Antioxidative Efficacy and Relative Accessible Hydrophobicity of Aromatic Residue Rich Peptides in Alfa-Chymotryptic Digests of Acid Casein

Wenjie Shao

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Antioxidative efficacy and relative accessible hydrophobicity of aromatic residue rich peptides in alfa-chymotryptic digests of acid casein

By

Wenjie Shao

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in the Department of Food Science, Nutrition and Health Promotion

Mississippi State, Mississippi

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Antioxidative efficacy and relative accessible hydrophobicity of aromatic residue rich peptides in alfa-chymotryptic digests of acid casein

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Four casein-derived peptides fractions of varying hydrophobicity were obtained from α-chymotryptic digest of acid casein using hydrophobic interaction chromatography, termed fractions one through four (abbreviated, F1, F2, F3, and F4). Four standard methods involving alkoxyl, peroxyl, 2, 2-diphenyl-1-picrylhydrazl (DPPH), and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTS$^{•+}$ radicals, were used to measure antioxidative properties. While significantly superior efficacy was exhibited by F2 for all tests except against DPPH, no correlation between antioxidant efficacy and surface hydrophobicity was found. By using capillary electrophoresis and high performance liquid chromatography, the detection of aromatic chromophores by ultraviolet at 280 nm in the fractions revealed that F2 contained the highest concentration of aromatic amino acids and a unique peptide. Result from circular dichroism exhibited remaining residual structure in F2 compared with undigested casein. The F2 possesses a high potential to be used in food industry as a natural source of antioxidant with pronounced antioxidant capacity.

Key words: casein, peptide, hydrophobicity, aromatic amino acids
DEDICATION

I would like to dedicate this thesis to my Father and Mother.
ACKNOWLEDGEMENTS

I would like to express my sincerely appreciation to Dr. Zahur Zee Haque, who has given his best guidance to my Master’s study and research. His experienced mind and profound thoughts guide me to overcome every difficulty that I have faced during my research.

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Finally, I want to express my deepest love to my parents, who gave birth to me, raised me up and always trust me.


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Antioxidant activity</td>
</tr>
<tr>
<td>ABAP</td>
<td>2,2’-azobis (2-methylpropionamidine) dihydrochloride</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>ANS</td>
<td>8-anilino-1-naphthalenesulphonic acid</td>
</tr>
<tr>
<td>AP</td>
<td>Antioxidant persistence</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BHA</td>
<td>Butylated hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxyl- toluene</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>CPP</td>
<td>Caseinophosphopeptides</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-Diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>F1</td>
<td>Fraction 1</td>
</tr>
<tr>
<td>F2</td>
<td>Fraction 2</td>
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<td>F3</td>
<td>Fraction 3</td>
</tr>
<tr>
<td>F4</td>
<td>Fraction 4</td>
</tr>
<tr>
<td>HAT</td>
<td>Hydrogen atom transfer</td>
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<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
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ix
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
</tr>
<tr>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PINO</td>
<td>Phthalimide-(N)-oxyl</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least squares regression method</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure activity relationship</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence unit</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light unit</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis Software</td>
</tr>
<tr>
<td>SET</td>
<td>Single electron transfer</td>
</tr>
<tr>
<td>SH</td>
<td>Surface Hydrophobicity</td>
</tr>
<tr>
<td>SS</td>
<td>Szeto-Schiller</td>
</tr>
<tr>
<td>TBHQ</td>
<td>Tertiary butylhydroquinone</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>TRAP</td>
<td>Total radical trapping antioxidant potential</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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CHAPTER I
INTRODUCTION

Oxidative degradation of food results in overall deterioration of food products including development of undesirable color and flavor and reduced shelf-life. This will cause considerable economic losses across the globe (Decker, Elias, & McClements, 2010). Due to large and increasing quantity of food loss in the U.S. every year, the United States Department of Agriculture (USDA) initiated research projects to reduce spoilage of fresh foods. This includes projects to develop antioxidants from rice hulls in an attempt to reduce lipid oxidation (USDA, 2015). Oxidation of food also results in the generation of reactive oxygen species in food systems – that may result in several life-threatening consequences such as cardiovascular diseases (Mügge, 1998) and certain types of cancers (Waris & Ahsan, 2006). Besides natural oxidation, food products often become susceptible to oxidative deterioration due to specific processing techniques they are subjected to or supplements that are added. For example, in iron-fortified food products like peanut snacks and sausages, the iron supplement can substantially catalyze lipid oxidation (Kanner, 2010) in spite of its beneficial role as a micronutrient. Oxidation also results in a considerable loss of the nutritional properties of food, for example, the damage induced by the singlet oxygen to vitamins D and E (Dyck, 2010).

Lipids and proteins in food systems are most vulnerable to oxidation. Among them, the consequences of lipid oxidation are more pronounced, which include rancidity, toxic by-
products and the development of undesirable color (Pokorny, Yanishlieva, & Gordon, 2001). In addition, it has been reported that microorganisms exhibit different preference to oxidation/reduction potential with respect to their fecundity, with aerobic microorganisms usually preferring conditions with greater oxidation-potential (Schmidt & Rodrick, 2003).

Antioxidants have proven to be of extreme importance to minimize the undesirable effects of food oxidation for a long period. The modes of action of antioxidants used for preservative purposes are diverse. For instance, antioxidants manifest their protective efficacy against lipid oxidation via the following two principal mechanisms: (1) by reacting with the peroxyl/alkoxyl radicals generated by lipid hydroperoxide decomposition, (2) by stabilizing lipid hydroperoxides and therefore inhibiting the formation of the peroxyl and alkoxy radicals (Pokorny et al., 2001). Some synthetic antioxidants with remarkable preservative efficacy [e.g. butylated hydroxyanisole (BHA; Pokorny et al., 2001)] have been developed and used in the food industry. However, current research has indicated their potentially harmful effects on consumer health following long-term use (Pokorny et al., 2001; Thompson & Moldéus, 1988). Bovine milk has been reported to contain a number of components with considerable antioxidative properties (Pihlanto, 2006). Peptides, while inert in intact proteins can exhibit dramatic antioxidative efficacy following enzymatic hydrolysis of the protein (Korhonen & Pihlanto, 2006; Park, 2009; Urista, Fernández, Rodriguez, Cuenca, & Jurado, 2011). The remarkable antioxidant capacity of whey and casein-derived peptides and their potential use as food preservatives have been reported by several authors (Díaz, Dunn, McClements, & Decker, 2003; FitzGerald, Murray, &
Walsh, 2004; Haque, Zhang, & Mukherjee, 2015; Mukherjee & Haque, 2015; Peña-Ramos & Xiong, 2003; Weerasinghe et al., 2013). Such peptides have already been used to develop several commercial products including cosmetics and foods and beverages (FitzGerald et al., 2004).

The close relationship between protein physiochemical properties and functionality has long been studied (Petsko & Ringe, 2004). Several published articles reported significant relationships between antioxidant ability and hydrophobicity of peptide fractions digested from soybean (Chen, Muramoto, & Yamauchi, 1995) and bullfrog skin (Qian, Jung, & Kim, 2008). Qian et al. (2008) reported greater efficacy of the peptides rich in aromatic amino acids (e.g., phenylalanine, tryptophan and tyrosine) to scavenge reactive oxygen species. The contribution of aromatic ring in phenolic antioxidant was studied and the kinetic research on the reaction of phthalimide-N-oxyl radical (PINO, a short-lived N-oxyl radicals) and phenols indicated that the process within this reaction is a hydrogen atom transfer (HAT) and the π-stacking of the PINO and phenolic aromatic ring contribute to the charge transfer for this reaction (D’Alfonso, Bietti, DiLabio, Lanzalunga, & Salamone, 2013).

1.1 Objective

1. To determine the relationship between antioxidant efficacy and accessible hydrophobicity.

2. To check whether there is a relationship between antioxidant efficacy and concentration of aromatic ring contained substances conceivably due to π-π stacking effect.
2.1 Reactive oxygen species (ROS) and oxidative stress

The ROS are a group of by-products generated during oxygen metabolism, which damage lipids, DNA and proteins and thus cause detrimental effect to cells (Klauer, 2005). ROS include Superoxide ($O_2^-$), Hydroxyl radical (‘OH), Peroxyl radical (ROO’) and Singlet oxygen ($^1O_2$) (Devasagayam et al., 2004). The formation of the superoxide radicals is by reaction of oxygen molecules with other compounds like vitamin folate (Sareen, Jack, & James, 2009). The hydroxyl radical is generated when tissue is exposed to some radiation (e.g. γ-ray; Sareen et al., 2009). The peroxyl radical is reactive and formed from the reaction between superoxide radicals with additional electrons (or with hydrogen; Sareen et al., 2009). The singlet oxygen is formed during photosensitization (Devasagayam et al., 2004).

Oxidative stress induced by ROS generated in food systems can potentially lead to morbidity and mortality due to their role in causing DNA mutations and damaging cellular machinery (Bartosz, 2013; Cooke, Evans, Dizdaroglu, & Lunec, 2003). For example, several researches have indicated that the diseases such as Alzheimer’s disease (Butterfield & Lauderback, 2002), and cataractogenesis (Augusteyn, 1981) are closely associated with the level of protein oxidation in the human body (Schuessler & Schilling, 1984). Other major health consequences resulting from the effects of ROS include...
cardio-vascular diseases (Sugamura & Keaney, 2011) and various types of cancers (Waris & Ahsan, 2006).

2.2 Oxidative degradation of Food

Oxidation leads to the degradation of food components, which in turn results in an overall deterioration of quality, such as development of rancidity, unfavorable color, decline in nutritional value etc. (Pokorny et al., 2001). All these factors adversely affect the shelf-life (Pokorny et al., 2001). During food processing and storage, a number of factors such as the type of packing material, temperature as well as the composition of a food product itself will determine the degree of oxidation in it (Bartosz, 2013). Lipids, proteins and carbohydrates are three major components of food. All of them are vulnerable to oxidation and can result in the formation of undesirable oxidation products. Many harmful substances are generated during lipid and protein oxidation, for example, lipid hydroperoxides and protein carbonyls, respectively (Stadtman & Levine, 2000). These substances tend to accumulate in the human body and cannot be removed solely by the actions of the antioxidative enzymes (e.g., superoxide dismutase) or the immune system (Terman & Brunk, 1998). Thus, in order to reduce oxidative deterioration of food as well as the detrimental consequences resulting from the consumption of food products that have undergone oxidation, appropriate levels of effective antioxidants must be present in food systems and consumed as dietary supplements (Pokorny et al., 2001).

2.3 An overview of antioxidants

During the past decades, some antioxidants, both natural and synthetic, have been discovered which differ considerably regarding their chemical structures and modes of
action. Synthetic antioxidants are more extensively used in the food industry because of their comparatively lower prices and more pronounced antioxidant properties compared to the natural ones (Case, 2000; Hand, 2000). Some examples of such antioxidants with extensive usage as food preservatives include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ). However, some toxicological data have indicated the potential health hazards resulting from the long-term consumption of food products treated with such agents (Thompson & Moldéus, 1988). Therefore, development of food preservatives from natural antioxidants that would be potentially free from such health concerns gained more scientific interest (Loliger, 1991; Velioglu, Mazza, Gao, & Oomah, 1998). Natural antioxidants are present in a diverse array of sources (such as fresh fruits, nut, vegetables, milk, etc.) and include but are not limited to phenolic compounds from plant origin (e.g. flavonoids; Kähkönen et al., 1999), and a wide range of peptides (e.g. carnosine) (Pokorny, 1999; Shahidi, 1997). There are many antioxidants from natural sources have been applied to food systems, such as tocopherols, ascorbic acid, erythrobic acid and extracts of rosemary or sage (Shahidi & Zhong, 2010).

2.4 The overview of antioxidants in bovine milk

Bovine milk is rich in its antioxidant contents, some of which include lactoferrin, β-carotene, vitamin E, ascorbic acid, some antioxidant enzyme like superoxide dismutase, glutathione peroxidase and catalase (Lindmark-Månsson & Åkesson, 2000). In addition, bioactive peptides resulting from enzymatic hydrolysis of specific protein components of bovine milk possess potent antioxidant properties and have gained considerable scientific interest in the current years (Urísta et al., 2011). Peptides resulting from enzymatic
hydrolysis of native proteins can exhibit different functionalities based on their amino acid sequences as well as the specific terminal residues they contain (Korhonen & Pihlanto, 2006). Such peptides may exhibit several important biological functions including, but not limited to antibacterial, anticytotoxic, antioxidative, antihypertensive and immunomodulatory activities (Park, 2009; Urista et al., 2011). Those bioactive peptides can also be used as additives in functional foods, cosmetics and drugs and until now a number of cosmetic products containing such bioactive peptides have already been manufactured commercially (Haque, Chand, & Kapila, 2008). Bovine milk, with its casein and whey protein contents of 80 and 20%, respectively, has currently become one of the most effective resources for the generation of the bioactive peptide (Urista et al., 2011).

2.4.1 Antioxidant peptide from whey protein hydrolysate

Previously considered to be a waste, whey protein, an inexpensive by-product of cheese manufacture is currently gaining importance for its potential use in food and drug industries (Smithers, 2008). Whey contains α-lactalbumin (α-La), β-lactoglobulin (β-Lg), serum albumin and other proteins like immunoglobulins and lactoferrin, some of which depict metal chelating properties (Haug, Hostmark, & Harstad, 2007; Meucci, Mordente, & Martorana, 1991). The whey protein hydrolysate exhibits better opioid (decrease the sensation of pain of the human body) and angiotensin I-converting enzyme inhibitory capacity (which is the capacity to lower blood pressure) when hydrolyzed by different enzymes separately (Pihlanto-Leppälä, 2000). Peña-Ramos, Xiong, and Arteaga (2004) reported the antioxidant ability of the chromatographic fractions generated by enzyme hydrolysis of whey protein against lipid oxidation, which can be potentially used to
prevent lipid oxidation in food products. A real application of whey protein hydrolyzed peptide was conducted by Peña-Ramos and Xiong (2003), who reported the remarkable efficacy of intact whey protein isolate and its hydrolysates in reducing cooking loss and oxidative degradation of cooked pork patties during refrigeration. Preservative properties of whey are further evident by its efficacy to protect the cubed beef steak from oxidative degradation (Haque, Shon, & Williams, 2009; Mukherjee & Haque, 2015; Weerasinghe et al., 2013).

2.4.2 Antioxidant peptides from casein hydrolysate

Casein is the principal constituent of milk protein and is composed of \(\alpha_{s1}\), \(\alpha_{s2}\) – casein, \(\beta\) – and \(\kappa\)–caseins (Pinnock, 2007). Casein is easily hydrolyzed by enzymes for concomitant release of bioactive peptides and, therefore, possess considerable potential applicability in dietary supplements and pharmaceutical industries (Schlimme & Meisel, 1995). Several peptides resulting from enzymatic hydrolysis of casein have been identified and their functionalities include mineral binding ability (e.g. caseinophosphopeptides) or immune-system promoting ability (e.g. glycomacropeptides; FitzGerald et al., 2004), etc. The ability of caseinophosphopeptides (CPP) to bind to transition metals can be attributed to the phosphorylated serine residues on the polar domain which remove positive metal ions by complexing with them (Díaz et al., 2003; Pihlanto, 2006). CPP also exhibits considerable efficacy to scavenge of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), hydroxyl and peroxy radicals (Chin & Kitts, 2004; Kim, Jang, & Kim, 2007). \(\beta\)-casomorphins, also resulting from the enzymatic hydrolysis of casein, are opiate peptides and known to impart anodynic effect on the nervous system, thus possessing potential use to help infants sleep (Matthies et al.,
Hydrolysis-derived peptides from casein are rich in amino acids that possess higher antioxidant capacity such as histidine, tyrosine, proline and lysine that exhibit dramatic radical scavenging efficacy (Pihlanto, 2006). Rival, Boeriu, and Wichers (2001) reported that casein and casein-derived peptides depict markedly efficacy to prevent lipid peroxidation by serving as preferred targets of the free radicals. Suetsuna, Ukeda, and Ochi (2000) reported the efficacy of several peptides resulting from pepsin-digested casein hydrolysate to scavenge superoxide, hydroxyl and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals. Currently, the application of novel enzymes and methods on hydrolysis of casein is being studied in details. Luo, Pan, and Zhong (2014) reported the antioxidant properties of the hydrolysates produced by subjecting casein to different enzymes (papain, pancreatin and trypsin) for various periods of digestion (10, 30 min, 1, 4 and 24 h). The results indicated a relationship between the enhancements of antioxidant properties with increased periods of digestion. Di Pierro, O’Keeffe, Poyarkov, Lomolino, and FitzGerald (2014) reported the potent antioxidant properties of the eight peptide fractions generated by hydrolyzing casein with the proteinase from *Ficus carica* latex.

**2.4.3 Applications of milk-derived peptides**

A rapidly expanding market and consumer demand has stimulated several industries to develop novel products containing natural substances with potentially enhanced health promotion effects (Capecka, Mareczek, & Leja, 2005; Phelan, Aherne, FitzGerald, & O'Brien, 2009). Several peptides originating from milk proteins can function as opioid receptor agonists, such as α-casein exorphins (from α-casein), β-casorphin (from β-casein), casoxin (from κ-casein), α-lactorphins (from α-lactalbumin),
β-lactorphin (from β-lactoglobulin) and lactoferroxins (from lactoferrin) (Teschemacher et al., 1997). A number of peptides from milk protein hydrolysates can also function as hypotensive substances (FitzGerald et al., 2004). Several such hypotensive products are being commercially manufactured or in their developmental stages, such as the C12 peptide (casein hydrolysate, manufactured by DMV internationals, The Netherlands) and Biozate series products (whey peptides, manufactured by Davisco, US; FitzGerald et al., 2004). Díaz and Decker (2004) reported the dramatic antioxidant ability of CPP and casein hydrolysate with respect to the reduction of lipid oxidation of ground beef and their potential use as food preservatives from natural origin. Sakanaka, Tachibana, Ishihara, and Juneja (2005) reported the potent radical scavenging ability of casein calcium peptides against superoxide, DPPH and hydroxyl radicals, as well as its strong efficacy to reduce lipid oxidation in ground beef, suggesting its potential use in muscle food industry to prevent rancidity of the products and thereby enhance their shelf lives (Sakanaka et al., 2005). Rossini, Norena, Cladera-Olivera, and Brandelli (2009) explored the efficacy of casein hydrolysate produced by digestion with alcalase and Flavourzyme (a peptidase derived from Aspergillus oryzae; Merz et al., 2015) to inhibit lipid oxidation in ground beef homogenates and mechanically deboned poultry meat, with the latter showing greater antioxidant properties.

2.5 α-Chymotrypsin

α-Chymotrypsin belongs to the serine proteases family and is synthesized in the pancreas and subsequently secreted into the digestive system (Di Cera, 2009; Kumar & Venkatesu, 2012). Kinetic studies elucidated that α-chymotrypsin specifically cleaves the peptide bonds on the C-terminal ends of the aromatic residues (tyrosine, tryptophan,
phenylalanine; Hedstrom, 2002; Tatsushi & Morihara, 1978). It also catalyzes the syntheses of peptides with hydrophobic amino acid residues at the C-terminal (Tatsushi & Morihara, 1978).

2.6 An overview of assays for the determination of antioxidant properties

Antioxidants in food systems have been defined as ‘the substances that can effectively preclude or inhibit the oxidation of food ingredients that are vulnerable to oxidation by applying the only small amount of this antioxidant’ (Chipault, 1962). Applied numbers of studies have been conducted to analyze the efficacies of added antioxidants in food and beverage systems (MacDonald – Wicks, Wood, & Garg, 2006). Several different classes of antioxidants are currently available that are used for preservative purposes, for example endocrines (N-acetyl-5-methoxytryptamine, angiotensin, etc.), macromolecules (transferrin, β-lactoglobulin, zein, etc.), micromolecules (anserine, vitamin c, nitric oxide, etc.) and enzymes (peroxidases, superoxide dismutase, etc.; Elias, McClements, & Decker, 2005; Kong & Xiong, 2006; Mancuso, McClements, & Decker, 1999; Prior, Wu, & Schaich, 2005). Many kinds of ROS and reactive nitrogen species (RNS) in biological and food systems have been identified and characterized, for example hydrogen peroxide, peroxyl radicals, superoxide anions, hydroxyl radicals, alkoxy radicals and singlet oxygens (MacDonald – Wicks et al., 2006). Due to the difference in biochemical properties of both the antioxidants and radicals, the mode of action for a particular antioxidant may take place either (1) by several different mechanisms in a reaction system; (2) by a specific mechanism determined by the reaction system itself (Ishige, Schubert, & Sagara, 2001; Prior et al.,
2005). A particular antioxidant may also act on one or more specific target radical(s) (Prior et al., 2005). Various biochemical assays to determine antioxidant properties of a test substance so far can be categorized into two classes based on the reaction mechanisms involved, namely: (1) hydrogen atom transfer (HAT) and (2) single electron transfer (SET) (Huang, Ou, & Prior, 2005).

2.6.1 Assays based on HAT

As the term indicates, HAT based assays are aimed to determine the hydrogen donating ability of an antioxidant to quench free radicals (Prior et al., 2005). The methods that utilize HAT reaction mechanisms include oxygen radical absorbance capacity (ORAC) assay, total radical trapping antioxidant potential (TRAP) assay, chemiluminescence assay and photochemiluminescence assay (Prior et al., 2005). Among those methods, ORAC and TRAP are considered to be the two most comprehensive and accurate methods that are (Prior et al., 2005).

2.6.1.1 ORAC assay

So far ORAC has been used to assess the antioxidant properties of several different types of protein hydrolysates, such as egg white (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004), α-lactalbumin and β-lactoglobulin (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005), casein (López-Expósito, Quirós, Amigo, & Recio, 2007), human milk (Tsopmo et al., 2011), algae protein (Sheih, Wu, & Fang, 2009), peanut (Su et al., 2011), salmon (Girgih, Udenigwe, Hasan, Gill, & Aluko, 2013), hemp seed protein (Girgih, Udenigwe, & Aluko, 2013), cowpea (Marques et al., 2015), cod (Girgih et al., 2015), soy (Zhang, Li, & Zhou, 2010). ORAC assay involves the use of a
specific fluorescent probe (FL, 3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-
xanthen]-3-one; Ou, Hampsch-Woodill, & Prior, 2001) that reacts with peroxyl radicals
and constitute a non-fluorescent substance. However, an antioxidant can prevent the
formation of this product by scavenging the peroxyl radicals from a system, thus the
quantitative change in fluorescence indicates its antioxidant capacity (Prior et al., 2005).
Initially limited only to the study of the hydrophilic antioxidants, this method is currently
modified for the analyses of the radical quenching efficacy exhibited by the hydrophobic
antioxidants as well (Prior et al., 2003).

2.6.1.2 TRAP assay

The TRAP assay was originally developed by Wayner, Burton, Ingold, Barclay,
and Locke (1987) and has subsequently been considerably modified resulting in the
development of several variants suited for a wide range of analytical purposes. Similar to
the ORAC assay, TRAP has been designed to observe the efficacy of an antioxidant to
inhibit the reaction between peroxyl (or alkoxyl) radicals and a target probe (Schlesier,
Harwat, Böhm, & Bitsch, 2002). In the TRAP assay, the azo compound 2, 2’-Azo-bis (2-
amidinopropane) (ABAP) is used as the inducer of radicals. So modifications of TRAP
was extensively employed to analyze the overall antioxidant capacity of biological fluids
(Chance, Sies, & Boveris, 1979; Lissi, Salim-Hanna, Pascual, & del Castillo, 1995;
Wayner et al., 1987). Since lipid peroxidation is initiated by the peroxyl radicals, TRAP
has also been proven to be useful in the study of the antioxidants that inhibit the process
(Dugas et al., 2000; Rubbo et al., 2000; Wayner et al., 1987).
2.6.2 Assays based on SET

The SET-based methods were originally designed to assess the capacity of an antioxidant to transfer electrons with the concomitant reduction of other compounds, as well as radicals (Wright, Johnson, & DiLabio, 2001). The probe used in these assays acts as an oxidant which results in a change in the color of the reaction mixture as it is reduced by the presence of an antioxidant (Huang et al., 2005). The rate of color change is governed by the concentration of an antioxidant. When the color of a system remains stable, the end point of the reaction can be considered to be attained. Finally a curve reflecting the linear relationships of antioxidant concentrations and absorbance is established and the differences of reducing capacities of various antioxidants are compared by using the rake ratios of different curves (Huang et al., 2005).

2.6.2.1 DPPH radical scavenging capacity assay

The DPPH is an organic nitrogen radical with an unpaired electron and has an absorption maximum at 515 nm (Prior et al., 2005). When dissolved in ethanol, it appears to have a purple color. The assay is designed to measure the reducing ability of an antioxidant, which is reflected by the change in the absorbance of the reaction mixture as it decolorizes from purple to yellow when DPPH is reduced by an antioxidant (Brand-Williams, Cuvelier, & Berset, 1995). The extent of the color change is proportional to the amount of antioxidants used and electrons absorbed (Mishra, Ojha, & Chaudhury, 2012). The DPPH assay is widely used to determine the antioxidative properties of peptides derived from different types of protein hydrolysates and food systems, including tuna cooking juice (Jao & Ko, 2002), casein (Suetsuna et al., 2000), oyster (Umayaparvathi et al., 2014), whey protein (Peng, Xiong, & Kong, 2009), shrimp
processing by-products (Zhao, Huang, & Jiang, 2013), corn peptide (Wang et al., 2014),
common bean (Luna Vital, Gonzalez de Mejia, Mendoza, & Loarca-Pina, 2015), yogurt
(Sah, Vasiljevic, McKechnie, & Donkor, 2014), rice residue protein (Yan, Huang, Sun,
Jiang, & Wu, 2015), rapeseed (He et al., 2012) etc.

2.6.2.2 Trolox equivalent antioxidant capacity (TEAC) assay

TEAC is one of the SET-based assays and widely used for the determination of 
antioxidant capacity of a wide range of food products, such as orange juice and milk
(Prior et al., 2005; Zulueta, Esteve, & Frígola, 2009). The compound 2,2’-azinobis(3-
ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) is used in the assay as the source of
ABTS$^{**}$ radical cations (Prior et al., 2005), which results in the formation of a blue-green
color in the reaction system (Re et al., 1999). The decolorization of the chromophore
following reaction with a test substance depicts the antioxidant properties of the latter,
which can be quantified by monitoring the change in absorbance of the reaction mixture
at 734 nm. Although the ABTS$^{**}$ radical is not generated naturally in the biological
systems, this assay has been proven useful for a variety of analytical purposes (Huang et
al., 2005; Prior et al., 2005).

2.7 Peptide hydrophobicity and antioxidant efficacy

Biological functions of a protein is known to be considerably governed by its
amino acid sequence and structural conformation (Buxbaum, 2007; Petsko & Ringe,
2004). Peptides resulting from the enzymatic hydrolysis of an intact protein differ
considerably with respect to their sequences, which conceivably provides them with
different physicochemical characteristics, including antioxidant properties (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998).

Antioxidant properties of peptides are known to have a close association with hydrophobicity (Chen et al., 1995; Qian et al., 2008). Kawashima, Itoh, Miyoshi, and Chibata (1979) found that dipeptides rich in branched chain amino acids on the N-terminal can exhibit higher antioxidant ability compared with those without branched-chain amino acids. These amino acids include leucine, isoleucine and valine, all of which are hydrophobic (Brosnan & Brosnan, 2006). Evelson et al. (2001) reported that in biological systems, antioxidants with different molecular weights and hydrophobicity can act as chain breaking agents and defend cellular damage caused by oxidative stress. It was hypothesized that the relatively higher hydrophobicity of these peptides can either (1) enhance their movements in the lipid phase, or (2) increase their contact with greater number of fatty acid molecules on the cell membranes – thus enhancing their biological activities, including efficacy to thwart oxidative stress in intracellular locations (Mendis, Rajapakse, Byun, & Kim, 2005; Murase, Nagao, & Terao, 1993; Saiga, Tanabe, & Nishimura, 2003). Li, Li, He, and Qian (2011) used the quantitative structure activity relationship (QSAR) modeling system to assess the association between the structure and antioxidant properties of a peptide and reported that the hydrophobicity of the N-terminus is the major contributing factor to its radical quenching efficacy. Udenigwe and Aluko (2011) used the partial least squares regression method (PLS) to explore the trend of different single amino acids or amino acid groups to contribute to the overall antioxidant ability of food protein hydrolysate and the results indicated that higher hydrophobicity resulted in enhanced radical scavenging ability of the food protein hydrolysate. Xie,
Wang, Jiang, Liu, and Li (2015) reported that chromatographic fractions of casein hydrolysate with the highest hydrophobicity also exhibited the most enhanced antioxidant property and bioavailability.

2.8 The overview of the relationship between antioxidant capacity and aromatic amino acid induced π-π stacking effect.

Peptides with aromatic amino acids (they are phenylalanine, tryptophan and tyrosine) have been shown to stabilize ROS for their better electron transfer ability (Qian et al., 2008). Tyrosine and dimethyl-tyrosine have been found to be the major contributors to the antioxidant properties of the peptide ‘Szeto-Schiller’ (SS) (Szeto, 2006) regarding to its efficacy to inhibit apoptosis caused by peroxidation. Several types of products (e.g. Di-tyrosine) may be generated during the reaction between aromatic amino acids and a given radical species (Davies, 2005). Davies (2005) investigated the possible mechanism and indicated the oxidation of aromatic rings is the major cause behind the formation of such products. The π-π stacking effect in chemistry defines as attractive, noncovalent interactions between aromatic rings (Du et al., 2013). Many reports have been done to analyze the relationship between aromatic amino acids induced π-π stacking effect and antioxidant capacity. D’Alfonso et al. (2013) did research on the kinetics of reactions between phthalimide N-oxyl (PINO) radical with a series of activated phenols which contains aromatic ring and suggested that those reactions having considerable degree of charge transfer is the consequence of π-π stacking effect of aromatic ring of the phenols and that of the PINO. Similar research was also done by Mazzonna, Bietti, DiLabio, Lanzalunga, and Salamone (2014) and conclude that the π-π stacking conformation between N-oxyl aromatic groups and phenolic aromatic groups
give a significant contribution in the HAT reactions from phenols to N-oxyl radicals. Du et al. (2013) evaluated the possible reason for the enhanced antioxidant capacity of surface-functionalized gold nanoparticles and suggest that the π-π stacking interactions existed between and among groups on the surface of test antioxidant contribute to the variation of the transition state of DPPH radical scavenging reaction.
CHAPTER III
MATERIAL AND METHODS

3.1 Material

Skimmed milk was obtained from the Mississippi State University Dairy Plant. Food grade lactic acid was purchased from ADM (Decatur, IL, U.S.). The food grade sodium hydroxide was purchased from Essential Depot (South Sebring, FL, U.S.). Acetic acid was purchased from Fisher Scientific (Pittsburgh, PA, U.S.). α-Chymotrypsin, N-ethylmorpholine, XAD-16 polystyrene resin, Whatman 4 filter paper, peptide molecular weight standards, 8-anilino-1-naphthalenesulphonic acid (ANS), 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (ABAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), trolox and Fluorescein (FL; 3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one) were from Sigma-Aldrich (Milwaukee, WI, U.S.). Food grade absolute ethanol was purchased from Department of Chemistry (Mississippi State University, MS, U.S.). Black, clear bottom Costar™ 96 well plates, and hydrochloride acid were purchased from Fisher Scientific (Hanover Park, IL, U.S.). The dialysis tube is purchased from VWR Scientific (Radnor, PA, U.S.).

3.2 Methods

3.2.1 Preparation of casein

Isoelectric precipitation method was used for the collection of casein. First, food-grade lactic acid was added to skimmed milk to adjust the pH to 4.6 to precipitate the
casein. Then the milk was centrifuged at 4307 g at 20 °C for 20 min. The supernatant was discarded and the dry casein precipitate was collected and dispersed in deionized water in a blender (Glen Allen, VA, U.S.). Then the casein was re-dispersed by adding food-grade sodium hydroxide to adjust the pH to 6.6 with continuous stirring for two hours. The solution was then filtered with a Whatman 4 filter paper and the filtrate was dialyzed at 10000 folds. Then the dispersion was pre-freezeed and lyophilized using a Freezone Triad freeze dry systems (Labconco, Kansas City, MO, U.S.) to obtain casein powder. It was then re-dispersed in deionized water and dialyzed. The dialyzed casein was again lyophilized and kept in freezer for later uses.

3.2.2 Hydrolysis of casein

Casein was dispersed in n-ethylmorpholine /acetic acid buffer at pH=8 with continuous stirring for one hour. The α-chymotrypsin (specific activity: 40 units/mg protein Unit, unit definition: One unit will hydrolyze 1.0 μmole of N-Benzoyl-L-tyrosine ethyl ester per minute at pH=7.8 at 25 °C) was added at a ratio of 1:200 (compared to the weight of casein) into the dispersion and stirred for one hour at 37°C. The reaction was terminated by adding 10 N hydrochloride acid to adjust the pH to 2. Precipitated casein was removed by centrifugation at 22000 g for 20 min at 4°C. The supernatant was filtered with a Whatman 4 filter paper, rotary evaporated in a Buchi rotavaper R-3 (Buchi, Flawil, Switzerland) and finally pre-freezeed and lyophilized to obtain the peptide powder.
3.2.3 Column separation

The peptide powder was dispersed in n-ethylmorpholine/acetic acid buffer at pH=6 and loaded on the GE-XK26 column (GE Healthcare, Little Chalfont, United Kingdom) packed with XAD-16 polystyrene resin. The column was pre-balanced with 2 bed volumes of 30% ethanol. The loading volume is 20 ml with hydrolysate concentration at 100 mg/ml. The column was gradient eluted using ethanol with concentrations ranging from 30-90%. The flow rate was 4.9 ml/min. A total of four bed volumes (2 liters) of eluent were used to elute the column. With an increase in ethanol concentration, the non-polarity of the eluted solution is expected to increase - as hydrophobicity is positively correlated with non-polarity. The fractions were rotary evaporated to eliminate the organic solvent and lyophilized for further analyses – with following measurement of yield.

3.2.4 Measurement of Surface Hydrophobicity (SH)

The SH of different peptide fractions was measured by using 8-anilino-1-naphthalenesulphonic acid (ANS). Black, clear bottom 96 well plate was used for the assay and 50 µL of each fraction as well as the undigested casein sample (2 mg/mL) and an equal volume of 300 µm ANS were added into each well. The fluorescence (in RFU, relative fluorescence unit) was recorded using a FlexStation 3 microplate reader with ‘emission’ and ‘excitation’ wavelengths of 300 and 500 nm, respectively. SH of all the samples tested was expressed as relative fluorescence unit. ANS probe is a well-known probe used to measure the SH of protein (Pica et al., 2012; W. Wu, Hettiarachchy, & Qi, 1998). The more hydrophobic regions a protein possess, the more chance this probe can
bind to the protein and, therefore, an increase in fluorescence can be observed (Alizadeh-Pasdar & Li-Chan, 2000).

3.2.5 **Contact angle measurement**

The contact angle values of all the samples were measured by utilizing a Rame-Hart 260-U1 Optical Goniometer. All the samples measured were at 2 mg/ml in phosphate buffer (75 mM, pH=7.0) and at room temperature.

3.2.6 **Antioxidative activity of peptide fractions**

3.2.6.1 **Oxygen radical absorbance capacity (ORAC) Assay**

The ORAC of the fractions was measured using the method described by Prior et al. (2003) and X. Wu et al. (2004) with modifications. A FlexStation 3 microplate reader (Molecular Device, Sunnyvale, CA, U.S.) equipped with SoftMax Pro data acquisition and measurement software was used for the assay. The reading chamber in the equipment was pre-heated to 37 °C. Phosphate buffer saline (PBS) (75 mM, pH=7.0) was used to prepare the trolox series standard solutions (12.5, 25, 37.5 and 50 μM), fraction samples (2 mg/mL, weight/volume – w/v), fluorescein (0.45 μg/mL, w/v) and ABAP (3.2 μM). Assay was run in black, clear bottom 96-well plates and 20 μL of the sample or standard solutions and 200 μL of fluorescein were added to each well. PBS by itself was used as the blank. The plate was pre-heated by putting into the plate reader for 30 min followed by addition of 20 μL of ABAP in each well. Fluorescence was recorded (in RFU) for 60 cycles with 30 seconds per cycle with excitation, emission and cutoff wavelengths of 485, 520 and 530 nm, respectively. A standard curve was constructed based on the differences in area under curve (AUC) between blank and serial standards as functions of
standard concentrations. The ORAC values were calculated and expressed as micromoles of trolox equivalents per gram of sample (µmol TE/g).

### 3.2.6.2 Total radical trapping potential (TRAP) Assay

The TRAP assay was performed using the method described by Lissi et al. (1995) as modified by Mukherjee and Haque (2015) and Haque, Mukherjee, Mukherjee, and Chang (2013). Four fractions and undigested casein were dispersed in PBS (2 mg/mL). Each reaction mixture contained 50 µL of 12.5 mM ABAP (peroxyl and alkoxy radical generator, 100 mM luminol (the inducer of chemiluminescence) and individual samples (2 mg/mL, dispersed in PBS), respectively. The buffer without samples served as the blank. Trolox (0.5%, w/v) dissolved in PBS buffer was the standard. Luminol-induced chemiluminescence, resulting from the presence of unquenched radicals in the reaction mixtures, was recorded (in RLU) every 1.5 min intervals at 37°C for one hour using a FlexStation 3 microplate reader. After one hour, another dose of ABAP with equal concentration and volume was added to each sample and chemiluminescence was recorded in same way for another hour. The data acquisition for the first and second hours indicated the antioxidant activity (AA) and persistence (AP) of the samples, respectively (Haque et al., 2013; Mukherjee & Haque, 2015). In TRAP experiment, a lower flux, evident by lower luminescence, indicates better antioxidative potential (higher TRAP; Haque et al., 2013; Mukherjee & Haque, 2015).

### 3.2.6.3 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Assay

The DPPH radical scavenging activity assay was conducted using the method described by Chen and Ho (1995) with modifications. DPPH (19.72 mg) was dissolved in
500 mL of absolute ethanol to prepare 0.1 mM DPPH solution. 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, Bristi, & Rafiquzzaman, 2013). Then 0.05 ml of individual sample solution (2 mg/ml) and 0.95 ml of 0.1 mM DPPH in absolute ethanol were mixed in 1.5 ml centrifuge tube by vortexing and the tubes were allowed to stand at room temperature in dark for 30 min. Clear bottom 96-well plates were used for the assay. Absorbance of the samples was immediately measured at 517 nm in a FlexStation 3 microplate reader. A blank was made with 0.05 mL of deionized water mixed with ethanol to replace the sample solutions. The mixture of deionized water with ethanol without DPPH was used as blank. The ratio of DPPH radical discoloration (the ratio of decrease in absorbance of sample compared with absorbance of blank) was expressed by the following equation:

\[
\text{Ratio} = 1 - \left( \frac{A_{\text{sample}}}{A_{\text{blank}}} \right)
\]  

A standard curve using absorbance as functions of trolox concentrations was established to calculate DPPH scavenging rate of the samples. The activity of scavenging was expressed as μmol of trolox equivalents/g of dry material (μmol TE/g).

3.2.6.4 TEAC Assay

The method of measuring the TEAC was following the method described by Re et al. (1999) with some modifications. 0.076 g ABTS was added into the 20 ml McIlvaine’s iso-ionic buffer to get seven mM ABTS solution. 0.013 g (2.45 mM) of potassium persulfate was added into the ABTS solution to prepare the ABTS•⁺ radical cation solution. The ABTS•⁺ solution was then kept at room temperature in the dark and stand
for 14 hours before use. The clear bottom 96-well plates were used for the assay. In each well, 50 μl of ABTS•+ mixture, 50 μl of each sample in 2 mg/ml (including casein and trolox) and 200 μl of McIlvaine’s buffer was mixed. The plate was read in the FlexStation 3 microplate reader for one hour, 37 °C with UV absorbance at 734 nm. The following equation is used to calculate the antioxidant capacity of each sample including casein and trolox:

\[
\frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100 \tag{3.2}
\]

3.2.7 Size Exclusion High-Performance Liquid Chromatography (HPLC)

Size exclusion HPLC of the selected peptide fractions of casein were conducted using an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Superdex Peptide 10/300 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Elution was performed using 30% (volume/volume, v/v) acetonitrile over a period of 80 min at a flow rate of 0.3 mL/min and the elute was monitored at 214 and 280 nm, respectively. The average molecular weight distribution of each fraction was determined by comparing its retention time with those of the molecular weight standards (Sigma-Aldrich, Milwaukee, WI, U.S.).

3.2.8 Capillary Electrophoresis (CE)

CE of the selected peptide fractions was conducted using an Agilent 7100 CE system (Agilent Technologies, Santa Clara, CA, USA) equipped with the Chemstation software; 20 mM borate (pH 9.3) was used as the running buffer. The applied voltage was set as 30 kV, and a pressure of 50 mbar was applied to the inlet capillary during the run. A G1600-60211 50 um x 48.5 cm standard capillary (Agilent Technologies, Santa
Clara, CA, USA) was used. The fraction was detected under UV wavelength at 214 and 280 nm, respectively.

### 3.2.9 Circular Dichroism (CD) analysis

All samples including undigested casein were prepared in 0.1 mg/ml in deionized water for analysis use. Jasco J-810 spectropolarimeter was used to acquire the CD data result. The sensitivity was set at low (1000 mdeg) and data path was set as 0.1 nm. The scanning mode is continuous and scanning speed is 100 nm/min. The response is 0.25 s and band width is 1 nm. The data was collected to analyze the possible remaining secondary structure of peptide fractions.

### 3.2.10 Statistical analysis

The analyses for all the assays and tests were conducted for two replications. A series of t-tests were conducted to assess whether for different assays and tests, the value for the blank, control and various fractions were significantly different. Fisher’s Least Significant Difference (LSD) Test was conducted to analyze the pattern of difference between means of the results. All the analyses were conducted by using the Statistical Analysis Software (SAS) version 9.3.
CHAPTER IV
RESULTS AND DISCUSSION

4.1 Column separation of peptides

The yields of various hydrolytic fractions resulting from α-chymotrypsin digestion of casein obtained following column separation and freeze drying were 899.9, 525, 373.2 and 115.1 mg for F1, F2, F3 and F4, respectively (Table 4.1). F1, F2, F3 and F4 yielded 45, 26, 18.7 and 5.8 percent compared with loading weight of hydrolysate (2 g). Yield of the hydrolytic fractions decreased as the concentration of the eluting ethanol increased (Table 4.1).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ethanol concentration (%)</th>
<th>Dry weight (mg)</th>
<th>Yield (% of loading weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>30-45</td>
<td>899.9</td>
<td>45.0</td>
</tr>
<tr>
<td>F2</td>
<td>45-60</td>
<td>525.0</td>
<td>26.0</td>
</tr>
<tr>
<td>F3</td>
<td>60-75</td>
<td>373.2</td>
<td>18.7</td>
</tr>
<tr>
<td>F4</td>
<td>75-90</td>
<td>115.1</td>
<td>5.8</td>
</tr>
</tbody>
</table>

4.2 SH deduced by 8-anilino-1-naphthalenesulphonic acid (ANS) fluorescence studies

As depicted by Figure 4.1, undigested casein and all the fractions exhibited increased relative fluorescence unit (RFU) compared to the blank. Compared to casein, all the chromatographic fractions depicted increased RFU, which indicates increased SH after α-chymotrypsin digestion of intact casein protein. The F1, F2 and F3 did not
significantly (p<0.05) different from each other in RFU. The F4 exhibited significantly (p<0.05) increase in RFU compared with other samples. The analysis that have been done by researchers indicated that the casein contains approximately 35-45% non-polar (hydrophobic) amino acids that contribute to the high degree of hydrophobicity of casein (Fox & McSweeney, 1998).

Figure 4.1 Surface hydrophobicity of blank, undigested casein and all the fractions. The values are expressed as relative fluorescence unit. Non-identical letters indicate significance at p=0.05.

4.3 Contact angle of undigested casein and its hydrolytic fractions

Undigested casein and F3 exhibit significantly (p<0.05) higher contact angle compared to other samples and their values were 24.0° and 23.9°, respectively (Figure 4.2). The F4 depicted the lowest contact angle value, which was significantly lower (p<0.05) than other samples. Based on contact angles analyses of two synthetic peptides, Shera and Sun (2009) reported that surface tensions of peptide solutions are determined
by the differences in their amino acid sequences. However, the results from the current study indicated a different pattern of SH of the hydrolytic fractions compared to the analysis involving ANS. One possible reason to explain this is that the ANS probe is a chemical probe that detecting the hydrophobic amino acids (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2014). Whereas the contact angle measurement considered the sample solution drop as a whole testing object and use physical measurement to measure the angle formed between the solid/liquid interface to indicate SH of the drop itself (Ramé-hart, 2015).

Figure 4.2  Contact angle analyses of undigested casein and its different hydrolytic fractions.

The X and Y axes indicate the samples under study and their contact angles (in degree, °), respectively. Non-identical letters indicate significance at p=0.05.
4.4 Oxygen radical absorbance capacity (ORAC) assay

Reaction mechanism of the ORAC assay is based on the efficacy of a test antioxidant for HAT (Ou et al., 2001). All hydrolytic fractions except for F1 showed significantly (p<0.05) increased ORAC values compared to undigested casein (Figure 4.3), with 260.8, 122.9 and 134.5% enhancements of ORAC for F2, F3 and F4, respectively. F2 exhibited significantly (p<0.05) higher ORAC with respect to all other samples tested (with augmentations of ORAC values of 177.5, 61.9 and 53.9% compared to the F1, F3 and F4, respectively). The ORAC values of the F3 and F4 were similar but nevertheless significantly higher (p<0.05) than those of the undigested casein as well as the F1. The ORAC method proved to provide an in-vitro model reaction system saturated with peroxyl and alkoxy radicals that can be used to simulate the behavior of lipid-based antioxidants (Prior et al., 2005). In addition, the study indicates the efficacy of the casein-derived peptides to terminate lipid peroxidation chain reaction by quenching peroxyl radicals (LOO•) which are formed during the lipid peroxidation process (Sheih et al., 2009). Based on the ORAC values of pepsin digested αs2 and κ-casein hydrolysates López-Expósito et al. (2007) hypothesized that the antioxidative properties of such peptides stem from the presence of two aromatic amino acids, namely phenylalanine and tyrosine. Kim et al. (2007) reported a trolox equivalent of 206 µmol/g for the hydrolysates exhibiting the highest antioxidant efficacy that were prepared from Alcalase-digested sodium caseinate at pH=3 (compared based on same sample concentration at 2 mg/ml). This value, however, was considerably lower than that of the F2 in the current study (453.7 µmol/g), concentration of the samples being equal (2 mg/ml).
The oxygen radical absorbance capacity of casein and its hydrolytic fractions (in µmol trolox equivalents/g) evident by their efficacy to quench alkoxyl and peroxyl radicals. The alkoxyl and peroxyl radicals are generated by the pyrolysis of ABAP. The X and Y axes indicate the test samples and oxygen radical absorbance capacity values in trolox equivalents (in µmol /g), respectively. Non-identical letters indicate significance at p=0.05.

4.5 Total radical trapping potential (TRAP) assay

All the hydrolytic fractions, as well as the undigested casein, exhibited significantly greater (p<0.05) AA (AA) compared to blank (Figure 4.6, Table 4.2). F2 showed markedly higher AA compared to all other samples, with 20.3, 60.7, 55.0 and 49.9% augmentations compared to undigested casein and F1, F3 and F4, respectively. F4 and casein did not show significant difference regarding AA, but the former showed significantly higher (p<0.05) AA compared to F3. F1 exhibited significantly lower AA (p<0.05) relative to all other samples. The F2 and casein showed significantly (p<0.05) greater antioxidative persistence (AP) compared to all other test samples (Figure 4.7,
Table 4.3). AP for individual samples exhibited results similar to those of AA. All the hydrolytic fractions as well as casein depicted significantly greater (p<0.05) AP compared to blank (Figure 4.7 and Table 4.3). F2 showed markedly higher AP compared to all other samples, with 46.4, 34.7 and 24.4% augmentations compared to and F1, F3 and F4, respectively. In summary, undigested casein and F2 showed considerably better AA and AP compared to other samples as well as the blank. F1, F3 and F4 showed a marked decrease in their viability as antioxidants, indicated by a considerable decline in AP.

It is noteworthy that both the assays to determine ORAC as well as TRAP are based on HAT reaction mechanism (Prior et al., 2005), and essentially depict the efficacy of a test sample to quench alkoxy and peroxyl radicals. In the current study, the samples except for the undigested casein exhibited markedly similar results when subjected to either of these assays – thus validating their efficacy to quench the above mentioned radicals.
Figure 4.4  Change in luminol induced chemiluminescence (in relative light unit) over time, following the first challenge.

The values of first challenge are recorded from undigested casein and its hydrolytic fractions every 1.5 min for a period of 60 min compared to the blank (the buffer by itself) and trolox standard. The X and Y axes indicate time (in min) and chemiluminescence in relative light unit, respectively.
Figure 4.5 Change in luminol induced chemiluminescence (in relative light unit) over time, following the second challenge.

The values of the second challenge are recorded from undigested casein and its hydrolytic fractions every 1.5 min for a period of 60 min compared to the blank (the buffer by itself) and trolox standard. The X and Y axes indicate time (in min) and chemiluminescence in relative light unit, respectively.
Figure 4.6   Determination of the AA of casein and its hydrolytic fractions.

The determination is evident from the point of maximal proliferation of pyrolysis induced free-radicals from the luminol emitted chemiluminescence maxima of the test samples [calculated by averaging five data acquisitions 1.5 minutes apart that corresponded to five highest chemiluminescence values detected from blank (the buffer by itself) following the first pyrolysis of ABAP (the 1st challenge)]. The X and Y axes indicate the test samples and chemiluminescence maxima in relative light unit, respectively. Non-identical letters indicate significance at p=0.05.
Figure 4.7  Determination of the AP of casein and its hydrolytic fractions.

The determination is evident from the point of maximal proliferation of pyrolysis induced free-radicals from the luminol emitted chemiluminescence maxima of the test samples [calculated by averaging five data acquisitions 1.5 minutes apart that corresponded to five highest chemiluminescence values detected from blank (the buffer by itself) following the second pyrolysis of ABAP (the 2nd challenge)]. The X and Y axes indicate the test samples and chemiluminescence maxima in relative light unit, respectively. Non-identical letters indicate significance at p=0.05.

Table 4.2  Percentage enhancement of AA.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Casein</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage (%) enhancement of AA compared to blank (buffer on its own)</td>
<td>45.2±1\textsuperscript{c}</td>
<td>23.6±2\textsuperscript{a}</td>
<td>59.0±1\textsuperscript{d}</td>
<td>37.3±2\textsuperscript{b}</td>
<td>45.8±1\textsuperscript{c}</td>
</tr>
</tbody>
</table>

The enhancement is recorded from the point of maximal proliferation of pyrolysis induced free-radicals from the luminol emitted chemiluminescence maxima of the test samples [calculated by averaging five data acquisitions 1.5 minutes apart that corresponded to five highest chemiluminescence values detected from blank (the buffer by itself) following the first pyrolysis of ABAP (the 1st challenge)] of the test samples compared to the blank. Non-identical letters indicate significance at p=0.05.
Table 4.3 Percentage enhancement of AP.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Casein</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage (%) enhancement of AP compared to blank (buffer on its own)</td>
<td>66.5±1d</td>
<td>32.2±4e</td>
<td>73.4±4e</td>
<td>40.7±5b</td>
<td>46.8±5c</td>
</tr>
</tbody>
</table>

The enhancement is recorded from the point of maximal proliferation of pyrolysis induced free-radicals from the luminol emitted chemiluminescence maxima of the test samples [calculated by averaging five data acquisitions 1.5 minutes apart that corresponded to five highest chemiluminescence values detected from blank (the buffer by itself) following the second pyrolysis of ABAP (the 2nd challenge)] of the test samples compared to the blank. Non-identical letters indicate significance at p=0.05.

4.6 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

As shown in Figure 4.8, all samples including undigested casein exhibited significantly (p<0.05) better antioxidant properties compared to blank based on their DPPH radical scavenging efficacy, though they did not differ significantly (p<0.05) from each other. The apparent deviation of the trend of results recorded from the DPPH radical scavenging assay from those obtained from the assays to determine ORAC and peroxyl and alkoxyl radical scavenging efficacy conceivably stemmed from the difference in reaction mechanisms of the assays and nature of radicals that were generated. The DPPH assay is based on single electron transfer (SET) reaction mechanism (Huang et al., 2005) and indicates the efficacy of a test antioxidant to scavenge DPPH radicals. The other two assays depend on HAT (Prior et al., 2005) and depict the efficacy of a sample to quench alkoxyl and peroxyl radicals. Also, the overall trolox equivalent value of the samples tested in DPPH assay are significantly lower (p<0.05) compared to the results from the ORAC assay. The limited electron donating capacity of casein and its hydrolysates has been reported previously (Luo et al., 2014). Coincidentally, Sheih et al. (2009) found that
the peptide from the hydrolysates of algae protein waste also only exhibit moderate DPPH radical scavenging ability. Prior et al. (2005) pointed out that a number of antioxidants react with DPPH radicals relatively slowly than they do with the peroxyl radicals due to the steric inaccessibility of the former to those antioxidants.

![Antioxidant property of undigested casein and its hydrolytic fractions determined by their efficacy to scavenge DPPH radicals.](image)

**Figure 4.8** Antioxidant property of undigested casein and its hydrolytic fractions determined by their efficacy to scavenge DPPH radicals.

The X and Y axes indicate individual treatments and trolox equivalents of the samples (in μmol/g), respectively.

**Table 4.4** Antioxidative capacity of undigested casein and its hydrolytic fractions, as determined by their efficacy to scavenge DPPH radicals

<table>
<thead>
<tr>
<th>Samples</th>
<th>TE (μM/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>64.46±15.9</td>
</tr>
<tr>
<td>F1</td>
<td>52.56±16.9</td>
</tr>
<tr>
<td>F2</td>
<td>56.37±11.5</td>
</tr>
<tr>
<td>F3</td>
<td>55.14±23.0</td>
</tr>
<tr>
<td>F4</td>
<td>56.27±9.6</td>
</tr>
</tbody>
</table>

The values are expressed as trolox equivalent (in μM per gram of sample). The abbreviation is as follows: TE, trolox equivalent.
Figure 4.9  Calibration curve representing the relationship of trolox concentration and free radical scavenging rate for deducing trolox equivalent of undigested casein and its various hydrolytic fractions.

The X-axis represents trolox concentrations (in μM) and the Y-axis indicates percent discoloration of DPPH indicating the concentration-dependent radical scavenging rate of trolox (evident by absorbance at 517 nm).

Table 4.5  Trolox equivalents (in micromole, μM) representing the relationship of trolox concentrations and DPPH radical scavenging rates.

<table>
<thead>
<tr>
<th>Trolox concentration (in μM)</th>
<th>percent discoloration of DPPH solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5</td>
<td>0.05</td>
</tr>
<tr>
<td>125</td>
<td>0.10</td>
</tr>
<tr>
<td>250</td>
<td>0.25</td>
</tr>
<tr>
<td>500</td>
<td>0.52</td>
</tr>
<tr>
<td>750</td>
<td>0.82</td>
</tr>
</tbody>
</table>

The values are used to obtain the calibration curve in Figure 4.9.

4.7  Trolox equivalent antioxidant capacity (TEAC) assay

Trolox exhibited significantly higher (p<0.05) antioxidant capacity compared to all other samples (Figure 4.10). All hydrolytic fractions exhibited significantly (p<0.05) enhanced antioxidant capacity compared to undigested casein with 64.0, 300.7, 191.2 and
153.1% augmentations for the F1, F2, F3 and F4, respectively. F2 exhibited significantly (p<0.05) higher antioxidant capacity relative to other treatments, with 75.0, 59.1, 27.3 and 36.8 % enhancements, with respect to undigested casein, F1, F3 and F4, respectively.

The results from the TEAC exhibited several similarities with those from the HAT-based assays, for instance, the F2 exhibiting markedly better antioxidant properties compared to other samples, various fractions depicting better antioxidant properties relative to undigested casein; F3 and F4 showing significantly better antioxidant efficacy with respect to the F1 and undigested casein, etc. However, these results deviated considerably from those from the DPPH radical scavenging assay. These observations conceivably resulted from the mechanism of TEAC, which is based on both HAT and SET (Jiménez, Selga, Torres, & Julià, 2004; Prior et al., 2005).
4.8 Size exclusion high performance liquid chromatography (HPLC) of peptide fractions

Three peptide fractions showed different HPLC absorbance profiles as shown in Figures 4.11 and 4.12 as well as the Table 4.6. Each fraction was separated with molecular weights between 7050-200 Da (Table 4.6) by using a Superdex Peptide 10/300 GL column. The F2 possesses 11 absorbance peaks which conceivably indicated richer peptide compositions than other two fractions. At 214 nm (Figure 4.11) the absorbance profiles difference indicated different peptide bond concentration and/or aromatic amino acid compositions. It is noteworthy that there is a peak at retention time of 58.6 min, which indicate a peptide component has a molecular weight of 854.5 Da and has high
area percentage in the entire peptide component in F2. Nevertheless, no absorbance was shown at this retention time in other fractions. This peptide component may need further purification in future studies to see if it contributes to the overall antioxidant efficacy of F2. While at 280 nm (Figure 4.12), the absorbance was mainly contributed by the presence of aromatic amino acids. Therefore, a higher absorbance signal indicated more aromatic amino acids present in the peptide fractions. The total peak area of F2, $4.8 \times 10^{-2}$ mAU, was significantly ($p<0.05$) higher than those of the others, followed by $8.2 \times 10^{-3}$ mAU and $5.7 \times 10^{-3}$ mAU, indicated by the F3 and F4, respectively. The presence of large amount of aromatic amino acids in F2 plausibly contributed to its highest antioxidative capacity exhibited by ORAC, TRAP and ABTS analyses resulting from potential π-π stacking effects among aromatic amino acids, which is expected in peptides with aromatic amino acid terminus that are generated following α-chymotrypsin digestion (Hedstrom, 2002; Tatsushi & Morihara, 1978).
Figure 4.11  Size exclusion high performance liquid chromatography elution profiles of F2, F3 and F4 on a Superdex Peptide 10/300 GL gel filtration column monitored at 214 nm.
Table 4.6  Calculated results of average molecular weight distribution of F2, F3 and F4 based on the absorbance at 214 nm.

<table>
<thead>
<tr>
<th>PK</th>
<th>F2 RT (min)</th>
<th>MW (Da)</th>
<th>ARP (%)</th>
<th>F3 RT (min)</th>
<th>MW (Da)</th>
<th>ARP (%)</th>
<th>F4 RT (min)</th>
<th>MW (Da)</th>
<th>ARP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34.9</td>
<td>7045.6</td>
<td>1.8</td>
<td>35.0</td>
<td>7028.6</td>
<td>20.1</td>
<td>35.0</td>
<td>7019.2</td>
<td>39.6</td>
</tr>
<tr>
<td>2</td>
<td>38.3</td>
<td>5228.1</td>
<td>43.6</td>
<td>38.4</td>
<td>5157.4</td>
<td>41.5</td>
<td>38.1</td>
<td>5294.2</td>
<td>26.1</td>
</tr>
<tr>
<td>3</td>
<td>41.0</td>
<td>4120.4</td>
<td>13.9</td>
<td>42.8</td>
<td>3498.6</td>
<td>0.9</td>
<td>42.2</td>
<td>3662.2</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td>45.2</td>
<td>2833.0</td>
<td>0.5</td>
<td>46.6</td>
<td>2502.7</td>
<td>5.5</td>
<td>56.5</td>
<td>1034.8</td>
<td>14.3</td>
</tr>
<tr>
<td>5</td>
<td>46.8</td>
<td>2452.0</td>
<td>4.6</td>
<td>64.3</td>
<td>516.3</td>
<td>23.6</td>
<td>64.3</td>
<td>516.0</td>
<td>11.3</td>
</tr>
<tr>
<td>6</td>
<td>48.4</td>
<td>2124.3</td>
<td>1.1</td>
<td>74.7</td>
<td>204.4</td>
<td>8.4</td>
<td>74.6</td>
<td>205.5</td>
<td>4.1</td>
</tr>
<tr>
<td>7</td>
<td>56.3</td>
<td>1047.4</td>
<td>2.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>58.6</td>
<td>854.5</td>
<td>11.8</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>9</td>
<td>64.0</td>
<td>527.8</td>
<td>13.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>68.6</td>
<td>350.9</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>71.3</td>
<td>276.8</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>74.2</td>
<td>212.4</td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations are as follows: PK, Peaks; RT, Retention time; MW, Molecular Weight; ARP, Area percentage.
Figure 4.12  Size exclusion high performance liquid chromatography elution profiles of F2, F3 and F4 on a Superdex Peptide 10/300 GL gel filtration column monitored at 280 nm.

4.9  High performance capillary electrophoresis (CE)

Results from the CE analyses were similar to those from the HPLC. At 214 nm (Figure 4.13), three fractions showed different CE profiles and within them the F2 depicting the richest peptide constitution. The same fraction also showed higher absorbance values at 280 nm compared to the F3 and F4, which was in accordance with the results of HPLC. It further confirmed that the F2 was considerably rich in aromatic amino acids and those aromatic amino acids that conceivably participated in π-π interactions and resulted in the enhanced antioxidative efficacy.
Figure 4.13  Capillary electrophoresis profiles of F2, F3 and F4 monitored at 214 nm.
Figure 4.14  Capillary electrophoresis profiles of F2, F3 and F4 monitored at 280 nm.

4.10  Circular dichroism (CD)

The CD result on analysis of peptide structure is very valuable where X-ray crystallography cannot be applied (Kelly, Jess, & Price, 2005). In CD analysis, the negative bands displayed at 222 and 208 nm indicates the existence of α-helix structure and the negative bands displayed at 218 nm indicates the existence of β-sheets (Greenfield & Fasman, 1969; Sreerama & Woody, 2004). While the positive bands exhibited at 193 and 195 nm indicates the presence of α-helix and β-sheets, respectively (Greenfield & Fasman, 1969; Sreerama & Woody, 2004). As shown in the Figure 4.15, the CD result of casein indicated a negative extrema of absorbance at 208 nm, which
showed the existence of $\alpha$-helix structures (Sreerama & Woody, 2004), but this extrema was not detected in the chymotrypsin-digested fractions. The negative extrema of peptide fractions shifted toward the lower wavelengths that indicated the disappearance of periodical secondary structures. The difference of CD profiles between peptide fractions and original casein indicated the F2, F3 and F4 have residual structure whereas the F1 does not.

![Figure 4.15](image)

Figure 4.15  The circular dichroism spectra of peptide fractions and undigested casein.
CHAPTER V
CONCLUSION

The current research evaluated the antioxidant properties of undigested casein and its hydrolytic fractions generated by α-chymotriptic digestion and chromatographic fractionation. Four different assays to evaluate antioxidant properties were conducted; that were based on entirely different reaction mechanisms. In addition, three classes of radicals were generated in-vitro (namely, peroxyl/alkoxyl, DPPH and ABTS⁺) to determine the radical scavenging efficacy of the test samples, which also differed markedly with respect to their chemical properties. F2, which was eluted by 45-60% ethanol exhibited the greatest antioxidant properties in response to all assays except for the one using DPPH radicals. No obvious correlation between antioxidant efficacy and SH was found. The size exclusion HPLC and CE were adopted to evaluate the concentration of amino acids containing aromatic ring in all the fractions and CE result indicates higher peak area of aromatic ring contained peptides in F2, which is also the fraction exhibits the best antioxidant capacity. The highest antioxidative ability of F2 conceivably attributes to its larger amount of aromatic amino acids compared to other fractions; this larger amount of aromatic amino acids may induce higher potential that π-π stacking effect happen which can contribute to charge transfer in antioxidant reaction. A particular peptide component was found in F2 and need further purification to measure its different properties. Future research approaches include determining the amino acid
sequences of the peptides belonging to various fractions – which would present further evidence of the association between antioxidant capacity and concentration of amino acids containing aromatic rings.
REFERENCES


