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Antioxidative and Antimicrobial Activity of Casein Hydrolyzate in Cheddar Whey-Based Edible Coatings

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By

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Hydrolysis of casein using chymotrypsin results in the formation of polypeptides (CH) with a hydrophobic aromatic amino acid on one end of the chain because the enzyme selectively cleaves the adjacent peptide-bond. Due to resonance of the aromatic micro-domain, thiols become redox-sensitive and actively participate in electron transfer. These types of amphipathic peptides also tend to be membrane-lytic. The two prong approach of this investigation was to, (1) assess antibacterial effect of the CH in beef steak, and (2), to determine its antioxidative efficacy as a constituent of Cheddar whey based edible coating mix. The edible coating prevented coliform growth even at a minute concentration range of 0.15-0.2 % (w/v). Marked antioxidative efficacy of the CH, particularly at a concentration of 0.3% (w/v), was also evident from its remarkable free radical scavenging ability and extended resilience in an abusive model system saturated with peroxyl-radicals generated through controlled pyrolysis.

Key words: Casein hydrolyzate, whey protein, antioxidant, antimicrobial agent.

DEDICATION

This dissertation is dedicated to all the people who spend their lives to eliminate human hunger.

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Since I was a child, I was deeply interested in science, and wanted to do something that would benefit mankind. Now that I am on the verge of completing my Master's Degree program, I feel like I have garnered new knowledge and skills. But at the same time, I faced many hurdles. I would like to profusely thank all those, who helped me not only to learn, but also to them who stood by me and taught me to overcome the obstacles that I faced during the tenure of my program.

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CHAPTER I

INTRODUCTION

Microbial and oxidative degradation cause a substantial amount of food spoilage, which remains a matter of grave concern across the world. Change of climate has a potential effect on food safety, including raw material microbiological contamination, foodborne diseases among different food chains, and substandard global trades (Tirado *et al.*, 2010). A number of other incidences also raise concerns regarding food safety issues. Deaths were reported in the United States due to infections caused by *Listeria monocytogenes* contamination in the year 2011 (Hoelzer *et al.*, 2012). Enterohaemorrhagic *E. coli* (EHEC) infections in Germany affected about 3,000 people the same year, causing deaths of more than 30 individuals (Robert Koch-Institute, 2011). An Ireland poultry feed contamination event affected almost the entire European population in 2011 (Patriarchi *et al.*, 2011).

According to the World Health Organization (WHO) statistics (Song *et al.*, 2005), approximately half of the population in developed countries (e.g., Europe and North America) suffers from various food-borne infections each year. These infections are the major causes of death in a number of developing countries (e.g., Middle East, North Africa and India), as well as across the world. Therefore, food safety is an extremely important issue, both in terms of economics, as well as medical points of view around the world. Food safety issues are causing challenges in food trade, and the availability of

high quality food products with reasonable prices. Food products with balanced nutritional status are extremely important for maintaining proper human health. Global food trade can considerably promote economic development as well as result in the improvement of people's living standards (Li *et al*., 2005).

Additions of synthetic food preservatives have been found to significantly reduce the extent of food spoilage both through oxidative and microbial degradations. However, it has been found that long term consumption of food items treated with such agents [e.g., butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT)] can cause unfavorable health related conditions in consumers (Pourmorad *et al.*, 2006).

A number of natural substances such as enzymatic hydrolyzate of casein (CH) are known to possess marked antioxidative property (López-Expósito *et al.*, 2007). This is presumably because of redox-activity of thiol groups in the peptides (Brandes *et al.*, 2002). These groups are particularly redox-sensitive when they are in the proximity of neighboring positively charged or aromatic residues (e.g., phenylalanine, tyrosine) (Antelmann & Helmann, 2011). Phelan *et al.* (2008) observed strong antioxidant effects of CH in terms of catalase activity.

The antimicrobial efficacy of CH has also been reported (López-Expósito *et al.*, 2007; Biziulevičius *et al.*, 2002; Benkerroum, 2010). Primarily, this property is due to the membrane-lytic activity of amphipathic peptides. Baranyi *et al.* (2003) showed strong efficacy of rabbit casein hydrolyzate to inhibit the growth of a number of gram positive bacteria.

Perishable food items, such as beef steak are highly susceptible to oxidative degradation, which is caused mainly by the presence of a larger surface area. Besides,

metal ions that are introduced during processing steps (e.g., cubing) further accentuate this problem. In addition, beef steak can be contaminated by a wide range of microbial genera such as coliforms. Consumption of food contaminated with such microorganisms can result in serious health related problems in humans, including death (López-Expósito *et al*., 2007).

Addition of antioxidants and antimicrobials can potentially reduce degradation which would be economically beneficial if the protection extends the refrigerated shelf life. Addition of even one to two days of additional storage life is a significant economic benefit for the retailer. It would be even more desirable if the preservatives are of natural origin due to increased consumer concerns of the potentially negative effects of long term consumption of synthetic preservatives. A number of natural agents have been identified with remarkable efficacy as preservatives. Haque *et al.* (2009) reported potential of sour whey protein to significantly extend shelf-life of cubed beef steak. Due to its remarkable antioxidative and antimicrobial properties, CH can conceivably be used as such a preservative to enhance the shelf-life of a wide range of food products such as beef steak.

Hypotheses: The current study accomplished two major goals:

1. Treating shoulder-cut beef steak with whey-based edible coating mix containing CH can significantly reduce coliform growth on it.
2. Addition of CH in a whey-based edible coating mix can significantly enhance its antioxidative efficacy.

Objectives: The objectives of this study were:

A. To determine the effect of casein hrdrolysate against coliform growth on shoulder cut beef steak.

B. To determine the intrinsic antioxidative efficacy and resiliency of CH in a whey-based edible coating solution by real-time luminometry study.

CHAPTER II

LITERATURE REVIEW

2.1 Microbial Degradation of Food and Antimicrobials in Food Preservation

It has been estimated that there are about 5000 deaths each year in the United States due to food-borne illness (Mead *et al.*, 1999). The main food contaminating organisms include *Escherichia coli*, *Listeria monocytogenes*, *Clostridium perfringens*, *Salmonella*, *Staphylococcus aureus* and so on. The antimicrobial agents that are commonly used for food preservation include nitrites, sulphites, and benzoic acids. However, long time consumption of food that has been treated with such chemical agents may have unfavorable effects on the consumers' health, and thus have been a subject of concern (Pokorny, 2007). Therefore, properties and applications of natural substances with antimicrobial functions are currently being investigated in greater details. Enzymatic hydrolyzate of casein (CH) and whey protein are examples of such natural agents. Due to the presence of specific peptides, they have been found to possess remarkable antimicrobial properties (Atanasova and Ivanova, 2010; Almaas *et al.*, 2011).

2.2 Oxidative Degradation of Food and Antioxidant Roles in Food Preservation

The process of oxidative degradation is responsible for the generation of free radicals in food systems. These are molecules with atoms that have unpaired electrons and are known to cause substantial reduction in the quality and shelf-life of various food

items (Li *et al*., 2005). The process is termed as oxidative degradation, which is also known to cause a number of serious health related problems in humans including atherosclerosis, aging and various types of cancer (Rival *et al*., 2001). Oxidative degradation particularly of lipid is known to be one of the main mechanisms in food quality deterioration, which, especially in meat products, has negative effects on flavor, color, texture and nutritive value (Gray *et al*., 1996). Addition of antioxidants (a substance with the potential of inhibiting the oxidation of other substances) to foods has been known to significantly reduce oxidative degradation resulting in considerable extension of shelf life of a diverse array of food products. At present, many synthetic antioxidants, such as 2-tert-butyl-p-cresol (used for preventing oxidative degradation of edible oil and dried fish products), hydroxyl ethers containing tert-butyl (for preserving edible oils and fats), vitamin E (baby food and powdered milk), vitamin C and its various derivatives (fish products and frozen food) and so on, are used for enhancement of food products. In addition, the United States Food and Drug Administration has also approved the use of a number of other antioxidants, such as ascorbyl palmitate, ascorbic acid, ethoxyquin, lecithin, sulfite, ascorbic stearic acid ester, sodium dithionite, sodium sulfite, stannous chloride, gallic acid, isoamyl and others.

Antioxidants must be added to food products in proper doses. For example, when tert-butyl hydroxyl anisole (BHA) is added at 0.01%, its effectiveness improves approximately 10% compared to a concentration of 0.02% (Parke and Lewis, 1992). In addition, mixing two or more antioxidants has been found to be better for increasing their efficacies. The addition of citric acid and butylated hydroxytoluene (BHT) increases the storage time of refined oil almost 100% than adding just BHT alone (Hu *et al*., 2004).

However, it needs to be mentioned that a number of synthetic antioxidants (e.g., BHT) have adverse effects on the health of the consumers (Wu *et al*., 2010).

BHA is known to induce second-stage fore stomach carcinoma and urinary bladder carcinogenesis in animal models, presumably by increasing the proliferation of epithelial cells and DNA synthesis in the target organs (Hirose *et al*., 1993). As a result, natural antioxidants are being studied in increasing detail in recent years. This CH has been known to possess strong antioxidative potential and has been studied for a long time (Li *et al*., 2005). In addition to its various potential uses including its use as a rich source of dietary protein, whey can also be used as a rich source of natural antioxidants – both in terms of dietary and preservative purposes (Bounous *et al*., 1989; Wu and Zhao, 2009).

2.3 An Overview of Whey Protein

Whey is a byproduct of the cheese manufacture. The variety of essential amino acids present in it can serve as a source of complete protein. Among the plant proteins, only those derived from soybeans can serve as the source of a complete protein, but adsorption of soy protein by the human digestive system is less compared to animal proteins. Whey protein contains amino acids in reasonable ratios that are required to fulfill the needs of human body, such as growth, development, and other important biological functions including reduction of the process of aging (Liu *et al*., 2004).

2.3.1 Industrial Source of Whey Protein

Whey protein makes up to 20% of milk protein (casein accounting for the remaining 80%). Whey protein is extracted after the process of coagulation of milk during cheese manufacture. It is further processed by drying and the protein content

increases after the removal of lipids and various non-protein components of milk (Pu and Liu, 2005).

2.3.2 Nutritional Characteristics of Whey Protein

Whey protein has the highest nutritional value among a wide range of proteins. It can be termed as the 'complete protein' due to the presence of essential amino acids in reasonable ratios (Sharon *et al*., 2009). It can also be more easily absorbed by human digestive tract. In addition, whey is rich in cysteine and methionine, which serve as glutathione and methyl-group donors. DNA hypomethylation is known to be an important factor that induces carcinogenesis in certain cells of the body. The glutathione precursors derived from the above mentioned sulphur containing amino acids present in whey conceivably play a role in the ability of dietary whey protein to stimulate both humoral and cell-mediated immune responses in animal models (Parodi, 2007). Growth factors present in whey (e.g., basic fibroblast growth factor and transforming growth factor-β) exhibit anti-carcinogenic activity at very low concentrations.

Whey contains very little fat and lactose, but is rich in β-lactoglobulin, α-lactalbumin, immunoglobulins, and a number of other active ingredients. These active components play important roles for preventing a number of health-related problems of the human body. It is therefore considered to be one of the required sources of high quality proteins for humans (Baer, 2009). Whey protein enzymatic extract (whey protein hydrolyzate) can lead to quicker synthesis of muscles in the human body (Parodi, 2007).

Many animal protein-based foods may contain excessive amounts of saturated fat, cholesterol and other harmful substances. Excessive consumption of which may lead to obesity and hypercholesterolemia, resulting in cardiovascular diseases. Consumption of

whey protein powders can complement protein requirements while substantially reducing the severity and onset of these health issues.

2.3.3 A Few Important Whey Protein Peptides

A few main proteins in whey protein are β-lactoglobulin, α-lactalbumin, immunoglobulin and lactoferrin (Marshall, 2004). B-lactoglobulin is rich in branched-chain amino acids, and plays an important role in increasing glucose and glycogen uptakes in L6 myocytes and isolated skeletal muscles, respectively (Hulmi *et al.*, 2010). It is also thought to increase bioavailability of carbohydrates and reduce muscle protein breakdown (Walzem *et al.*, 2002). A-lactalbumins are also excellent sources of essential amino acids and branched chain amino acids (Marshall, 2004). They are the only peptides derived from whey protein that can be combined with calcium ions (Lönnerdal and Glazier, 1985). Recent studies have found that they may have anti-cancer properties (López-Expósito *et al.*, 2007). Furthermore, the amino acid sequences and functional properties of α-lactalbumin and breast milk are highly identical (Marshall, 2004). Clinical studies have shown that α-lactalbumin can be safely administered in infant formula (Marshall, 2004). Immunoglobulins are antibody proteins that play important roles in mucosal immunity (Pilette *et al.*, 2001). Lactoferrins have antioxidative activity, and play a critical role in destroying or inhibiting bacterial infection, thus improving immunity (Mulder *et al.*, 2008; Actor *et al.*, 2009). They stimulate the immune system and decrease inflammatory tissue damage (Ha and Zemel, 2003) – which are of crucial importance to athletes, who are vulnerable to such situations due to the immunosuppressive effects of vigorous training (Nieman, 2000). In addition, lactoferrins also play a vital role in iron metabolism (Iyer and Lönnerda, 1993) and development of bones (Naot *et al.*, 2005).

2.3.4 Antimicrobial Properties of Whey Protein

Whey proteins are rich in glutamine precursors, providing raw materials for gluconeogenesis, thus maintaining levels of glutamine and protecting the immune cell functions. In addition, the lactoferrin and immunoglobulin components of whey protein have marked antibacterial and antiviral properties (Pourmorad *et al.*, 2006).

Almaas *et al.* (2011) reported that the human gastrointestinal (GI) tract enzymes generate different peptides by hydrolyzing caprine whey compared to enzymes derived from non-human origin, and the hydrolyzates exhibit better antibacterial effects compared to the pure peptides. The GI tract enzymes are conceivably more complex, possessing multiple cleavage sites in their protein chains which are absent in the purified non-human enzymes. It has also been shown that the peptides derived from whey protein by means of digestion with the human GI tract enzymes exhibit their antimicrobial effects against the pathogens, but not against the probiotic organisms (Almaas *et al.*, 2011).

A number of peptides present in whey (e.g., β-lactoglobulin, α-lactalbumin, lactoferrin and glycomacropeptide) are known to play main roles against the development of diseases and infections (Gauthier *et al.*, 2006). Peptides derived from the digestion of β-lactoglobulin with trypsin and chymotrypsins are known to possess remarkable activity against Gram-positive bacteria (Pellegrini *et al.*, 2001), while lactoferrin plays a role against both Gram-negative and Gram-positive bacteria such as *Listeria monocytogenes* (Bellamy *et al.*, 1992).

2.3.5 Antioxidative Properties of Whey Protein

Two polypeptides present in whey, namely lactoferrin and lactoferricin play the pivotal roles in the antioxidative efficacy of whey via their ability to bind with ferrous

ions (Ha and Zemel, 2003). Lactoferrin inhibits the process of oxidation stress by means of its chelating capacity. Another contributing factor to the antioxidative efficacy of whey is its rich content of cysteine containing proteins. These are converted to glutathione which functions as a major intracellular antioxidant (Walzem *et al*., 2002). Plasma glutathione content was found to significantly increase in HIV patients who regularly consumed whey supplements (Micke *et al*., 2001).

2.4 An Overview of Casein and Casein Hydrolyzate

The unique amphipathic nature of β-casein is thought to be responsible for its functional properties (Dickinson, 1999). A wide range of bioactive peptides are synthesized upon its enzymatic hydrolysis. Bioactive peptides can be defined as protein fragments that are inactive in precursor forms but are able to regulate the biochemical and physiological functions upon enzymatic digestion (Meisel, 1997). Biologically active peptides are also recommended as functional food components (Korhonen and Pihlanto, 2003). Enzymatic hydrolyzate of casein phosphopeptides (CPP) are used for preventing the loss of minerals, dental caries, osteoporosis, rickets. They are also important for maintaining the proper balance of various mineral elements such as calcium, magnesium, iron, nickel, zinc and copper. In addition, they serve as an extremely good mineral carrier (Walzem *et al*., 2002). Also the angiotensin converting enzyme (ACE) inhibitory peptides also exhibit potent anti-hypertensive properties (Darewicz *et al*., 2006). These are released by enzymatic digestion of food proteins and are adsorbed and transported to the cardiovascular system. ACE-inhibitory activity is particularly enhanced by the enzymatic digestion of fermented casein solutions (Vermeirssen *et al*., 2003). These types of antihypertensive peptides generated from α s1- and β-casein are known as casokinins

(Masuda *et al*., 1996; Yamamoto *et al*., 2003). Casein hydrolyzate also contains opioid peptides, which exert a number of other important biological functions, like changes in body temperature, sensation of satiety and regulation of food intake (Tome and Ledoux, 1998; Meisel, 1997; Raha *et al*., 1998; Gill *et al*., 2000).

2.4.1 Antimicrobial Properties of Casein Hydrolyzate

It has been reported by some that pathogens are becoming increasingly resistant to antibiotics (Teuber, 1999; Food and Drug Administration, 2011). So development of new antimicrobial agents is of extreme importance. Antimicrobial efficacy of peptides derived from casein hydrolyzate is currently being extensively studied (Phelan *et al*., 2008). Such antimicrobial peptides are examples of food-borne bioactive compound that can potentially serve as new antibacterial agents for food preservation. Numerous authors have studied the marked antimicrobial properties of casein hydrolyzate. Both the glycosylated and non-glycosylated forms of a peptide derived from casein hydrolyzate were found to effectively inhibit the growth of *Enterococcus faecalis* (Liu *et al*., 2012). Antibacterial peptides are also released upon proteolysis of casein. Chymosin digestion of casein leads to the release of casecidins, a group of basic, glycosylated and high molecular weight (~ 5 kDa) polypeptides, which exhibit high bactericidal efficacy against *Staphylococcus aureus* (Lahov and Regelson, 1996). Another peptide isracidin, exerting *in vivo* protective actions against *Staphylococcus aureus, Streptococcus pyogenes*, and *Listeria monocytogenes* corresponds to the N-terminal end of α s1-casein (Lahov and Regelson, 1996).

2.4.2 Antioxidative Properties of Casein Hydrolyzate

The antioxidative properties of the casein hydrolyzate have been studied by many authors. Rival *et al*. (2001) reported an inhibition of enzymatic and nonenzymatic lipid peroxidation mediated by caseins and casein-derived peptides indicating their roles as targets for various free radical species. Dreher and Junod (1996) reported an oxygen radical absorbance capacity of the bovine κ-casein hydrolyzate. Peptides derived from ovine κ-casein were found to be strong inhibitors of linoleic acid oxidation (Gómez-Ruiz, 2008).

2.4.3 Advantages of combined use of whey protein and casein

A new clinical trial by Rasmussen *et al*. (2006) showed that eating a combination of proteins (soy, whey and casein protein) after exercise had the best muscle tissue building potential (Gómez-Ruiz *et al*., 2008). The combined consumption of these three proteins was found to extend the 'anabolic window' of the body (the time required to start building muscle tissue after exercise).

The rates of digestion and absorption of the above mentioned proteins vary considerably. Whey protein is known to be the 'fast' protein as it is absorbed at the fastest rate, while casein is called the 'slow' protein, because it takes the longest to digest (Hall *et al*., 2003). The conveying capacity of amino acids of soybean protein is in the 'medium' range, meaning that the concentration of soy protein in the blood reaches a peak later than whey protein, but before casein. Therefore, the combined effect of these three proteins can extend the time of conveyance of amino acids to muscle tissue for muscle development (Halliwell, 1994).

2.5 Antioxidant measurement methods

2.5.1 TRAP Assay

The total radical-trapping potential (TRAP) method is one of the most employed techniques to evaluate the antioxidative efficacy of a biological substance as developed by Wayner *et al.* (1985). It is relies on the measurement of induction times during oxidation of a lipid dispersion subjected to a source of free radicals that generates a constant and known rate of radicals in aerobic condition. 2, 2'-Azo-bis (2-amidinopropane) (ABAP) is used as the source of free peroxyl radicals, and luminol is used as the inducer of chemiluminescence. The general mechanism of luminol induced chemiluminescence has been described by Yamamoto *et al.* (1987) as follows:

$$\begin{aligned} &LOOH + \text{microperoxidase} \rightarrow LO^{\cdot} \\ &LO^{\cdot} + \text{isoluminol } (QH^{-}) \rightarrow LOH + \text{semiquinone radical } (Q^{\cdot -}) \\ &Q^{\cdot -} + O_2 \rightarrow Q + O_2^{\cdot -} \\ &Q^{\cdot -} + O_2^{\cdot -} \rightarrow \text{isoluminol endoperoxide} \rightarrow \text{light} \end{aligned} \qquad (2.1)$$

The luminescence values are used to compare the antioxidative efficacies of the test antioxidants. Lissi *et al.* (1995) studied the mechanism of luminol induced chemiluminescence mediated by ABAP pyrolysis and concluded that it is driven by the production of luminol derived radicals generated from its reaction with peroxyl radicals:

$$\begin{aligned} &ABAP \rightarrow 2\,R^{\cdot} \\ &R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \\ &ROO^{\cdot} + LH2 \rightarrow ROOH + LH^{\cdot} \end{aligned} \qquad (2.2)$$

At the final stage of the reaction, the luminol derived radicals (LH·) comes to equilibrium with the concentration of radical anions (L-). The ability of a component to quench the luminol-derived luminescence is defined as its capacity to 'trap' luminol-derived and/or ABAP-derived radicals. The effect exhibited by antioxidants at low concentrations conceivably takes place due to the quenching of LH· radicals (derived from luminal) resulting from the rapid quenching of peroxyl radicals generated by ABAP by luminal (Lissi *et al*., 1995).

2.5.2 DPPH (2, 2-diphenyl-1-picrylhydrazyl) Assay

The DPPH method has been found to be highly effective in determining antioxidative efficacy of food products (Mishra *et al*., 2012). It can be performed easily and the results are considerably reproducible and can be compared to those from other techniques such as ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)], reduction of superoxide anions and inhibition of lipid peroxidation. The structure of DPPH and the process of how it gets reduced by an antioxidant are shown in Fig. 2.1. A purple color is emitted by the unpaired electron present in the DPPH free radical which shows a strong absorption maximum at 517 nm in methanol. DPPH gets reduced to DPPH-H as its unpaired electron gets paired with an H+ ion from an antioxidant. This leads to a reduction in the molar absorptivity of DPPH, and thus its color changes from purple to yellow. This change in color is equivalent to the amount of reactants and products from the reaction and the number of electrons absorbed. This method has been extensively applied to the study of antioxidative activities of food items, such as olive oil, fruits, juices and wines (Gorinstein, *et al*., 2003; Heinonen, *et al*., 1998; Llorach, *et al*., 2003; Sánchez-Moreno, *et al*., 2003).

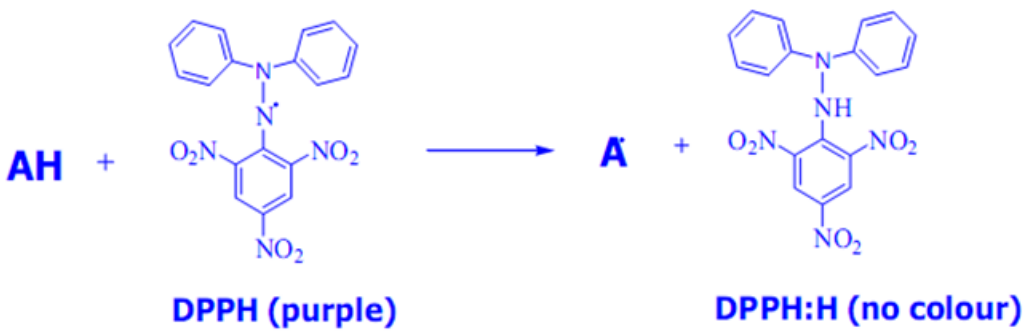

Figure 2.1 Structure of DPPH and its reduction by an antioxidant

AH: antioxidant compound; DPPH: 2, 2-diphenyl-1-picrylhydrazyl

CHAPTER III

MATHERIALS AND METHODS

3.1 Materials

3.1.1 Antimicrobial Activity of Casein Hydrolyzate (CH)

Calcium chloride, peptone, proteose-peptone, β-lactose, sodium chloride, crystal violet, neutral red, bile salts and bacteriological agar were purchased from Sigma-Aldrich (Milwaukee, WI). The cheddar whey powder was obtained from the Mississippi State University dairy plant. Konjac root glucomannan was obtained from Konjac Food (Sunnyvale, CA). Sorbitol was purchased from Archer-Daniels-Midland (Decatur, IL); carboxymethyl cellulose was purchased from FMC-BioPolymer (Princeton, NJ). CH was prepared by chymotrypsin (EC 3.4.21.1) digestion of freshly prepared acid casein curd by the method described by Haque *et al*., (1993). PetrifilmTM Coliform Count Plates were purchased from 3M Microbiology Products (St. Paul, MN) and the Coliform Test Kits were obtained from LaMotte Co. (Chestertown, MD). Fresh shoulder-cut beef steak was purchased with different batches from a local grocery, Piggly-Wiggly (Starkville, MS).

3.1.2 Antioxidative activity of CH

2, 2'-Azo-bis (2-amidinopropane) (ABAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Trolox were purchased from Sigma-Aldrich (Milwaukee, WI). Black, clear

bottom 96 well plates were purchased from Fisher Scientific (Hanover Park, IL). All the other reagents were obtained from the same sources as mentioned above.

3.2 Methods

3.2.1 Preparation of the Coating Solution

The whey-based edible coating solution was prepared by the method described by Shon and Haque (2007) (Fig. 3.1). The components of the coating solution included 5% (all the concentration mention in this following paragraph is in weight/volume–w/v) cheddar whey powder, 2.5% sorbitol, 0.25% glucomannan, 0.125% calcium chloride, 0.25% carboxymethyl cellulose and different concentrations of CH. The components and CH (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4%, w/v) were dissolved in distilled water. A vacuum aspirator was used for degassing the solution, followed by heating it at 90°C for 30 min. The solution was then homogenized with a sonicator for 2 min and filtered through five layers of cheese cloth, cooled to 22°C, degassed for a second time and the final pH was adjusted to 7.0 with 0.1 and/or 1M NaOH. It was stored at 4°C for a short period of time (about 3 – 4 hours) until to be used for coating specific food products for preservative purposes. The process is depicted in Fig. 3.1.

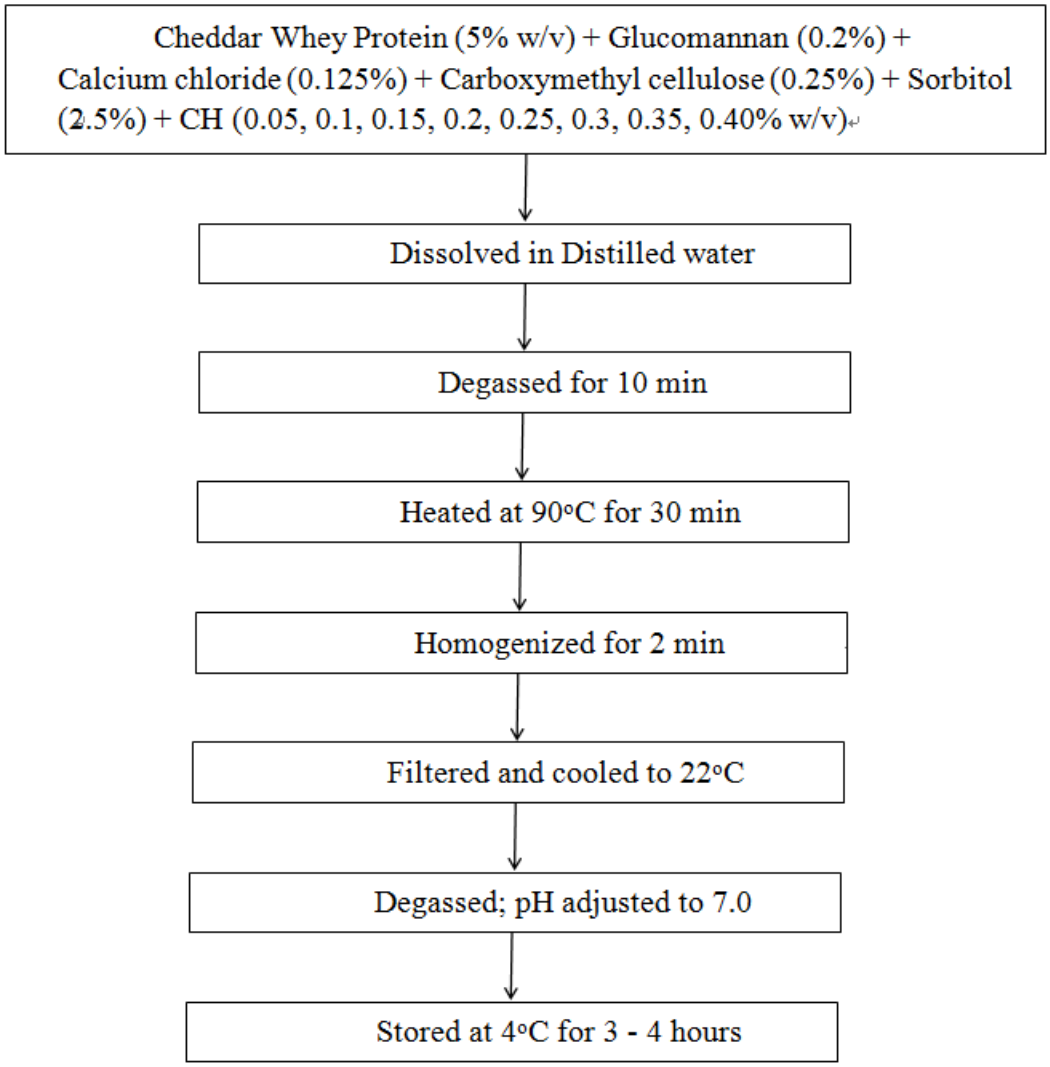

Figure 3.1 Preparation of whey-based edible coating solution (Shon and Haque, 2007)

3.2.2 Preparation of MacConkey Agar

MacConkey agar (MacConky, 1908) was used for culturing coliform bacteria. The components of the media including 17 g peptone, 3 g proteose-peptone, 10 g β-lactose, 5 g sodium chloride, 1 mg crystal violet, 30 mg neutral red, 1.5 g bile salts and 13.5 g bacteriological agar were dissolved in distilled water and the final volume was brought to 1 L in a 1 L volumetric flask with distilled water. The pH was adjusted to 7.1

± 0.2 with 0.1M and/or 1M HCl. Finally it was autoclaved at 121°C for 15 min for sterilization (Allen *et al.*, 2005).

3.2.3 Sample Preparation

Fresh shoulder cut beef steak (chuck) samples (different batches) of equal weight (10 g) and uniform geometry were immersed for five minutes in the whey based edible coating solution containing varying concentrations of CH as described previously. Samples immersed in the coating solution containing no CH were used as controls. Three replicates were used for each treatment. Treated samples were air dried for five minutes, incubated at 37°C in a Thermolyne 5000 incubator (Thermolyne, Dubuque, IA) for 48 hours in sterile plastic petri-plates (Fisher Scientific, Hanover Park, IL), blended using a sterile Oster food processor (Sunbeam Products, Boca Raton, FL) in 90 mL of distilled water (leading to a final dilution of 10%, w/v) and extracted by filtration.

3.2.4 Coliform Counts

Each aqueous extract was used to produce three samples of different dilutions (10^{-2}, 10^{-3} and 10^{-4}, respectively) and plated on McConkey agar plates. The plates were incubated at 37°C for 48 hours. Coliform counts for each sample were determined by the total plate count method. Presence of coliform was further confirmed by using PetrifilmTM Coliform Count Plates (3M Microbiology Products, St. Paul, MN) and the Coliform Test Kits (LaMotte Co., Chestertown, MD).

3.2.5 Data Analysis

The completely randomized design was used on three replicates and the ANOVA table was conducted to assess whether the increase in CH concentrations had any

negative impact on coliform growth. They were conducted using the statistical software program package Statistical Applicatory System (SAS) version 9.2 [SAS Institute Inc., (2006), SAS Institute SAS 9.1.3 Language Reference Concepts 3rd Edition, Cary, NC].

3.3 Antioxidative Activity of Casein Hydrolyzate

3.3.1 Total Radical Antioxidative Potential (TRAP) Assay

3.3.1.1 Sample Preparation

Black, clear bottom 96 well plates were used for running the assay. Coating solutions with three different concentrations of CH (0.1, 0.2 and 0.3%, w/v) were prepared by the method described in section 3.1.2.1. Each reaction mixture contained 100 µL each of whey-based edible coating solution (with no CH), 400 mM ABAP - used as a peroxyl radical generator (Sigma-Aldrich), 10 mM luminol (Sigma-Aldrich) dissolved in 20 mM McIlvaine's iso-ionic buffer (pH 7.0) - as the inducer of chemiluminescence and the coating solutions with dissolved CH (which were used as the test samples). McIlvaine's buffer containing no CH was used as the control. Two standards were used for the study,

1. The same whey-based edible coating solution containing no CH (the negative standard), and
2. 0.05% Trolox (w/v) – a water soluble vitamin E derivative dissolved in the coating solution (the positive standard).

3.3.1.2 Measurement of Chemiluminescence

Chemiluminescence was measured by a modification of the method described by Haque *et al.*, (2013). An IVIS 100 Imaging System (Caliper Life Sciences, Inc.,

Hopkinton, MA) was used to record the chemiluminescence at 3 minute intervals, at 37°C for 1 hour. Total flux (number of photons emitted per second, p/s) obtained from the luminescence studies was used to directly measure the TRAP of the samples studied. A lower flux, evidenced by lower luminescence, indicates better antioxidative potential (higher TRAP).

The following two parameters, deduced from the total flux data, were used to analyze the antioxidative potential of CH.

1. QT_F (final quenching time) - This can be defined as the time required for the test antioxidant to reach the maximum luminescence (luminescence maxima, L_{max}). This value reflects the period of time until which it shows viability, i.e. maintains its antioxidative efficacy. A more extended antioxidative potential (prolonged viability) is indicated by a higher QT_F value. The period of time between the first recorded luminescence to the QT_F can be referred to as the radical "leakage" time of the test antioxidant (Haque *et al.,* 2013; Mukherjee *et al.,* 2012).
2. ΔQT_F - This value indicates the difference in the time required to attain luminescence maxima by the test antioxidant sample relative to that of the control. ΔQT_F can be used for an accurate measurement of the period of time until which the activity of the test antioxidant is effective, compared to the control. Antioxidant activity is denoted by a positive ΔQT_F value (Haque *et al.,* 2013; Mukherjee *et al.,* 2012).

$$\Delta QTF = QT_{F\text{-Sample}} - QT_{F\text{-Blank}} \quad (3.1)$$

3.3.2 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Assay

3.3.2.1 Sample preparation

3.3.2.1.1 Standard samples

DPPH solution (0.1 mM) was prepared by dissolving 39.43 mg of DPPH in 1L of absolute ethanol. Trolox standard stock solution (10 mM) was prepared by dissolving 25.3 mg of Trolox in 10 mL of absolute ethanol. Next 200, 150, 100, 75 and 37.5 µM series solutions were prepared from the same Trolox stock solution (Alam *et al.*, 2012). A calibration curve with linear relationship between Trolox concentration and free radical scavenging rate was established according to the Colorimetric Method (Caillet *et al.*, 2006).

3.3.2.1.2 Test samples

The same whey-based edible coating solution containing no CH prepared by the method described in the section 3.1.2.1 was used as the control. Samples containing varying concentrations of CH (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5%, w/v) were prepared by dissolving CH in McIlvaine's iso-ionic buffer (pH 7.0).

3.3.2.2 Experimental Procedures

The radical scavenging capacity of the coating solutions and CH samples were evaluated according to the method described by Xu and Chang (2007). A dose of 0.2 mL of the test sample (coating solutions with different concentrations of CH) was added to 3.8 mL of DPPH-ethanol solution. The mixture was shaken vigorously and continuously for 1 minute by vortexing and left to stand in the dark for 30 min in an incubator at 22°C (Thermo-Scientific, Marietta, OH). After that, the absorbance of the sample (A_{sample}) was

measured using the Thermal BIOmate spectrophotometer (Thermo Electron Corp., Madison, WI) at 517 nm against the ethanol blank. A negative control ($A_{control}$) was taken after adding 3.8 mL DPPH solution to 0.2 mL of the 100% ethanol. The percent of DPPH discoloration (free radical scavenging rate) of the sample was calculated according to the following equation:

$$\text{Percent discoloration} = [1 - (A_{sample}/A_{control})] \times 100 \quad (3.2)$$

The free radical scavenging activities of the samples were expressed as equivalents to that of Trolox. Three replicates were taken for each of the samples, and the results were calculated and expressed as micromoles (μmoles) of Trolox equivalents (TE) per gram of sample using the calibration curve of Trolox. Linearity range of the calibration curve was 0 to 400 μ M (r = 0.99).

3.3.2.3 Data Analysis

The analyses using a completely randomized design were conducted on eight samples of each treatment for three replications. A series of t-tests were conducted to assess whether the total flux values for the control and the various samples (including the standards) were significantly different. Duncan's multiple range tests was carried out to test any significant differences between different CH concentrations ($\alpha = 0.05$). These analyses were conducted using the statistical software program package SAS version 9.2.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Coliform counts

The results showed a gradual decrease in coliform counts in MacConkey agar with increases in CH concentration. This trend continued up to a CH concentration of 0.2% (w/v) and then the number of colonies gradually increased (Fig. 4.1). This shows a negative correlation of CH concentration and coliform growth at concentrations ≤0.2% (w/v). At the lowest concentration of CH (0.05%), no marked effect of CH on coliform growth was observed. The average number of CFUs of the plates representing beef steak samples treated with 0.05 and 0.1% CH were 1.15×10^5 and 3.07×10^4 CFUs /mL, respectively (Appendix I). The best results were observed at CH concentrations of 0.15 – 0.2%, which resulted in least number of colony growth (< 10 CFU/mL). These results were significantly lower than all the other CH concentrations. However, at concentrations above 0.2%, CH seemed to have lost its effectiveness in inhibiting coliform growth since marked gradual increases in colony growth were noted at the CH concentrations ranging from 0.25 – 0.4%. On an average, 4.83×10^3, 6.30×10^2 and 4.10×10^4 CFUs/mL were counted from plates prepared from beef steak samples treated with 0.25, 0.3 and 0.35% CH, respectively (Fig. 1).

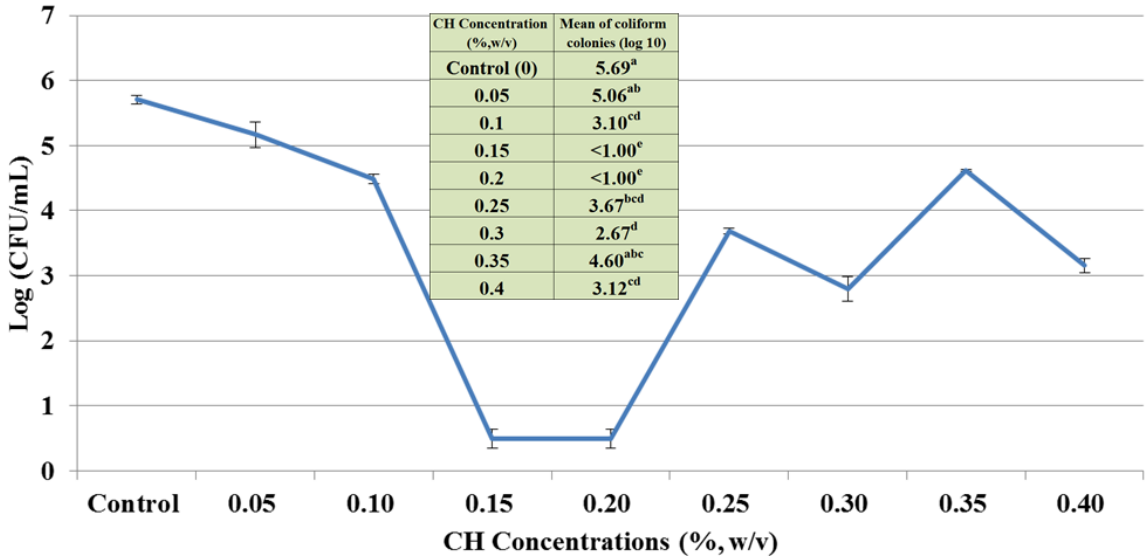

Figure 4.1 Coliform counts [Log (CFUs/mL)] in MacConkey agar in response to increasing concentrations of casein hrdrolyzate from 0 to 0.40 percent

A, b, c, d, e means with the same letter are not different ($P<0.05$).

It can be theorized from the results that beyond a certain concentration of CH (0.15 – 0.2%, w/v), clustering or micelle formation of the peptides in the CH takes place (Donato *et al.*, 2007). Thus, the total number of particles in CH is reduced resulting in a reduction in bacterial cell lysis.

4.2 Results from the Chemiluminescence Studies

Two parameters are relevant in the chemiluminescence study, namely (1) luminescence maxima (p/s), and, (2) the time required reaching these maxima. A lower maximum indicates better antioxidative activity, and time, reflects antioxidative resilience (Haque *et al.*, 2013). A sharp initial increase in luminescence (photon yield) was observed for all the samples studied. This was termed the initial maxima (I_{max}) that occurred due to an initial unquenched increase in peroxyl radical concentration (Haque *et al.*, 2013; Mukherjee *et al.*, 2012) (Figs. 4.2 and 4.3). This photon yield decreased

shortly after, followed by a second, more prolonged increase in photon yield (luminescence maxima, L_{max}). This slowly led to peroxyl radical depletion over time as evidenced by the gradual decrease of luminescence.

A series of t-tests were conducted to assess whether the mean luminescence (total flux) values for the control were different ($p \leq 0.05$) from the treatments (the coating solutions containing different concentrations of CH), as well as whether the values for the various treatments were significantly different from both the standards (the coating solution without CH and Trolox). The results are summarized in Tables 4.1 (comparison of mean photon yield in the CH samples with those of the blank, control and Trolox standard) and 4.2 [comparing the mean total flux values of CH, with only the control and Trolox standard (with the photon yield value in the blank eliminated)].

Table 4.1 Total Radical Trapping Potential of CH compared to blank, control and Trolox standard

Samples	Mean total flux (p/s) value
Blank	$(2.9\pm0.08)*10^{7\ a}$
Coating solution without CH (Control)	$(3.7\pm0.13)*10^{5\ b}$
CH (0.1%, w/v)	$(5.7\pm0.15)*10^{5\ b}$
CH (0.2%, w/v)	$(5.4\pm0.12)*10^{5\ b}$
CH (0.3%, w/v)	$(5.2\pm0.08)*10^{5\ b}$
Trolox (0.05%, w/v)	$(4.0\pm0.10)*10^{5\ b}$

A, b means with the same letter are not different ($P<0.05$).

Table 4.2 Total Radical Trapping Potential of CH compared to the control and Trolox standard

Samples	Mean total flux (p/s) value
Coating solution without CH (Control)	$(3.7\pm0.13)*10^{5\ c}$
CH (0.1%, w/v)	$(5.7\pm0.15)*10^{5\ a}$
CH (0.2%, w/v)	$(5.4\pm0.12)*10^{5\ ab}$
CH (0.3%, w/v)	$(5.2\pm0.08)*10^{5\ b}$
Trolox (0.05%, w/v)	$(4.0\pm0.10)*10^{5\ c}$

A, b, c means with the same letter are not different ($P<0.05$).

Results from the t-tests indicated that the mean luminescence for the control and the various samples (including the standards) were significantly different (Table 4.1). These analyses suggest that addition of CH can significantly alter the antioxidative efficacy of the coating solution by postponing the time required for maximum radical quenching in an abusive condition of free peroxyl radical proliferation.

The change in mean photon yield of the blank, control, CH samples and Trolox standard over time is depicted in Fig. 4.2.

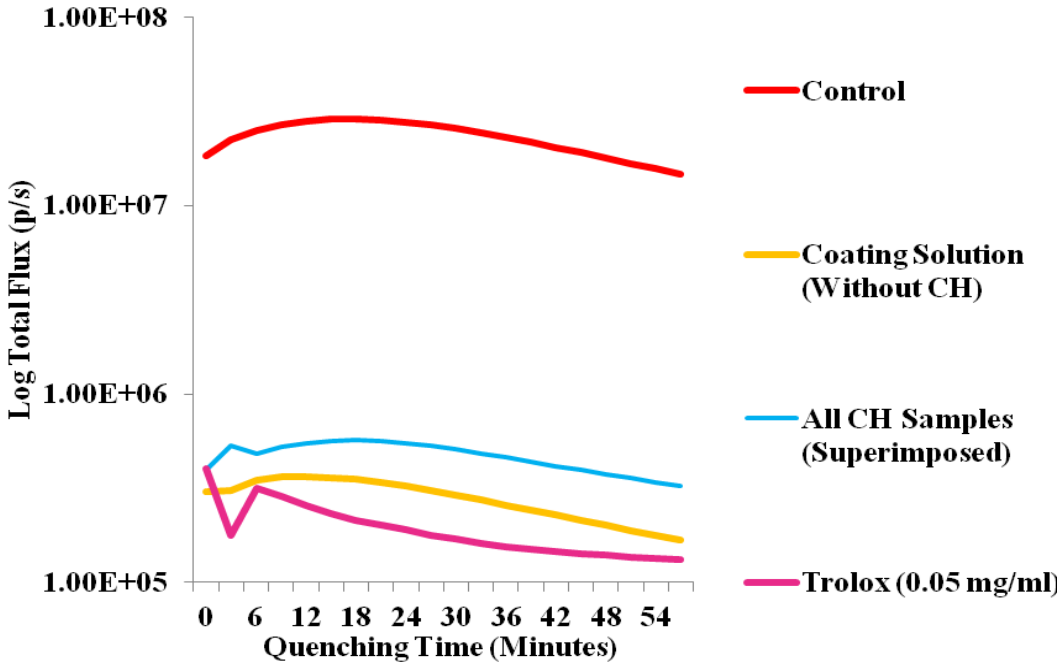

Figure 4.2 Change of total flux (p/s) over time in coating solutions with and without casein hydrolyzate (CH) compared to control.

The L_{max} (p/s) of control, coating solution without CH, CH (0.1%, w/v), CH (0.2%, w/v), CH (0.3%, w/v) and Trolox are $2.89*10^7$, $3.66*10^5$, $5.66*10^5$, $5.44*10^5$, $5.22*10^5$ and $4.01*10^5$, respectively.

The luminescence values of the individual CH samples were found to be superimposed on each other when their total flux values were compared to the control (Fig. 4.2). Since the total flux of the blank was two orders of magnitude higher than the treatments and the control, its trace was removed from Fig. 4.3 for better comparison.

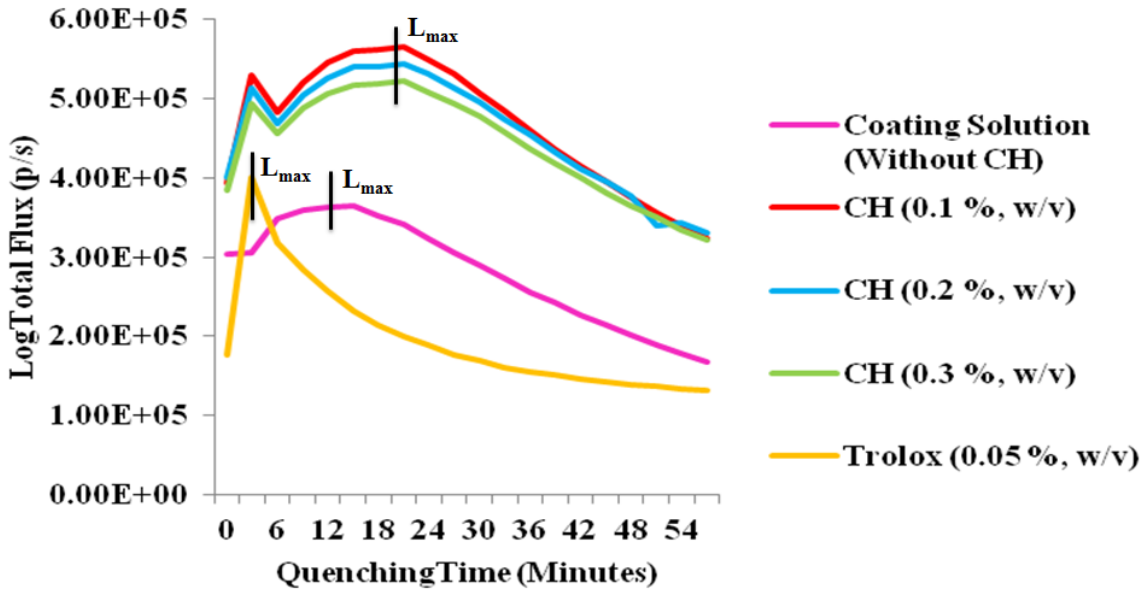

Figure 4.3 Change of total flux (p/s) over time in coating solutions with and without CH compared to the synthetic antioxidant Trolox

Both the control and the Trolox standard showed lower photon yields compared to the CH samples (Fig. 4.3). However, the treatments with CH reached luminescence maxima (L_{max}) much later (21 mins) than the control (18 mins) (Fig. 4.2) and Trolox (3 mins) (Fig. 4.3). This indicated better resiliency as antioxidants. A dose-dependent enhancement of antioxidative efficacy of the CH samples could also be observed.

A comparison of the time required by the control and the various samples to reach the L_{max} (final quenching time, QT_F) further exhibited the better antioxidative resiliency of the test samples (Fig. 4.4).

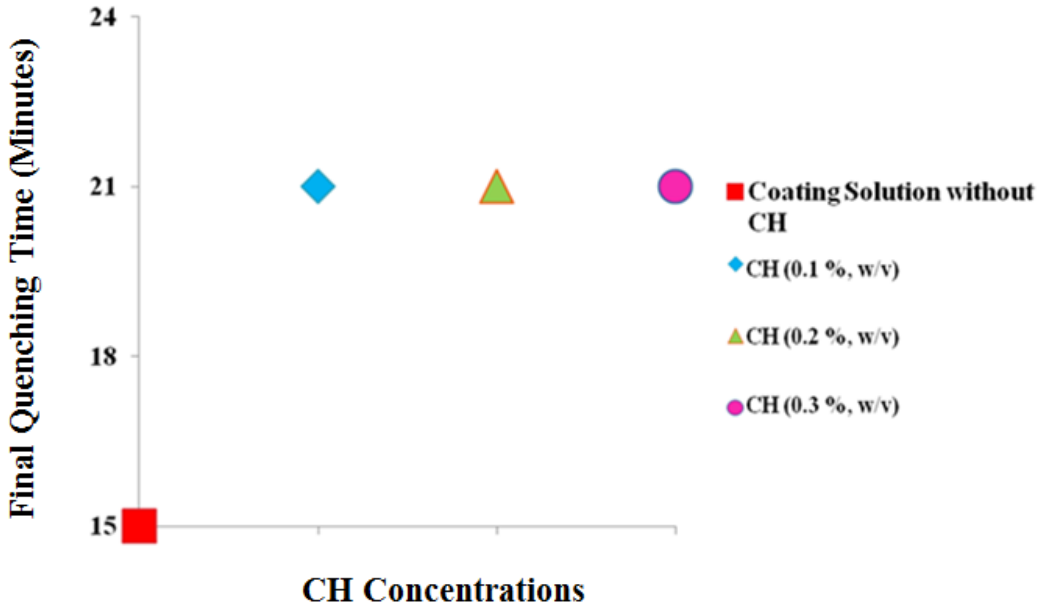

Figure 4.4 Change of final quenching time [QT_F - time required for the test antioxidant to reach the maximum photon yield, or luminescence maxima (L_{max})] in the coating solution with or without CH

The L_{max} at QT_F for all the CH samples was observed at 21 min after the induction of pyrolysis (total flux values of 5.66×10^5, 5.44×10^5 and 5.21×10^5 p/s, for samples containing 0.1, 0.2 and 0.3%CH, respectively), whereas the coating solution containing no CH reached the L_{max} 15 min after the initiation of reaction (total flux value of 3.65×10^5 p/s) (Fig. 4.5). This reflects the markedly greater antioxidative longevity of the CH containing samples. The addition of CH delayed the point of maximum free radical generation by 6 min even at the lowest concentration, which is a considerable extension of efficacy in such an abusive condition of artificial free radical proliferation.

The QT_F for control, various treatments with CH and the Trolox standard are shown in Figure 4.5. These values can be obtained by comparing the QT_F for individual treatments and that for the control ($QT_{FControl}$ - $QT_{FTreatment}$). The fact that CH possesses a

better antioxidative potential compared to the standards was further confirmed by ΔQT_F values of the various samples (Fig 4.6).

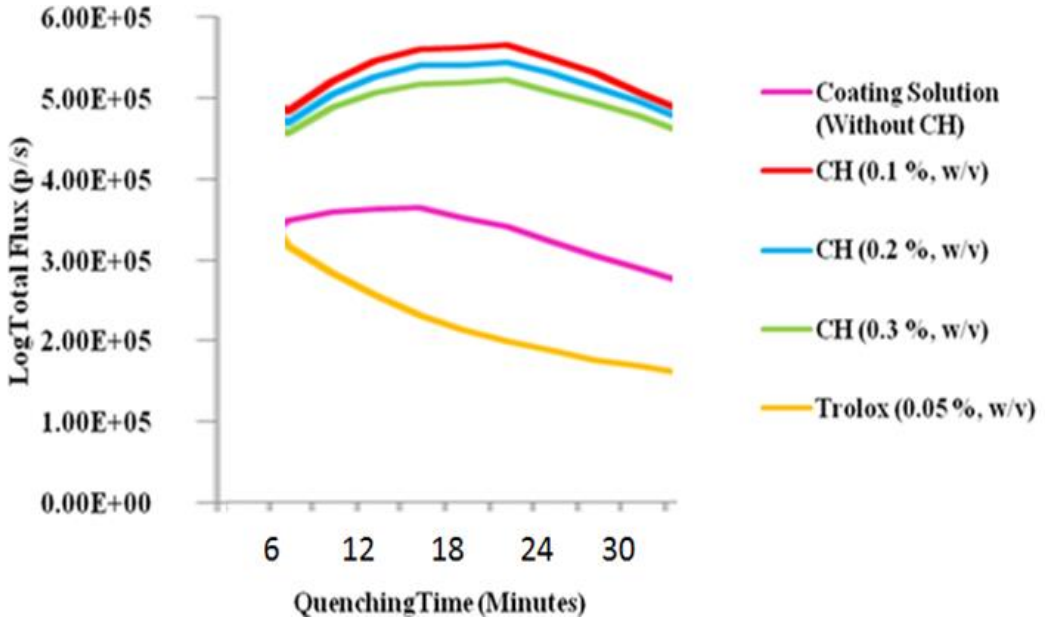

Figure 4.5 L_{max} of the CH samples compared to the control and the Trolox standard

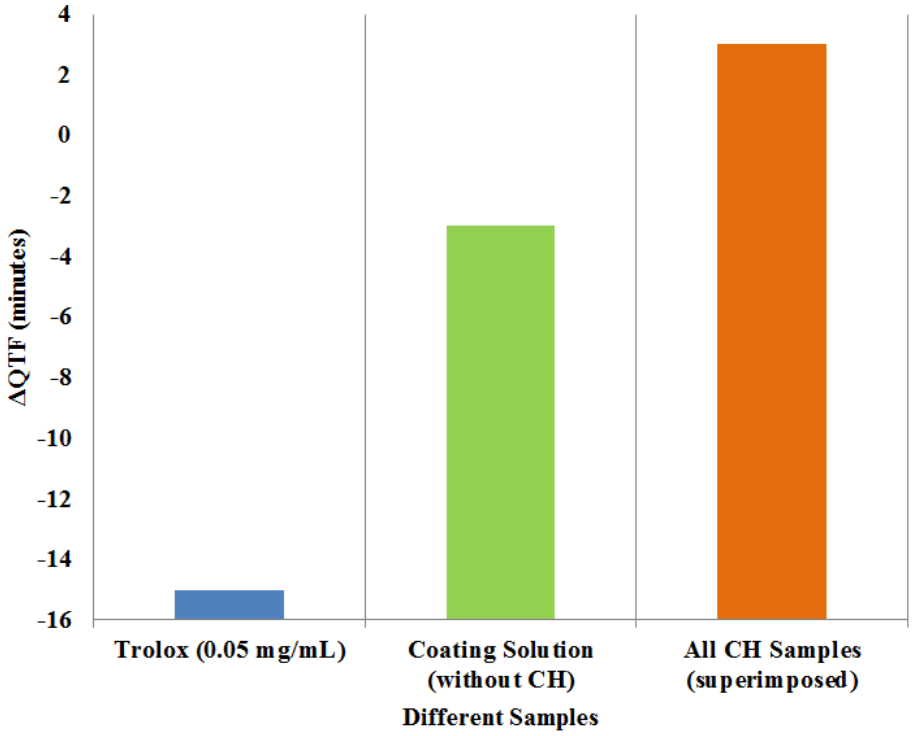

Figure 4.6 Difference in time required to attain the final quenching time (QT_F) by the various samples under study, compared to the control (ΔQT_F)

The ΔQT_F value for the coating solution without CH was found to be -3 min [QT_F of the coating solution without CH (15 min) – QT_F of the McIlvaine's iso-ionic buffer (18 min) (control)], whereas for all the three CH samples, it was determined to be 3 min. Even the standard (Trolox) showed a ΔQT_F of -15 (QT_F was found to be at 3 min), which revealed that CH as an antioxidant was much more resilient than the standard chemical antioxidant (Trolox). Trolox showed greater initial antioxidative potency, but quickly lost its viability under the proliferation of peroxyl radicals. The whey-based edible coating solution without CH also showed better resiliency as an antioxidant compared to the sample containing Trolox, evident by its comparatively higher ΔQT_F value. CH samples conceivably exhibited greater antioxidative potential, compared to both the standards as

the ΔQT_F for the CH samples were found to be positive, and considerably higher than both the control and the standard (Fig. 4.6). This phenomenon further indicated better antioxidative resiliency of the CH samples. In spite of its greater initial antioxidative potential, Trolox was found to be less resilient as an antioxidant even compared to the coating solution containing no CH, which were much more persistent (Haque *et al.*, 2013).

Hetero-polymerization of the proteins or peptides that might lead to reduced particle numbers, thus resulting in reduced numbers of side chains may be responsible for the initial radical leakage in the whey based coating solution containing different concentrations of CH. This concept was proposed by Haque *et al.* (1993) using Quasielastic light scattering studies.

4.3 Results from the DPPH Studies

The DPPH values of the antioxidant samples are presented in Table 4.3. The control and CH treatments differed ($P < 0.05$) in their DPPH radical scavenging activities as was the case with the TRAP studies. The DPPH radical scavenging activity of the controls and CH samples differed from 24.1 to 35.4%. This represents an increase of approximately 47%. There were no significant differences among the samples that contain more than 0.1 % (0.2, 0.3, 0.4 and 0.5%) CH. There was a consistent positive relationship between DPPH radical scavenging activities and CH concentrations until the latter exceed 0.3%. The sample with 0.3% CH showed the highest antioxidantive efficacy (35.4% of DPPH inhibition).

Table 4.3 Results of DPPH radical scavenging activities of coating solution at varied CH concentrations.

CH Concentration (%,w/v)	DPPH radical scavenging (%)
Control (0)	24.1±2.5[a]
0.05	26.3±1.3[a]
0.10	30.8±1.5[b]
0.20	31.1±0.1[bc]
0.30	35.4±1.3[c]
0.40	33.2±0.8[bc]
0.50	31.5±0.8[bc]

A, b, c means with the same letter are not significantly different ($P<0.05$).

In order to calculate Trolox equivalent of CH, a calibration curve representing the relationship of Trolox concentration and free radical scavenging rate was determined (Fig. 4.7). The Y value stands for radical scavenging rate which was obtained in Table 4.3 while the X value, which means Trolox equivalent, can be calculated with equation 4.1. The calculated Trolox equivalent of the various concentrations of CH is given in Table 4.4.

$$y = 0.3906x + 0.1184 \qquad R^2 = 0.9998 \qquad (4.1)$$

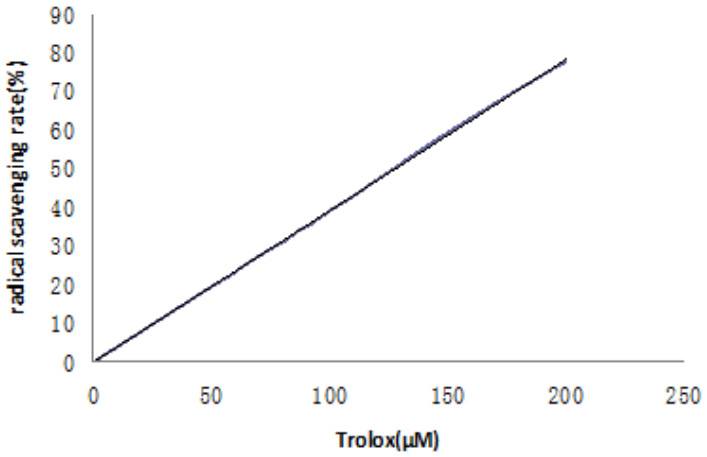

Figure 4.7 Calibration curve representing relationship between Trolox concentration and free radical scavenging rate

Table 4.4 Results of Trolox equivalent calculation of coating solution and varied CH concentrations.

CH Concentration (%,w/v)	Trolox Equivalent Calculation (uM)
Control (0)	61.35
0.05	67.11
0.10	78.50
0.20	79.45
0.30	90.25
0.40	84.64
0.50	80.21

When CH concentration was 0.30 %, w/v, the Trolox equivalent reached the peak (90.25 uM), implying that the antioxidative ability of CH at this concentration is equivalent to 90.25 uM of Trolox (Table 4.4).

CHAPTER V

CONCLUSIONS

The current study exhibited the enhanced antimicrobial and antioxidative properties of a natural, healthy and inexpensive chymotryptic casein hydrolyzate when combined with Cheddar whey and used as an edible coating to protect beef steak. Addition of the hydrolyzate at a concentration as low as 0.15 – 0.2% resulted in a five log reduction (logCFU<1) of coliform growth, virtually eliminating it when all other conditions were uniform. The study also demonstrated the antioxidative efficacy of hydrolyzate-whey mix as studied by two time-tested and sophisticated methods; the TRAP and DPPH methods. The TRAP method was applied for the qualitative determination of antioxidative property while the DPPH method was used to satisfy the objective for quantitative assessment. One method substantiated the other in that there was a two order magnitude greater reduction of free radicals. This was reflected by a decrease in the magnitude of the luminescence maxima, which is caused by unquenched radicals, and its delayed onset by 40% (from 15 min to 21 min) as the antioxidant-combination provided the electron to quench the lone pairs of the radicals. This reflected the outstanding resilience of the antioxidant combination as being significantly effective compared to the controls, and even, Trolox, the gold standard of antioxidants. The study clearly depicts the potential of casein hydrolyzate to be used as a preservative to reduce

oxidative and microbial degradation of cubed beef steak and thus to enhance its retail-cut display life.

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APPENDIX A

COLIFORM COUNTS

Number of coliform colonies (CFU/ml) counted from the McConkey agar plates which were inoculated with different concentrations of sample extracts prepared from beef steaks treated with coating solutions with different CH concentrations.

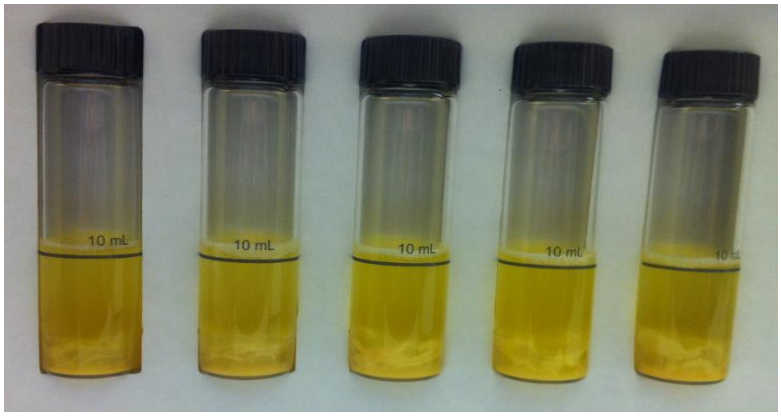
		R1	R2	R3	Total	Mean
Control	10^{-2}	300	300	300	900	300.00
	10^{-3}	300	300	300	900	300.00
	10^{-4}	53	62	37	152	50.60
0.05 g CH	10^{-2}	300	300	300	900	300
	10^{-3}	300	16	29	345	115.00
	10^{-4}	4	23	17	44	14.67
0.10 g CH	10^{-2}	300	300	300	300	300
	10^{-3}	57	0	35	92	30.67
	10^{-4}	0	0	2	2	0.67
0.15 g CH	10^{-1}	0	0	0	0	0.00
	10^{-2}	0	0	0	0	0.00
	10^{-3}	0	0	0	0	0.00
	10^{-4}	0	0	0	0	0.00
0.20 g CH	10^{-1}	0	0	0	0	0.00
	10^{-2}	0	0	0	0	0.00
	10^{-3}	0	0	0	0	0.00
	10^{-4}	0	0	0	0	0.00
	10^{-2}	41	61	43	145	48.30

0.25 g CH	10^{-3}	4	3	3	10	3.30
	10^{-4}	0	0	0	0	0.00
0.30 g CH	10^{-2}	13	2	4	19	6.30
	10^{-3}	0	0	0	0	0.00
	10^{-4}	0	0	0	0	0.00
0.35 g CH	10^{-2}	300	300	300	900	300.00
	10^{-3}	42	43	38	143	41
	10^{-4}	9	9	11	29	9.67
0.40 g CH	10^{-2}	17	7	19	43	14.33
	10^{-3}	5	3	0	8	2.67
	10^{-4}	0	0	0	0	0

APPENDIX B

COLIFORM TEST KITS

Coliform test kits (LaMotte) - 24 hours after inoculation with untreated (control) beef steak sample. Bright yellow colors of the solution in the vials with bubbles at the surface indicate positive growth for coliforms.



APPENDIX C

RESULTS FROM THE CHEMILUMINESCENCE STUDIES

Total flux (p/s) values for different samples and standards under study at different time intervals. The mean (μ) and standard deviation (S.D.) values from each treatment are also summarized.

Time (Min)	**Control 10^7**	**Coating Solution (without CH) 10^5**	**CH (0.1 mg/mL) 10^5**	**CH (0.2 mg/mL) 10^5**	**CH (0.3 mg/mL) 10^5**	**Trolox (0.05 mg/mL) 10^5**
0	1.85	3.04	3.94	4.01	3.85	1.77
3	2.23	3.06	5.29	5.14	4.93	4.01
6	2.51	3.49	4.83	4.70	4.57	3.18
9	2.71	3.60	5.21	5.06	4.89	2.85
12	2.820	3.64	5.45	5.26	5.06	2.56
15	2.88	3.66	5.61	5.40	5.17	2.31
18	2.89	3.52	5.61	5.41	5.18	2.14
21	2.85	3.41	5.66	5.44	5.22	2.00
24	2.78	3.24	5.50	5.31	5.08	1.90
27	2.69	3.06	5.32	5.14	4.93	1.77
30	2.57	2.90	5.07	4.95	4.77	1.70
33	2.44	2.73	4.84	4.75	4.58	1.60
36	2.31	2.55	4.60	4.54	4.37	1.54
39	2.18	2.42	4.37	4.33	4.19	1.50

42	2.04	2.27	4.15	4.12	4.00	1.45
45	1.91	2.13	3.96	3.95	3.81	1.42
48	1.79	2.00	3.76	3.77	3.65	1.39
51	1.68	1.88	3.56	3.40	3.51	1.36
54	1.58	1.78	3.40	3.44	3.34	1.34
57	1.48	1.67	3.24	3.30	3.21	1.31
μ	2.78	2.80	4.67	4.57	4.41	1.96
S.D.	0.471	0.670	0.810	0.710	0.670	0.713