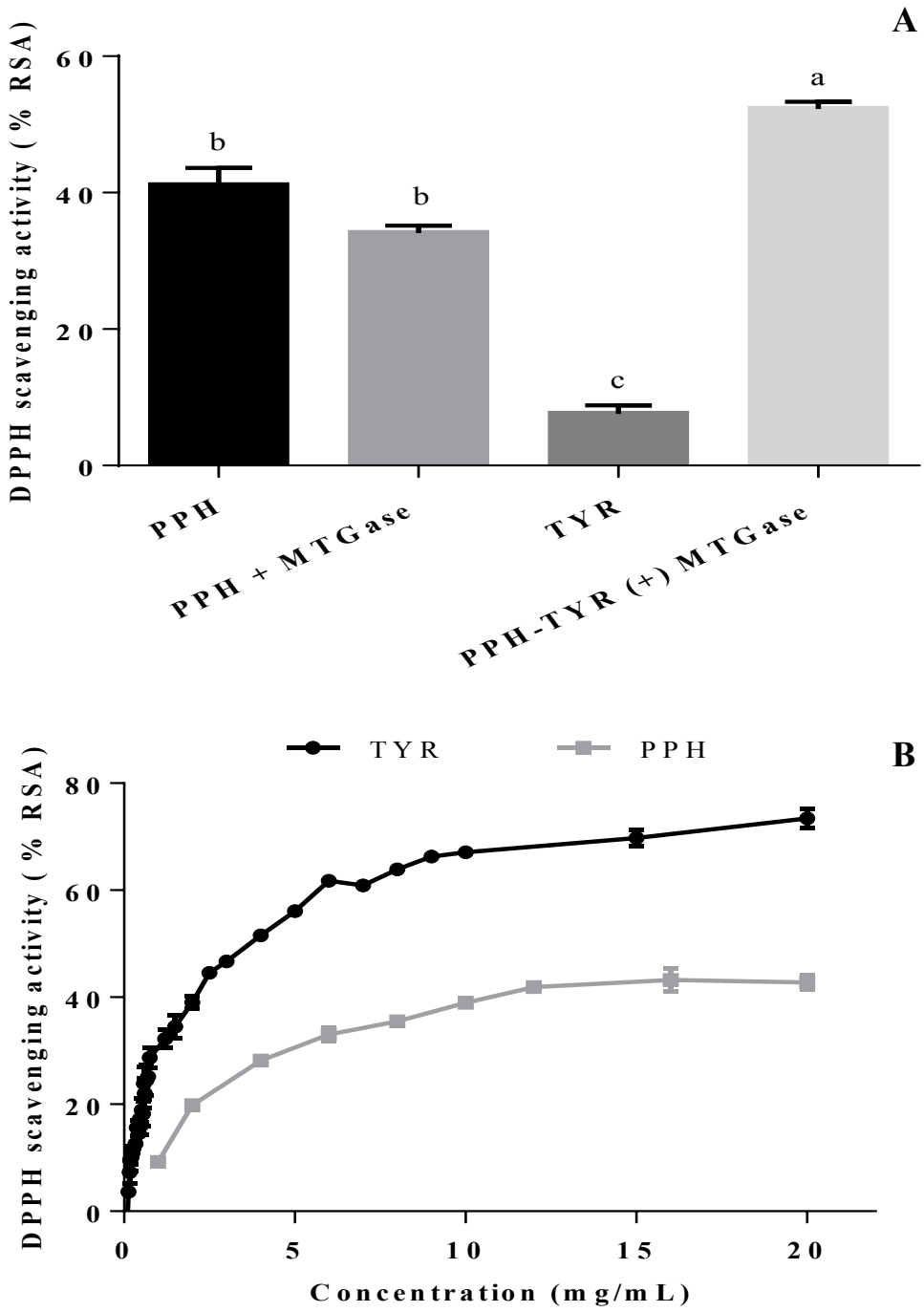

Figure 2.4: DPPH free radical scavenging activities of (A) PPH-TYR conjugates produced via MTGase and control treatments (PPH, PPH + MTGase, TYR). The vertical bars represent the standard deviation (n = 6) of each data point. Different letters above the bars represent significant differences ($p < 0.05$). (B) A plot of TYR and PPH concentration (mg/mL) vs. DPPH scavenging activity (% RSA).

2.3.5. Inhibition of linoleic acid oxidation

The effect of MTGase amination on antioxidant activity of PPH was also tested using the ferric thiocyanate method to measure the peroxidation of linoleic acid. During linoleic acid peroxidation, peroxides are formed and are able to induce oxidation of Fe^{2+} to Fe^{3+}, the later forms a colored complex with ammonium thiocyanate, which has a maximum absorbance at 500 nm (Sultana and Przybylski, 2007). Therefore, the degree of linoleic acid oxidation is directly proportional to the absorbance intensity. A lower absorbance of reaction complexes represents a greater degree of inhibition of linoleic acid oxidation. As shown in Figure 2.5, the blank solution (0.1 M phosphate buffer) showed a rapid increase in the absorbance and reached a maximum value on the fourth day, indicating the maximum formation of linoleic acid peroxides. Subsequently, a gradual decline started as the incubation period progresses suggesting the instability and decomposition of peroxides into secondary lipid oxidation products (Jayaprakasha et al., 2001; Chen et al., 2007). TYR is credited with a strong antioxidant effect on the peroxidation of linoleic acid, similar to α-tocopherol (Yen and Hsieh, 1997). The ultrafiltrated TYR treatments were more effective against linoleic acid oxidation at all incubation times as compared to that of the blank solution. Previous studies reported that peptides containing hydrophobic amino acids produced by alcalase hydrolysis exhibited a strong lipid oxidation inhibition (Hong et al., 2014). PPH samples showed a 49.1% reduction in lipid oxidation at 2 d of incubation compared to the blank. However at day 4 the percent of inhibition decreased to only 23.7% compared to day 2. It is possible

that PPH peptides gradually lose the ability to adequately prevent free radical formation with continuous incubation (Alashi et al., 2014). A greater percentage of inhibition in PPH + MTGase treatment was observed at all incubation times as compared to PPH, suggesting that cross-links-induced conformational alterations via MTGase reaction increased the ability to reduce lipid oxidation. The greatest inhibitory effects of PPH-TYR (+) MTGase samples (Figure 2.5) were evident at all incubation times relative to the other treatments, with the only exception being the positive control containing the synthetic antioxidant BHT. These effects are likely due the introduction of TYR into the peptide sequences. The results are in line with the DPPH results and suggest that amination of PPH increases the overall antioxidant activity.

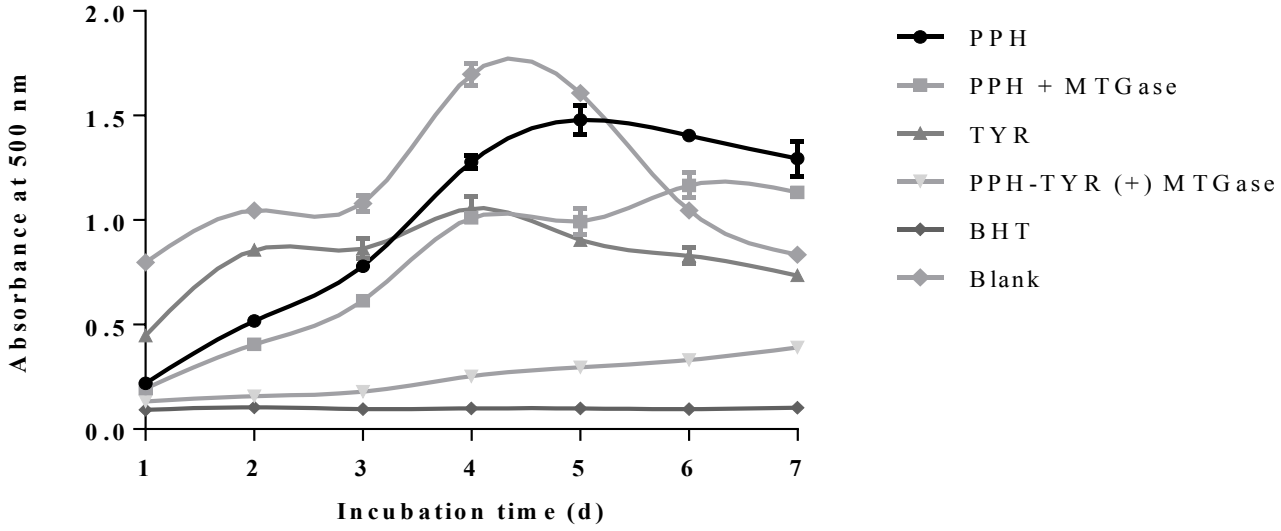

Figure 2.5: Inhibition of linoleic acid oxidation by PPH-TYR conjugates produced via MTGase and control treatments (PPH, PPH + MTGase, TYR). Measurements were performed over time from 0 to 7 d and recorded at absorbance of 500 nm. Data represent the mean of three independent experiments. Bars with different letters are significantly different ($p < 0.05$).

2.4 Conclusions

Biogenic amines are able to conjugate to pea protein hydrolysates using MTGase-mediated enzymatic reaction; hence reduction of their toxicity can be achieved. The conjugation of tyramine via MTGase also improved the ability of the pea protein hydolysate to scavenge free radicals and reduce linoleic acid peroxidation. The method presented in this study represents a practical solution to reduce levels of biogenic amines in food and beverages, and at the same time produce novel chemical compounds with antioxidant capacity to control lipid oxidation.

[2]Chapter 3 (Study 2): Incorporating tyramine with transglutaminase weakens gelatin gels – a rheological investigation [2]

3.1 Introduction

Gelatin is a denatured fibrous protein made by the partial hydrolysis of collagen, a major component of animal connective tissues. During the production of gelatin, many bonds break randomly and the resulting gelatin represents a broad spectrum of smaller molecular fragments, as well as larger unhydrolyzed main chains (Kaur et al., 2002). Commercial gelatins for food consumption are mainly derived from bovine bones and porcine skins due to their low cost and availability (Cho et al., 2005). Gelatin's unique properties are highly regarded in food manufacturing; as an additive it can improve functional properties of food products due to its elasticity, consistency and stability (Hashim et al., 2010). The quality of a gelatin depends largely on its rheological properties, which are normally characterized by gel strength, gel elasticity, gelation point and gel melting point (Karim and Bhat, 2009). Gelatin has accessible functional groups which can be coupled with various cross-linkers or targeting-ligands to bear multiple modification opportunities (Elzoghby et al., 2012). Introducing these chemical cross-links, or a combination of cross-linking agents within or between gelatin chains, is a mean of manipulating the characteristics of any given gelatin. This approach has been reported in earlier studies for maltodextrin (Kasapis et al., 1993), gellan (Wu et al., 2001) and caffeic acid (Mohtar et al., 2014).

[2] *A version of this chapter has been submitted for publication in the LWT – Food Science and Technology.*

Incorporation of these multifunctional molecules into gelatin chains leads to a three-dimensional covalently cross-linked network with altered rheological properties.

Tyramine (TYR) is one of the most important biogenic amines (BAs) naturally present in a variety of foods and beverages (Spano et al., 2010). As reported in a previous research (Lu et al., 2016), this aromatic and antioxidant BA can be incorporated into pea protein peptides using a GRAS (generally recognized as safe) microbial enzyme - transglutaminase (MTGase). MTGase is a Ca^{2+}-independent enzyme industrially produced from the *Streptoverticillium species. It* catalyzes an acyl transfer reaction between the γ-carboxyamide of peptide- or protein-bound glutamine and the ε-amino group of lysyl residues. It also catalyzes aminolysis of the γ-carboxamide group of peptide-bound glutamine residues by the variety of primary amines (Han and Damodaran, 1996). A recent report also highlighted the high reactivity of MTGase for the incorporation of the aromatic TYR into protein in model systems (Gundersen et al., 2014). In this context, we anticipate that MTGase would introduce TYR onto glutamine residues of gelatin and alter its functionality. The action of MTGase has been demonstrated to be largely responsible for cross-linking gelatins (Fuchsbauer et al., 1996; Dickinson and Yamamoto, 1996; Lim et al., 1999; Crescenzi et al., 2002), but, to the best of our knowledge, no information is available on the MTGase-catalyzed amination of gelatin, and its subsequent specific effects on rheological properties. It is hypothesized that incorporation of BAs into the gelatin molecule would affect the gel network formation during the cooling phase. The TYR conjugation would

increase surface hydrophobicity of the gelatin and thus may induce the faster formation of hydrogen bonding of gelatin chains (inter-molecular hydrogen bonds) to speed up gel formation. This faster reaction would be desirable in gelatin applications in both the food and pharmaceutical industries. On the other hand, the incorporation of TYR could decrease inter-molecular hydrogen bonds due to steric hindrance and protein folding. In this case the formation of intra-hydrogen bonds would be dominant over the inter-molecular ones, and as a consequence, the gelatin chains would fold in such a manner to reduce the interactions of between the gelatin chains thus negatively affecting the rheological properties. The overall aim of this study was to evaluate and compare the viscoelastic properties of aqueous MTGase-treated gelatin dispersions in the presence or absence of TYR. It was important to understand the impact of incorporating TYR on rheological characteristics of gelatin.

3.2 Materials and Methods

3.2.1 Materials

Commercial gelatin extracted from porcine skin (type A) was from Sigma-Aldrich (St. Louis, MO, USA). Gelatin prepared by acid treatment (type A) and not by the base treatment (type B) was chosen, because treatment with base hydrolyzes the amide groups of glutamine residues and thus suppresses enzymatic reactions (Crescenzi et al. 2002). Tyramine hydrochloride (TYR, ≥ 98.0%), N-Benzyloxycarbonyl-L-Glutaminylglycine (Z-Gln-Gly), L-glutamic acid γ-monohydroxamate and hydroxylamine were purchased from Sigma-Aldrich.

MTGase (Activa-TI; 99% maltodextrin and 1% MTGase) was from Ajinomoto Food Ingredients (Eddyville, IA). All other chemicals and reagents were from Sigma-Aldrich or Fisher Scientific (Fair Lawn, NJ, USA) and were of analytical grade.

3.2.2 Experimental Design

Porcine skin gelatin (G) solutions were used at final concentrations of 5% and 6.67% (w/v) and were subjected to TYR amination through MTGase catalysis reactions conducted at 50°C for 4 h. The latter concentration of gelatin is typical for determining the "Bloom" strength measurement. Two concentrations of TYR (0.5 and 1% w/w) were added to the gelatin solutions in the presence or absence of MTGase. Thus, for each of the two gelatin concentrations 6 treatments were produced: a control gelatin without MTGase and Tyr (G), a treatment of gelatin and MTGase (G + MTGase), a treatment of gelatin, MTGase and addition of 0.5% TYR (G + MTGase + 0.5% TYR) and its respective control (G + 0.5 % TYR), a treatment of gelatin, MTGase + 1% TYR (G + MTGase + 1% TYR) and its respective control (G + 1% TYR). The whole experiment was repeated three independent times using gelatin from different batches. For each trial two tubes per treatments (including controls) were incubated at 50°C for 4 h.

3.2.3 Determination of enzyme activity

MTGase activity was measured using CB

Z-Gln-Gly and hydroxylamine as substrates following the method of Folk (1970) with some modifications. The final 0.23 mL reaction mixture contained

174 mM Tris buffer, 31 mM CBZ-Gln-Gly, 87 mM hydroxylamine, 8.7 mM glutathione (reduced form), 4 mM calcium chloride and 0.06 unit transglutaminase. The reaction mixture was incubated at 37°C and 50°C for 10 min and terminated by adding 500 µl of ferric chloride/trichloroacetic acid reagent (0.7% w/v). After centrifugation for 5 min at 1000 × *g*, the absorbance of the resulting supernatant was recorded at 525 nm (V-530, Jasco Corporation, Tokyo, Japan). L-glutamic acid-γ-monohydroxamic acid was used as a standard for calibration. One unit of enzyme activity was equivalent to a change in absorbance of 0.29/min at 525 nm, corresponding to the formation of 1 µmol of hydroxamate/min from CBZ-Gln-Gly and hydroxylamine at pH 6.0. The enzyme MTGase activity was expressed as the initial reaction rate (U/g) determined for the maximum slope of the plot of absorbance versus time.

3.2.4 Preparation of the gelatin treatments

Gelatin solutions were prepared by dissolving gelatin powder in Milli-Q water to obtain a final concentration of 5 and 6.67% (w/v). Gelatin + TYR solutions were prepared by adding different amounts of TYR into each gelatin solutions to achieve final TYR concentrations of 0.5 and 1.0% (w/w).

For the enzymatic treatments, MTGase was dissolved in Milli-Q water to prepare a stock solution of 10 mg/mL. Any insoluble particles were removed by centrifugation at 1000 × *g* for 5 min. Enzyme stock solutions were prepared freshly for use. MTGase activity was determined as described in Section 2.3 and obtained 45 U/g at 50°C. Adequate volumes (~100 µL) of MTGase stock solution

were added to each G + TYR solutions to achieve a final enzyme activity of 0.2 U/g gelatin. Samples were named as G + MTGase + 0.5% TYR and G + MTGase + 1% TYR, respectively. The quantity (0.2 U/g) of MTGase and the reaction temperature of 50°C were chosen based on a preliminary study, where higher level of enzyme (> 0.2 U/g protein) and lower reaction temperature (37°C) led to the inability of enzyme-treated gelatins to undergo characteristic thermally reversible transitions.

All gelatin treatments (including controls) preparations were incubated at 50°C for 4 h. After incubation, the enzymatic treatments were heated to 80°C for 10 min to inactivate MTGase enzyme and cooled to the room temperature. Obtained samples were stored at 4°C prior to the rheological analyses.

3.2.5 Dynamic viscoelastic measurements

Small deformation viscoelasticity was investigated by dynamic oscillatory rheometry using a Physica MCR 301 rheometer (Anton Paar GmbH, Ashland, VA) according to Du et al. (2013). A stainless steel flat plate (25 mm diameter) was lowered to form a gap of 0.5 mm. Oscillatory measurements were performed at a fixed frequency of 1 Hz and a strain amplitude of 0.5%, which was within the linear viscoelastic region (LVR) as determined by the preliminary tests. To optimize for LVR, the frequencies were tested from 0.01 to 10 Hz, while the strain sweeps were performed from 0.1 to 10%. To prevent excessive evaporation, an Anton Paar H-PTD200 hood was used. Each treatment was heated to 45°C for 10 min in a water bath and the 1 mL gelatin solution was transferred to the

rheometer, where the temperature maintained at 45°C to allow for equilibration. The temperature was decreased from 45°C to 10°C at a rate of 2°C/min. The evolution of G' (storage modulus, a measure of elastic rigidity) and G'' (loss modulus, a measure of the viscous rigidity) were recorded simultaneously through the cooling and presented as a function of temperature. The gelation temperature of treatment was defined as the temperature at which the G' increased above that of the G'' (the G'/G'' cross over point) (Nijenhuis, 1981).

To ensure the formation of gel networks, the gels were also kept for 3 h at 10°C. During this time, time sweep measurements were conducted at a constant strain of 0.5% and a constant frequency of 1 Hz. The evolution of G' (Pa) over time during the holding phase of gelation was fitted to a logarithmic equation according to Fonkwe et al. (2003):

$$G_t = K_{gel} \ln (t_{gel}) + C \qquad (1)$$

G_t is the value of G' at time t, C is a constant, t_{gel} is gelation time and K_{gel} is the gelation rate constant.

A frequency sweep measurements were performed to characterize the cross-linking behavior of the gelatin at a constant strain of 0.5% and a constant temperature of 10°C. G' and G'' (Pa) were both measured over a frequency range from 0.01 to 10 Hz. All tests were performed within the identified LVR. The evaluation of the gel strength was based on the frequency response of gel samples. The storage compliance J', like the storage moduli, reflects the propensity of a sample to retain a supplied mechanical energy and to restore it in the form of an

elastic strain (Gnanou and Fontanille, 2008). J' was evaluated in as described by Fonkwe et al. (2003):

$$J' = \frac{G'}{G'^2 + G''^2} \qquad (2)$$

The gel strength G_N was obtained according to Ferry (1980):

$$G_N = \frac{\mathbf{1}}{J'} \qquad (3)$$

At the end of the frequency sweep, the temperature of the treatment gel was increased back from 10°C to 45°C at a rate of 2°C/min.

3.2.6 Statistical analyses

Data are reported as mean ± standard deviation of the three independent experiments. The effect of MTGase amination on gelatin's gelation temperatures and melting temperatures was analyzed by three-way analysis of variance (ANOVA) using the PROC MIXED procedure of SAS (v. 9.4, SAS Institute Inc., Cary, NC, USA). The model, in this case, tested gelatin solution concentrations (5%, 6.67% (w/v)), tyramine concentrations (0.5 and 1% (w/w)) and presence/absence of MTGase were considered fixed effects. The effect of MTGase amination of gelatin's gel rate and gel strength was determined by one-way analysis of variance (ANOVA) using the PROC ANOVA procedure of SAS. For both models, post-hoc analysis for significance was obtained using Tukey's HSD ($p < 0.05$). Graphing of gelation temperatures and melting temperatures of treatments were conducted using GraphPad Prism 4 (GraphPad Software, Inc. San Diego, CA).

3.3 Results and Discussions

3.3.1 Effect of MTGase amination on gelatin's gelation temperature

The temperature of gelation (T_{gel}) is one of the most important functional attributes associated with the applications of gelatins for food manufacturing. The temperature at which *G′/G″* cross over represents the transition phase from a liquid to a more solid-like material occur during cooling of gelatin (Gudmundsson, 2002). This transition involves the formation of triple helical sequences randomly connected by hydrogen bonds (Crescenzi et al. 2002). The gelation temperatures of the different treatments are shown in Figure 3.1. In general, the treatment with 6.67% gelatin resulted in a higher gelation temperature than the ones produced using 5% gelatin. This is likely due to the greater concentration of proline (Pro) and hydroxyproline (HyPro) in the 6.67% treatment; it is known that the content of Pro and HyPro affect thermal and rheological properties including melting and gelling temperatures of gelatin (Cheow et al., 2007), where the higher contents of Pro and HyPro result in higher gelling and melting temperature of gelatin (Haug et al., 2004). A previous study from Sarbon et al. (2015) has also shown that increasing concentrations of chicken skin gelatin from 3, 5 and 10% increased gelling temperatures to 21.0, 23.3 and 27.2°C, respectively.

The addition of MTGase alone (G + MTGase) led to an increase in gelation temperatures and was observed at 24.1 and 27.6°C, respectively for 5 and 6.67% gelatin, respectively. Moreover, 6.67% G + MTGase produced a gel with the highest gelling temperature among all of the treatments tested. This indicates that

cross-linking of gelatin occurred via the action of MTGase. The effect of MTGase on the T_{gel} of the control treatment at 5% G + MTGase was not statistically different compared to the T_{gel} of control treatment 6.67% G. This suggests that the addition 0.2 U/g MTGase can replace an equivalent amount of 1.67% gelatin relative to the gelation temperature. The earlier onset of gelation for MTGase-treated gelatin compared to the other treatments may indicate the formation of covalent cross-links within or between gelatin chains, hence reduced the time required for gelation. These observations are in agreement with the MTGase-catalyzed gelation of fish gelatins (cod and hake) (Fernandez-Dıaz et al., 2001) and surimi extracted fish gelatin (Norziah et al., 2009). The T_{gel} of treatments G + MTGase + 0.5% TYR and G + MTGase + 1% TYR were not significantly different as compared to gelatin alone (G) at both concentrations. This means that the MTGase-catalyzed amination with TYR is not effective in changing the gelation temperature of pig gelatin compared to the control treatments. The addition of TYR to the G + MTGase treatment negatively affects the cross-linking reactions between the gelatin chains. This is likely due to the competition of the different sources of amino groups (lysine vs. tyramine) as a substrate for MTGase. When an exogenous source of -NH_2 groups is provided by adding TYR, it shifts the reaction towards gelatin amination rather than gelatin cross-linking, which involves the acyl group of a glutamine and the ε–NH_2 group of a lysine.

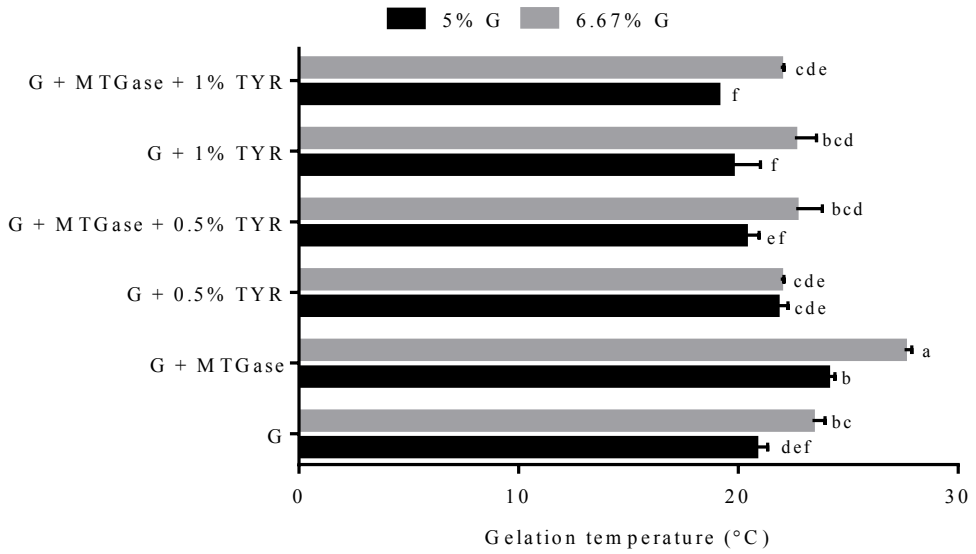

Figure 3.1: Gelation temperatures (T_{gel}) of treatments with gelatin concentrations of 5 and 6.67% (w/v). The values were obtained during cooling of the respective treatments from 45 to 10°C. Data are presented as mean ± standard deviation (n = 6). Values with different alphabets are significantly different ($p < 0.05$).

3.3.2 Effect of MTGase amination of gelatin on storage (G') and loss modulus (G'')

The changes of the storage modulus (G'), the loss modulus (G'') and the phase angle (δ) during gelatin temperature sweep are presented in Figure 3.2. During the cooling phase, both the elastic (Figure 3.2A, D) and loss (Figure 3.2B, E) moduli of all samples increased rapidly from 20 to 10°C. In general, all the treatments at 6.67% gelatin showed better gelling abilities and greater G' and G'' values compared to 5% gelatin preparations (Figure 3.2 (B, D); Figure 3.2 (A, C)). This also confirmed that the gelatin gel elasticity is concentration-dependent since greater frequency of encounters among strands would likely occur with

greater concentrations of gelatin (Tosh and Marangoni, 2004). The addition of MTGase without TYR to both gelatin controls did not affect the G' values during the temperature sweep (Figure 3.2A, B). This suggests that introduction of covalent cross-links produced by MTGase did not result in a more elastic gelatin gel network compared to control treatments. MTGase-catalyzed amination decreased the G' values from 3620 Pa (gelatin alone; G) to 2670 (G + MTGase + 0.5% TYR) and 2640 Pa was observed in its control (G + 0.5% TYR). A similar pattern was also observed with 1.0% TYR (data not shown). This indicates that MTGase amination with TYR negatively affects the elasticity of the gelatin gels. It is possible that TYR interfered with the gel-forming ability of gelatin and hence weakened the gelatin gels (Walkenström and Hermansson, 1996; Norziah et al., 2009). The formation of gelatin gels may rely on a perfect protein network, mainly driven by the complex network of hydrogen bonds. Introduction of TYR functional group to the gelatin molecule may alter this perfect phenomenon.

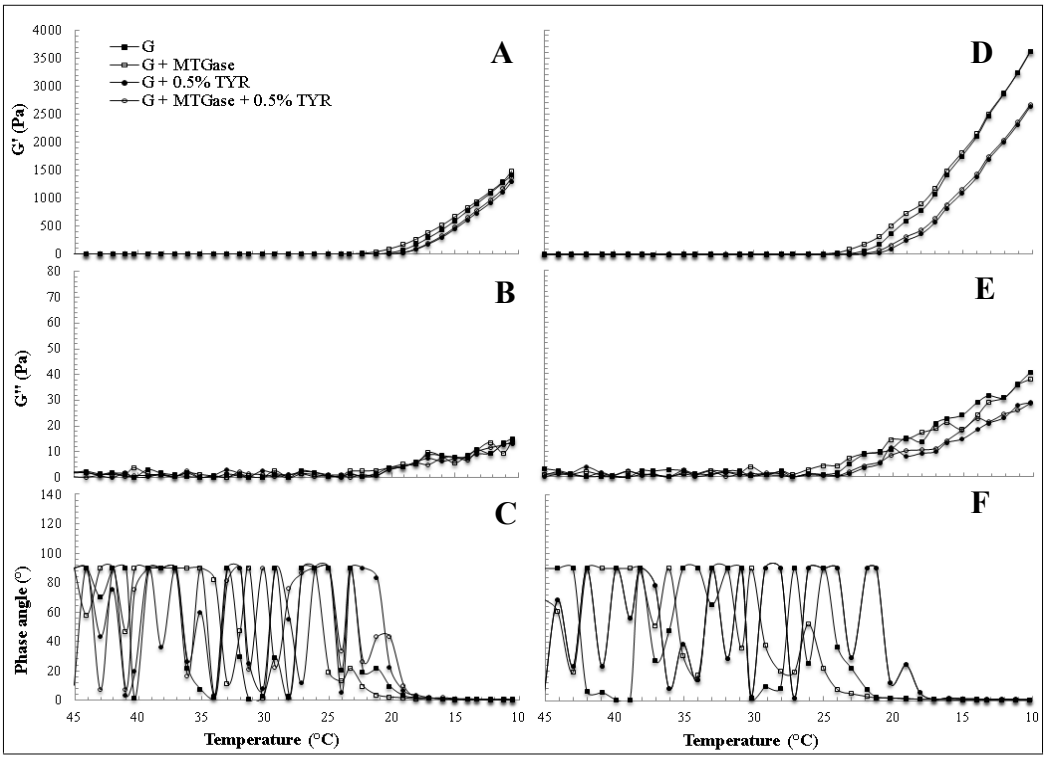

Figure 3.2: Development of elastic (*G′*) and viscous (G″) moduli and phase angle (δ) during cooling from 45 to 10°C of treatments G, G + MTGase, G + 0.5% TYR and G + MTGase + 0.5% TYR at 5% (w/v) (A-C) and 6.67% (w/v) (D-F) of gelatin concentrations. Changes in the storage modulus *G′* (A, D), loss modulus *G″* (B, E), and phase angle δ (C, F) were monitored at a frequency of 1 Hz and plotted against time.

3.3.3 Effect of MTGase amination of gelatin on gelation rate (K_{gel}) and gel strength (G_N)

Determining the gelation rate and gel strength from kinetic models has been extensively used to understand the kinetics of structural changes during the gelation process (Eq. (1); Eq. (3)). A lower K_{gel} value predicts a longer time before the *G′* and *G″* reach equilibrium values (Kohyama and Nishinari, 1993), corresponding to the formation of a stable gel. The effect of the different

treatments on gelation rates and gel strength are summarized in Table 1. Overall, the $K_{gel,}$ for treatments with a gelatin concentration of 6.67% was greater than those with 5%. The addition of MTGase in the presence of absence of TYR at gelatin concentrations of 5 and 6.67% did not significantly reduce the K_{gel}. The gelation rate was mainly affected by the gelatin concentration rather than the addition of MTGase + TYR or TYR alone. Gel strength (G_N) analysis (Table 3.1) showed that, in general, the gel strength was greater in samples with a gelatin concentration of 6.67% compared to those with 5%. For instance, a G_N value of 7852 Pa for gelatin alone (G) at 6.67% gelatin concentration was greater ($p < 0.05$) than a G_N of 5055 Pa for 5% gelatin. Pang et al. (2014) reported that increased gelatin concentrations led to stronger gels due to more triple helical structures. The addition of MTGase (gelatin + MTGase) significantly decreased G_N, indicating MTGase negative impact on the gel strength. This is presumably due to the geometrical restrictions of the gelatin chain flexibility as a result of MTGase cross-links (Bode et al., 2011). These restrictions would disturb the normal formation of hydrogen-bonded triple-helix junction zones during cooling, despite the presence of a high density of the chemical network. Hence, the development of a physical polymeric network with elastic properties is inhibited. The results were consistent with those reported by Babin and Dickinson (2001) for acid-processed gelatin pre-treated with MTGase. Also MTGase-catalyzed amination reduced the gel strength of treatments (G + MTGase + 0.5% TYR; G + MTGase + 1% TYR) when compared to gelatin alone. These observations suggested that the incorporation of TYR in the gelatin molecule also have a

negative effect on the gel strength, likely due to the interference with a regular triple helix formation.

Figure 3.3 shows the time dependence of the storage (G') and loss (G'') moduli for four selected treatments: gelatin alone (5% (w/v) G, 6.67% (w/v) G) and MTGase-treated gelatins (5% (w/v) G + MTGase, 6.67% (w/v) G + MTGase) at 10°C, respectively. For all treatments G' was greater than G'' by more than 10-fold. During the 3 h holding time, G' rapidly increased in the first 23 min, where the treatments undergo a sol-gel transition due to partial refolding of gelatin single strands into triple-helix (Djabourov, 1988). Next, the evolution of G' over time reached a plateau, indicating the formation of a stable gel. Treatments with a gelatin concentration of 6.67% (G and G + MTGase) had greater G' at all times compared to those at 5%. This is consistent with findings from previous studies which have shown that the samples containing more concentrated gelatins exhibited a greater rigidity (Simon et al., 2003; Haug et al., 2004). TYR treatments in the presence or absence of MTGase did not significantly affect the evolution of G' (data not shown). This is consistent with the results reported in Table 3.1 and reflects the important effect of MTGase on the gels rheological properties.

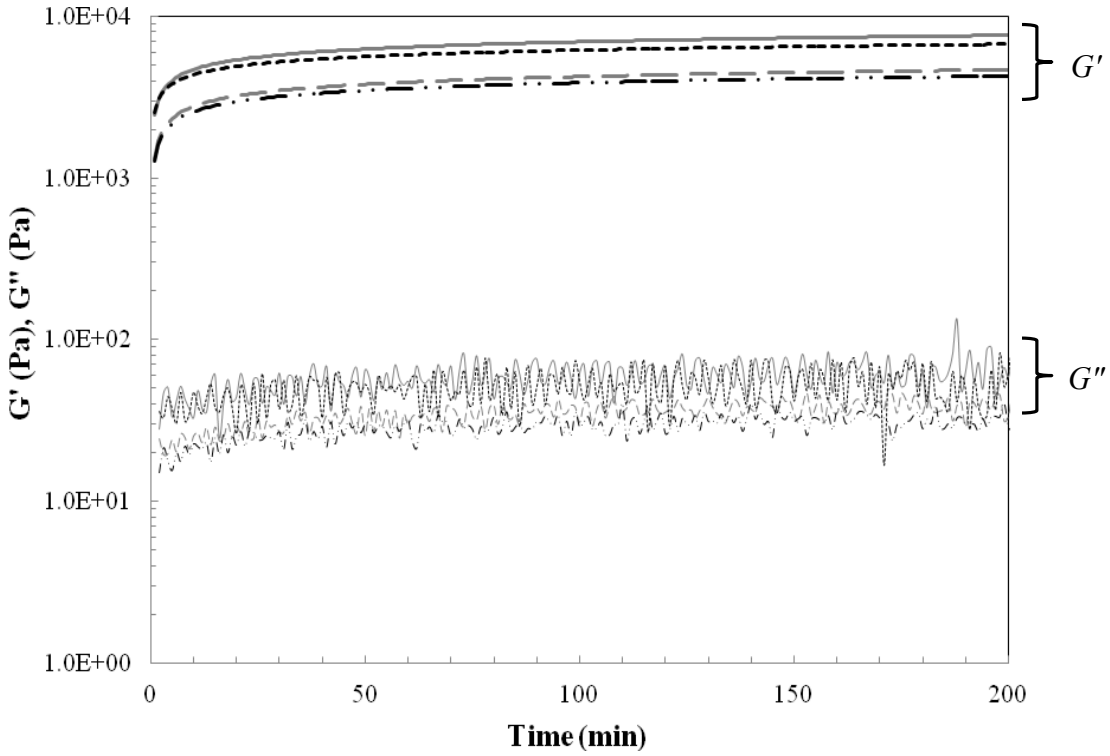

Figure 3.3: Time sweep curves of treatments: 5% (w/v) G (- - -), 5% (w/v) G + MTGase (-..-..-..), 6.67% (w/v) G (-) and 6.67% (w/v) G + MTGase (- - -) at a holding temperature of 10°C for 3 h. Evolution of storage modulus *G′* plots (thick lines) are always above the corresponding loss modulus evolution *G″* plots (thin lines).

Figure 3.4 shows the results of the frequency sweep measurements of selected treatments: gelatin alone (5% (w/v) G, 6.67% (w/v) G) and MTGase-treated gelatins (5% (w/v) G + MTGase, 6.67% (w/v) G + MTGase), respectively. In general, both moduli varied only slightly during the frequency sweep. The storage (*G′)* modulus value dominated the loss (*G″*) modulus, and the tan δ were much lower than 0.1, indicative of a solid-like nature for the formed gels. Greater values of both moduli were observed in treatments at 6.67% gelatin concentrations at every frequency, as a result of a greater concentration of helical

structures. This is consistent with the findings from previous studies, which have shown that an increased gelatin concentration contributes to a greater storage modulus (Gómez-Guillén et al., 2002; Simon et al., 2003). The addition of MTGase (G + MTGase) decreased the *G′* of gelatin gels (by about 500 Pa) in contrast to those treatments without MTGase (G). As explained before, the restriction in gelatin chain flexibility as a consequence of MTGase cross-linking may be the cause of this phenomenon.

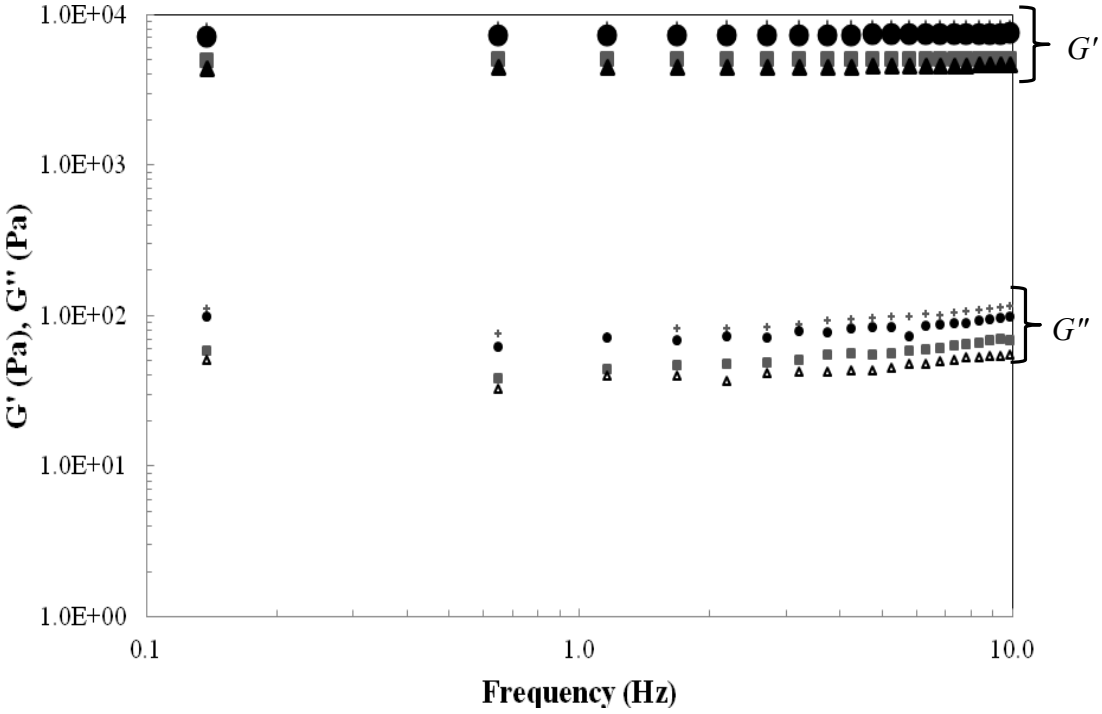

Figure 3.4: Frequency dependence of storage (*G′*, large symbols) and loss (*G″*) moduli of treatments: 5% (w/v) G (■), 5% (w/v) G + MTGase (▲), 6.67% (w/v) G (✚) and 6.67% (w/v) G + MTGase (●). The frequency was tested over the range from 0.01 to 10 Hz recorded at 10°C.

Table 3.1 Gelation rate (K_{gel}) and gel strength (G_N) of treatments during holding phase at 10°C for 3 h.

Treatments	K_{gel}		G_N	
	5% G	6.67% G	5% G	6.67% G
G	637 ± 13	1011 ± 37	5055 ± 28^{a}	7852 ± 15^{a}
G + MTGase	584 ± 31	912 ± 85	4568 ± 21^{d}	7343 ± 12^{c}
G + 0.5% TYR	607 ± 16	927 ± 59	4653 ± 15^{b}	7363 ± 28^{c}
G + MTGase + 0.5% TYR	626 ± 21	934 ± 33	4424 ± 20^{e}	7697 ± 15^{b}
G + 1% TYR	631 ± 24	903 ± 29	4622 ± 13^{bc}	7252 ± 45^{cd}
G + MTGase + 1% TYR	622 ± 5	923 ± 53	4575 ± 15^{cd}	7297 ± 26^{cd}

Logarithmic model equation: $G_t = K_{gel} \ln (t_{gel}) + C$ where K_{gel} = gelation rate constant.

Gel strength equation: $G_N = \frac{1}{I'}$ where G_N = Gel strength at end of 3 h holding period.

G_N values within the column with the same letter are not significantly different ($p > 0.05$).

All data is expressed as mean ± standard deviation (n = 6).

3.3.4 Effect of MTGase amination on gelatin's melting temperature

Figure 3.5 shows the melting temperature (T_m) of different treatments. No significant differences in the melting temperature of 5% and 6.6.7% (w/v) G were found with the values of 32.0 and 33.1°C, respectively. The addition of MTGase (G + MTGase) led to an increase in the melting temperatures to 34.1 and 34.5 °C for 5% and 6.67% gelatin, respectively. Norziah et al. (2009) reported that the addition of MTGase significantly increased the melting temperature of surimi extracted fish gelatin (EFG) to 17.5°C at the enzyme concentration of 5.0 mg/g as compared to untreated EFG (16.7°C). Consistent with those results, the current study shows an increasing trend of melting temperature due to the presence of covalent bonds induced by MTGase. In general, an increase of gelling and melting temperatures was expected due to transglutaminase-catalyzed amide bonds formation (Saito et al., 2007).

The T_m of treatments G + MTGase + 0.5% TYR and G + MTGase + 1% TYR and their controls (G + 0.5% TYR; G + 1% TYR) were not significantly different compared to gelatin alone (G) at both gelatin concentrations. This indicates that TYR did not induce a major change in the transition of gelatin from a helical network to single strands. However, MTGase amination with TYR decreased T_m values compared to treatment G + MTGase. This suggests that the introduction of cross-links between gelatins and TYR may have further hindered the triple helix junction zones. The resulting cross-link formation may have

caused coagulations inside of the gels; these coagulated chains were resistant to heat as indicated by lower melting temperatures (Kaewdang and Benjakul, 2015).

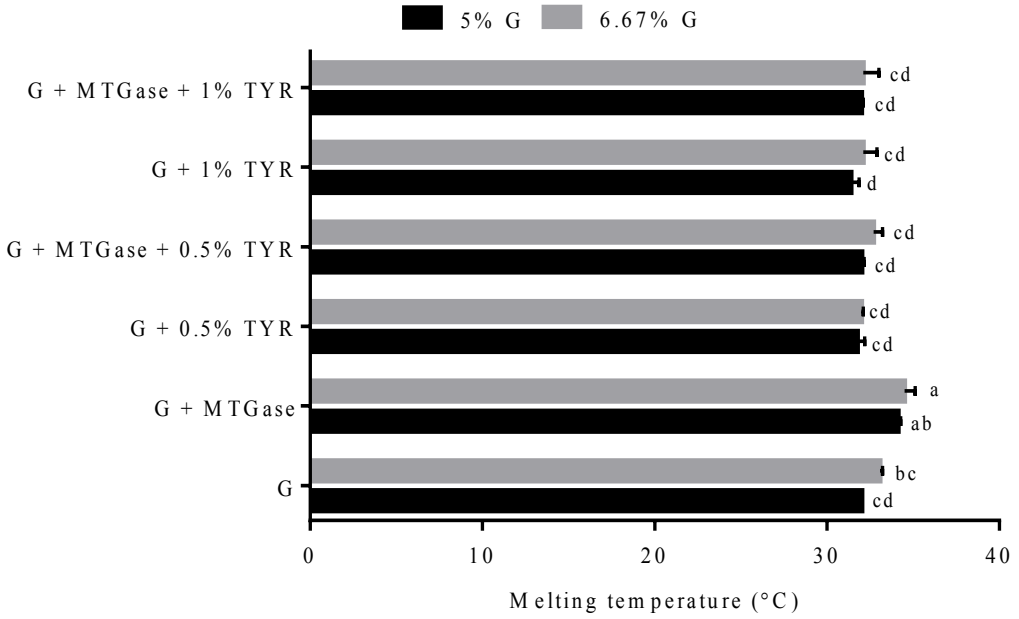

Figure 3.5: Melting temperatures (T_m) of the treatments with gelatin concentrations of 5 and 6.67% (w/v). The values were obtained during heating of the respective treatments from 10 to 45°C. Data are presented as mean ± standard deviation (n = 6). Values with different alphabets are significantly different ($p < 0.05$).

3.4. Conclusions

Viscoelastic properties of modified gelatins were investigated to determine the effects of MTGase-catalyzed amination of gelatin with TYR. The MTGase-catalyzed covalent incorporation of TYR perturbs the regular triple helical network in gelatin chains, where covalent cross-linking normally occurs randomly. The altered structure of the TYR-modified gelatins did not contribute

to the improvement of the gelatin gel rheological properties. However, incorporating a biogenic amine like TYR into these gels may increase their antioxidant activity as previously investigated by Lu et al., (2016).

Chapter 4: Conclusions, implications and future work

This research was conducted to modify food proteins and peptides by conjugating them with biogenic amines catalyzed by microbial transglutaminase (MTGase). MTGase is a commercially available food-grade enzyme that is widely applied to modify the structure and function of food proteins. Biogenic amines (BAs) are present in various food products and elevated consumption can lead to various types of food-borne diseases, including histamine-induced "scombroid syndrome" and "the cheese reaction" triggered by tyramine. The use of BAs as acyl acceptor substrates for MTGase catalysis (enzymatic amination) could be a practical solution to reduce BA content in foods, while at the same time changes the protein functionality and bioactivity in a food system. This research demonstrated that MTGase could be used to incorporate both heterocyclic and aromatic amines. Protein systems that were used included pea protein peptides produced through alcalase hydrolysis and pigskin gelatin, one of the most widely used animal hydrocolloids in both food and pharmaceutical industries. The studies conducted proved that both histamine and tyramine can be conjugated to pea protein peptides and tyramine can be conjugated to gelatin. The antioxidant capacity of pea protein peptides was improved as consequence of amination. The rheological properties of the gelatin were reduced from the MTGase-catalyzed amination. This research widens the application of MTGase catalysis for the food industry.

The objective of the first study was to produce, identify and characterize the formation of BA-pea protein peptides conjugates by using MTGase. For this purpose, histamine was added in the presence of MTGase and the conjugation was determined by liquid chromatography. Overall, 76% of histamine was covalently incorporated to pea protein peptides by the enzymatic reaction. The study also revealed the conjugation of TYR via MTGase improved the antioxidant ability of peptides by acting as free radical scavengers and reducing linoleic acid peroxidation.

The second study investigated the incorporation of TYR into porcine skin gelatins. The evaluation of MTG amination of gelatins was based on determining the rheological properties of gelation and melting temperatures, as well as gelation rate and gel strength. In order to accomplish this, two levels of TYR (0.5%, 1% (w/w)) and two levels of gelatins (5%, 6.67% (w/v)) were mixed in aqueous media at 50°C in the presence of MTGase. The gelation temperatures of MTGase cross-linked gelatins were markedly increased and reduced the time required for gelation, but these decreased upon TRY incorporation. Weaker gels were produced when gelatins were subjected to both TYR and MTGase.

Overall, this research demonstrated BAs can be potential substrates by MTGase and introduced into food proteins and peptides. A combination of glutamine-containing peptides together with MTGase has the potential to decontaminate fermented foods and beverages from BAs. Future work needs to focus on applications in fermented foods such as cheese, salami, beer and wine.

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