Deriving and evaluating alternative antioxidants from corn coproduct proteins

by

Ruijia Hu

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College of Agriculture

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Approved by:

Major Professor
Yonghui Li

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Abstract

Corn is one of the most cultivated crops worldwide and is an important source for food, feed and biofuel in the U.S. Corn gluten meal (CGM) and distillers' dried grains with solubles (DDGS) are two major protein-rich co-products from corn processing, which are potential sources to produce high-value bioactive peptides. However, limited information is available on the production and antioxidant performance of CGM and DDGS protein hydrolysates. The objectives of this study were to produce hydrolysates from those corn co-products via enzymatic hydrolysis, fractionate and identify antioxidant peptides, and evaluate their antioxidant performances with chemical assays and in different model systems.

In the first part of our experiment, we screened and evaluated nine different microbial-, plant-, and animal-derived proteases for corn antioxidant production and found that CGM protein hydrolyzed with Neutrase at enzyme-to-substrate ratio of 0.4 Au/g and reaction time of 4 h had the most promising antioxidant properties and yield. The 1-3 kDa ultra-filtrated fraction of the hydrolysate exhibited the highest antioxidant capacities with respect to DPPH and ABTS free radical scavenging activity and metal ion (Fe^{2+}) chelating activity. The fraction was further purified through RP-HPLC, and peptide composition and sequences were identified using MALDI-TOF/TOF MS. Addition of this fraction in an oil-in-water emulsion system significantly reduced the amount of primary and secondary oxidation products. It also led to 49.2% reduction of lipid peroxidation compared with the control (i.e., no antioxidant) when incorporated at 1 g/kg in ground pork. In addition, the hydrolysate significantly inhibited cancer cell growth when tested with a human hepatocarcinoma (HepG2) cell model, with cell growth reduction of 64.2% and 71.6% compared with the control (i.e., no antioxidant treatment) when added at 50 and 200 μg/mL, respectively.

The second part was focused on enzymatic hydrolysis of CGM using three plant proteases (i.e., papain, ficin, and bromelain) for antioxidant production and process optimization. Optimum enzyme-to-substrate ratios for papain, ficin, and bromelain were found to be 60 U/g, 90 kGDU/g, and 180 kGDU/g, respectively. Optimum hydrolysis time for papain was 3 h, and that for ficin and bromelain was 4 h. The 5-10 kDa peptide fraction produced by papain, <1 kDa fraction produced by ficin, and 3-5 kDa fraction produced by bromelain showed the strongest antioxidant activity and yield in respective hydrolysates. These peptide fractions greatly inhibited lipid oxidation by reducing the formation of thiobarbituric acid reactive substances (TBARS) when added into ground pork.

In the last part, hydrolysates were prepared from CGM and DDGS proteins with Neutrase and Alcalase, respectively, and the antioxidant performances of those hydrolysates in bulk oils, ground pork, canine pet food, and pig feed were evaluated by measuring peroxide value (PV) and TBARS. Alcalase-hydrolyzed CGM (CPH-A) and Neutrase-hydrolyzed CGM (CPH-N) had stronger DPPH radical scavenging activity than Alcalase-hydrolyzed DDGS (DPH-A) and Neutrase-hydrolyzed DDGS (DPH-N). CPH-N showed better prevention of lipid oxidation in both corn oil and fish oil compared with other corn antioxidants. The best oxidation prevention in ground meat was observed with 2 g/kg of CPH-N. Lipid oxidation in pet food containing 2% DPH-A was efficiently retarded by 37.8% reduction at the end of incubation, and TBARS value of pig feed containing 2% CPH-N was reduced the most compared with other treatments.

This study generates useful knowledge to produce antioxidative hydrolysates from CGM and DDGS, which adds value to the co-products from corn processing industries and provides alternative naturally-derived antioxidant options for food, pet food, and animal feed uses. It revealed that bioactive peptides from corn could inhibit lipid oxidation through scavenging free

radicals as well as chelating metal ions. The antioxidants also demonstrated potential anticancer properties based on HepG2 cell study.

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Chapter 1 - Introduction

1.1 General background

1.1.1 Corn and corn co-products

Corn (Maize) is one of the mostly cultivated crops worldwide. International Grains Council has reported that the production of corn was approximately 1074 million tons in 2017/2018, and more than half of this is produced in the United States. Dent corn, which is also called filed corn, is the major variety grown in the U.S. It is widely used for livestock feed, food ingredients, and industrial products (Li et al., 2019). Corn contains approximately 60-70% starch and 10-15% protein varied among different varieties and sources, and most of proteins are in the endosperm (Delcour & Hoseney, 2010). Corn proteins can be classified into four fractions based on their different solubility. Prolamins (also called zein) and glutelin are the main seed proteins which account for about 68% and 28%, respectively. Zein is insoluble in water but exhibits good solubility in ethanol, and glutelin has good solubility in dilute acid or alkali solutions (Zheng et al., 2015). Albumins are the only type of corn protein that is hydrosoluble, and globulins are soluble in salt solutions (Zheng et al., 2015). Comparing to other cereals or legumes, corn contains lower amount of lysine and tryptophan (Drajana et al., 2015). In addition, only albumins and globulins are considered to be nutritionally favored because they have better digestibility than prolamins and glutelin (Margarita, 2017).

With high content of hydrophobic amino acids, like alanine, leucine, and phenylalanine (Liang et al., 2017), peptides from corn protein is still a potential source with biological activities, such as antioxidant activity (Jin et al., 2016), enzyme-inhibitory activity (Suh et al., 2003), and facilitating alcohol effect (Yamaguchi, 1997). Although corn is a staple food in many parts of world, few of these corns are directly consumed as human foods. Most of them are used for ethanol production, animal feed or other related products. During

corn processing, there are huge amounts of corn co-products generated each year. Corn gluten meal (CGM) is one of the major co-products of corn wet milling and contains about 60% crude protein (Wang et al., 2016). Distiller's dried grains (DDG) or distillers' dried grains with soluble (DDGS) are other two high nutrient co-products produced during corn ethanol production (Belyea, Rausch, & Tumbleson, 2004). Traditionally, those co-products are mainly used as feeding materials or otherwise discarded due to low water solubility and imbalanced amino acid composition (Li, Han & Chen, 2008). In recent years, there is a growing interest in the modification of corn proteins and therefore adding value to those protein rich corn co-products. Previous studies showed that hydrolysis could be a feasible approach to increase bioactivity of proteins from low value sources, such as poultry industry residues (Rossi et al., 2009), fish byproducts (Bougatef et al., 2008), and algae waste (Shei, Wu, & Fang, 2009), as well as CGM (Wang et al., 2014; Yang, et al. 2007; Zhou et al., 2015; Zhu, He, & Hou, 2018). Those hydrolyze proteins and peptides showed an improvement of functional properties and enhanced biological values.

1.1.2 Production of bioactive corn peptides

Enzymatic hydrolysis

Preparation of protein hydrolysates is the predominant approach to release bioactive peptides from insoluble corn byproducts. It has been reported that enzymatic hydrolysis is the predominant way to prepare bioactive protein hydrolysates, and the bioavailability of CGM can be improved significantly (Wang et al. 2014). Enzymatic hydrolysis is most commonly used, since it is easy to process and control, and high in reaction efficiency. Several factors such as enzyme specificity, hydrolysis time, enzyme-to-substrate ratio could affect hydrolysis efficiency.

Enzyme used for hydrolysis can be simply classified into three types, acidic proteases, alkaline proteases, and neutral proteases. It has been founded that different enzymes can yield different functional bioactive peptides, and the enzyme-to-substrate interaction showed increase trend during hydrolysis until reached a stable point (Tavano, 2013). Different commercially available enzymes, such as Alcalase (Li et al., 2008; Zheng et al., 2006; Zhuang et al., 2013), Neutrase (Zhou, Sun, & Canning, 2012), Flavorzyme (Liu et al., 2015; Zhuang et al., 2013), trypsin (Zhuang et al., 2013), and papain (Zhuang et al., 2013) were reported could be used for protein hydrolysates production.

It is reported that antioxidant properties of corn peptides are highly related to the hydrophobic amino acids, such as Tyr, Try, Phe, and Leu (Li et al., 2019; Rajapakse et al., 2005; Zhou et al. 2015). Hence, the specificity of enzymes is critical for functional properties of corn hydrolysate. Alcalase is the most common enzymes used for corn hydrolysates production because it prefers to clave Tyr, Trp, Phe, and Leu position which are high in corn proteins (Li et al., 2007). Laing et al. (2017) found that the corn peptides from CGM prepared with Alcalase possessed high antioxidant properties against DPPH scavenging. Wang et al. (2014) also reported that Alcalase-hydrolyzed CGM showed higher antioxidant performance than Protamex-hydrolyze CGM against DPPH, ABTS and hydroxyl radicals. Besides using individual enzyme, combination of multiple enzymes has also been applied to produce corn peptides. The multiple-enzyme methods base on the combined action of several proteases, and optimum ratio of the various enzymes along with the optimal reaction conditions for each enzyme should be considered. Compared with using single enzyme, the multiple-enzymes approach is more efficient with higher antioxidant yield. Jin et al. (2016) reported using Alcalase, Flavourzyme, and combination of those two enzymes for the hydrolysis of CGM. They found that using of two enzymes was more effective than single enzyme, and

hydrolysates prepared by sequential hydrolysis with Alcalase followed by Flavourzyme showed better antioxidant performance.

Chemical hydrolysis

In addition to enzymes, alkaline, acid or other chemical reagents treatments can be used to prepare bioactive peptides from different proteins, such as zein (Cabra et al., 2007), whey (Matemu et al., 2012), and soy (N'Guyen et al., 1992). It was reported that soaking CGM with 4% sodium carbonate solution at 40 °C for 10 min could increase protein contents to 58.24±0.11% (Liu et al., 2015). Combination the treatment with starch removal, the protein content of CGM increased to 77.70±0.11% (Liu et al., 2015). Casella and Whitaker (2010) found that sodium dodecyl sulfate (SDS) had a similar function as sodium carbonate ($NaCO_3$) at pH of 8.0 to increase the solubility and emulsifying activity of zein. Besides, alkaline deamidation of zein using NaOH at 90 °C could also denature the secondary and tertiary structures of α-zein (Cabra et al., 2007). Another study reported the effect of heating and alkali treatment on DDG (Wang et al., 2016), and the results showed that the degree of hydrolysis and the conversion rate of protein are increased with heat treatment. Overall, chemical hydrolysis is more difficult to be controlled compared with enzymatic hydrolysis. It may produce several undesirable side products such as salt and D-amino acids from L-amino acids, and some of those products are difficult to be removed (Samaraweera et al., 2013). In addition, the use of alkali reactants in protein hydrolysis usually cause poor functionality and results in loss of nutritive value of the hydrolysate (Kim et al., 2004).

Microbial fermentation

Microbial fermentation is another approach which could also be used to produce bioactive peptides. The microorganisms commonly used include mold, bacteria, and yeast (Li

et al., 2019). Microbial fermentation usually costs less, and it has high level of protease activity (LeBlanc et al, 2002). In recent years, some researchers have reported that microorganisms could help release peptides during fermentation, such as dipeptides, tripeptides, as well as some peptides with bioactive property (Cao et al., 2009; LeBlanc et al., 2002). Qian et al. (2011) reported that peptides produced from skim milk with *Lactobacillkus delbrueckii* ssp. *Bulgaricus* LB340 possessed antioxidant and antihypertensive activities. Besides, pea seeds (Jakubczyk et al., 2013) and soybean (Iwai et al., 2012) could also generate bioactive peptides though microbial fermentation. There are some studies focusing on fermentation of CGM. Zhang et al. (2009) found that CGM fermented with B. *subtilis* Is-45 at 41 °C for 63 h could produce 82.7% of corn peptides. The *Aspergillus* was also reported could produce corn peptide from CGM with about 36% conversion (Li, Cui, & Tan, 2012). Microbial fermentation is cheap and safe method with little safety concern, and it does not cause loss of amino acids, but it is still a new method with less understanding compared with other approaches (He et al., 2012).

1.1.3 Identification and characterization of bioactive corn peptides

Corn hydrolysates usually contain various peptides with a wide range of MW distribution. To study the correlation of antioxidant property and MW distribution, and further analyze the specific peptides, corn hydrolysates are usually separated into several distinct fractions using membrane ultrafiltration and column chromatography (Lin et al., 2010; Wang et al., 2014; Zhuang et al., 2013). Fractionation with sequential membrane ultrafiltration is easy to control and has high efficiency which is commonly used for the separation of corn peptides (He et al., 2013; Wang et al, 2014). Ion exchange chromatography (Wang et al., 2014) and gel permeation chromatography (Zhou et al., 2015; Zhuang et al.,

2013) are also used to separate protein hydrolysates. The initial hydrolysates could be separate into several fractions with different MW by UF and then further purified by gel permeation chromatography with a Sephadex column (Jin et al., 2004; Wang et al., 2014). Corn protein hydrolysates were purified and isolated using high-performance liquid chromatography (HPLC) (Chen et al., 2005; Wang et al., 2014). Wang et al., 2014 reported the purification and characteristics of corn antioxidant peptides using sequential purification steps. The initial hydrolysates were first ultrafiltrated into four fractions, and then the fractions were further purified by anion exchange and size exclusion chromatography. The purified peptides were finally identified by RP-HPLC.

The characterization of hydrolysate peptides could be conducted by electrophoresis analysis such as sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE could reveal the MW distribution of peptides (Margarita et al., 2017). The mass spectrometry (MS) is another common technique for identification of peptide sequences (Chen et al., 2005; Wang et al., 2015; Wang et al., 2016; Zhuang et al., 2013). The primary structure of corn antioxidant peptides could be identified by HPLC-MS/MS using electrospray ionization (ESI), especially for low MW peptides (Li et al., 2004; Ma et al., 2012; Wang et al., 2015; Yang et al., 2007). For examples, a novel antioxidant peptide from corn protein hydrolysate was determined by RP-HPLC-MS/MS as Gln-Gln-Pro-Gln-Pro-Trp with MW of 782.34 Da (Wang et al., 2014). Yang et al. (2007) used the same method and identified the corn peptide of Ala-Tyr. The quadrupole time-of-flight mass spectrometer (Q-TOF) coupled with ESI was also used by some researchers for identification of bioactive

peptides (Jin, et al., 2016; Lin, et al., 2010; Tang & Zhuang, 2014; Zheng et al., 2006). MALDI-TOF/TOF mass spectroscopy had also been used for peptide sequence identification (Bythell et al., 2007; Wang, Chen, et al., 2015; Wang, Ding et al., 2015). Moreover, the iTRAQ-LC/MS/MS method and a JEOL JAS-47K peptide sequencer can perform the same role for peptide identification (Kasper et al., 2009; Suh et al., 1999).

1.1.4 Biological activities of corn peptides

Peptides can potentially terminate the radical chain reaction by donating protons or react with free radicals to convert them into more stable products. It could remove superoxide anions generated from pyrogallol autoxidation and exhibits scavenging activity of superoxide anion (Li et at., 2019). Corn peptides were reported to serve as a reducing agent to eliminate hydroxyl radicals before they attacked rhodamine B, scavenging radicals, and possessed reducing power (Li et al., 2010). Corn peptides were commonly examined for their scavenging activities toward 2, 2-diphenyl-1-picrylhydrazyl (DPPH $^{\cdot}$), 2, 2'-azino-bis (2-ethylbenzothiazoline-6-sulfonic acid) (ABTS $^{\cdot}$), and superoxide anion ($O_2^{\cdot}$). There are various mechanisms that oxidation can be inhibited by antioxidant compounds, such as scavenging of free radicals, inactivation of reactive oxygen species, reduction of hydroperoxides, chelation of metal ions, and enzymatically elimination of specific oxidants (Elias, Kellerby & Decker, 2008). Since antioxidative mechanisms and reaction conditions may differ between assays, different results may be obtained depending on the assay system. To get a comprehensive understanding of antioxidant activity, multiple assays are usually performed, but they may show opposite results due to the different mechanisms (Jin et al., 2016). However, no specific or standard assay has been developed to measure the antioxidant

capacities of peptides. Thus, most researches used combination of different assays which commonly used for non-peptide antioxidants to measure antioxidant activities of peptides.

According to the results of previous researches, the free radical scavenging activity of corn protein hydrolysates or peptides was related to both the molecular weight and their hydrophobicity. Low MW peptides have been reported to interact more effectively with radical intervening than high MW fractions (Chi et al., 2015; Jin et al., 2016). In the study of Zhuang et al., the enzymatic hydrolysates were separated into three fractions, below 1 kDa, 1 to 3 kDa, and above 3 kDa, and the fraction with MW < 1 kDa was found to exhibit the highest inhibitory activity. Zhou's research (2012) has indicated similar results. The 1-3kDa peptide fraction from zein exhibited the highest activity in scavenging peroxyl radicals. Corn peptide fractions were also studied by Wang et al. (2015), and their results showed that two fractions (MW below 1 kDa and 1 to 3kDa) exhibited good hydroxyl radical, superoxide anion radical and $ABTS^{\cdot}$ radical scavenging activities.

Interpretations have been attempted to explain the antioxidant properties of corn peptides. Amino acids such as His, Leu, Lys, Met and Tyr are widely accepted to be antioxidative and contribute to the scavenging of free radicals (Li, et al., 2010; Wang & Chen et al., 2015; Wang & Ding et al., 2015). Saito et al. (2003) reported that tripeptide containing Trp or Tyr residues at the C-terminus has strong radical-scavenging activity. Pro residues of peptides also contribute to the radical scavenging because of its unique structure (Wang et al., 2014). Zhang et al. (2012) reported that the antioxidant peptide Phe-Leu-Pro-Phe showed the highest DPPH radical scavenging activity. There was another research indicated that antioxidant peptide Tyr-Phe-Cys-Leu-Thr also showed good antioxidant capacity (Zheng et al., 2006). Both peptides contained Thr and hydrophobic amino acid residues Phe and Leu. Phe is an aromatic residue which could working as a proton donator in antioxidant activities to transfer proton to free radicals (Rajapakse, 2005; Zhou et al., 2015).

Besides antioxidant capacity, other studies have investigated the anti-obesity performance of corn protein hydrolysates or peptides. Lu et al. (2016) studied the effects of corn peptides as supplement on fat loss and blood lipid profile in obese rats. Compared with the control group, the group treated with corn peptides showed significant decrease on body weight. The protein levels of adipose triglyceride lipase (ATGL) in livers and lipoprotein lipase (LPL) in adipose tissues were significantly increased in the group treated with exercise and the group treated with corn peptides plus exercise. The rat treated with corn peptides showed more obvious increase. It indicated that corn proteins had the penitential to control body weight and that may relate to the change of protein levels of ATGL in livers and LPL in adipose tissues. In addition, corn hydrolytes or peptides were also reported to possess antihypertensive activity (Acharya et al., 2003; Huang et al., 2011; Wang, Chen et al., 2015; Wang, Ding et al., 2015), hepatoprotective activity (Lv et al., 2013; Wu et al., 2014; Zhang et al., 2012), and alcohol-metabolism facilitating (Haseba & Ohno, 2010; Li et al., 2011; Lu & Cederbaum, 2008).

1.1.5 Potential applications of bioactive corn peptides

In recent years, there is a growing interest in developing alternative antioxidants from protein sources due to the low cost and less safety concern. Protein hydrolysates and antioxidant peptides can be used in food systems to prevent oxidation during storage. Several studies have shown that the hydrolysates or bioactive peptides produced from rice bran protein (Cheetangdee & Benjakul, 2014), milk casein (Blanca et al., 2007), soy protein (Oliveira et al., 2014), sorghum kafirin (Xu et al., 2019a; 2019b), egg yolk (Psrk et al., 2001), marine blue mussel (Rajapakse et al, 2005), as well as corn proteins (Liu, et al., 2015; Zhu, He, & Hou, 2018) exhibited high antioxidant activities in both *vitro* and *vivo* models, and they had similar or even higher oxidative inhibition capability compared with some

commercial synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG). Corn peptides have high bioactivities as reviewed above, such as antioxidant, anti-hypertension, and anti-cancer properties. Therefore, bioactive corn peptides can be potentially used as natural addictive in beverages, dairy products, and other oil/fat rich food, feed or pet food products to enhance shelf stability. Although some studies have been carried out previously to produce bioactive peptides from corn proteins, there still lacks information regarding process optimization, composition-activity relationships, and applications of peptide antioxidants derived from corns, especially utilizing corn co-products such as CGM and DDGS.

1.2 Objectives

The objectives of this study were to produce and characterize protein hydrolysates from CGM and DDGS and evaluate their antioxidant activities as well as antioxidant performances in several model systems, in order to add value to corn processing and deliver alternative novel natural antioxidants. The specific objectives were to：

(1) investigate the effects of various factors (types of proteases, enzyme-to-substrate concentration, and reaction time) on the production of CGM protein hydrolysate and antioxidative properties of the hydrolysates;

(2) study the correlation between antioxidant activity and MW as well as peptide sequences of produced hydrolysates;

(3) investigate the antioxidant performance of peptides with promising antioxidant properties in several model systems, including HepG2 cancer cell, emulsions, bulk oils, ground meat, pet food, and animal feed.

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Chapter 2 - Optimization of Antioxidant Peptides Preparation from Corn Gluten Meal Using Enzymatic Hydrolysis and Their Applications

2.1 Abstract

Hydrolyzed bioactive peptides from food proteins are potential alternative natural antioxidants for food and feed industries. In this study, corn gluten meal (CGM) was hydrolyzed by several enzymes to obtain antioxidant peptides. Neutrase-hydrolyzed CGM protein prepared with enzyme-to-substrate ratio at 0.4 Au/g and reaction time of 4 h was selected for further analysis because of promising antioxidant properties and yield. The selected hydrolysate was fractionated by ultrafiltration, and the 1-3 kDa fraction exhibited highest antioxidant capacities regarding 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, ABTS scavenging activity, and metal ion (Fe^{2+}) chelating activity. The addition of selected peptide fraction into an oil-in-water emulsion system reduced the amount of primary and secondary oxidation products with an average of 82.17% and 90.71%, respectively. The hydrolysate also resulted in 49.17% inhibition of lipid peroxidation when incorporated at 1000 mg/kg in ground pork system. The peptides from 1-3 kDa were identified using RP-HPLC analysis followed by MALDI-TOF/TOF MS. The anti-cancer performance of 1-3 kDa peptide fraction was evaluated in the human hepatocarcinoma (HepG2) cell model. The cancer cell treated with 50 and 200 μg/mL of 1-3 kDa peptide fraction showed significant growth prevention with reduction of 64.2% and 71.6% after 144 h incubation, respectively. This study demonstrated that corn gluten meal is a potential source to produce antioxidant peptides which may be used in different areas.

2. 2 Introduction

In recent years, antioxidants are widely used in human foods, pet foods, animal feed, and many other industrial products to improve their quality and shelf stability. However, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate have caused many concerns regarding their health risks and toxicity (Li et al., 2010; Shahidi & Zhong, 2008). Thus, alternative antioxidants from natural sources, especially antioxidative peptides from different proteins, have drawn attention of researches because of their strong activities but low health concerns (Li et al., 2010; Pan, Jiang, & Pan, 2011; Zhou et al., 2015; Zhuang, Tang, & Yuan, 2013). Hydrolyzed proteins from several animal and plant sources as well as low-value commercial sources, such as frog skin (Qian, Jung, & Kim, 2008), milk casein (Blanca et al., 2007), rice protein(Zhou, Canning, & Sun, 2013), soy protein (Oliveira et al., 2014), fish by-products (Bougatef et al., 2009), and algae waste (Sheih, Wu, & Ayub, 2009) have been reported to possess antioxidant activities.

Previous researches reported that corn proteins contained abundant bioactive peptides and structural domains which performed critical functions in human body including antihypertension, anti-obesity and antioxidant (Huang et al, 2011; Yang et al., 2007; Lu et al., 2016; Zhuang t al., 2013; Jin et al., 2016). However, corn proteins have low water solubility which limits its performance and utility in food systems. Prolamins (also called zein) and glutelin are the main seed proteins in corn which account for 68% and 28%, while zein is insoluble in water but exhibits solubility in ethanol (Coleman and Larkins, 1989), and glutelin has good solubility only in dilute acid or alkali solutions (Zheng et al., 2015). Corn is the third crop cultivated worldwide with production of approximately 1074 million tons in 2017/2018, and 34% is cultivated by United States (Zhu, He, & Hou, 2019). Large amount of corn co-products was produced each year from corn processing. Corn gluten meal (CGM) is one of the major coproducts from corn wet milling and contains about 60%-70% crude

protein. Distiller's dried grains (DDG) or distillers' dried grains with soluble (DDGS) are other two high nutrient coproducts generated during ethanol production from corn (Wang et al., 2016). Traditionally, those products are mainly used as feeding materials or otherwise discarded due to low water solubility (Li et al., 2008), while researchers have found that preparation of hydrolysates can release bioactive peptides from insoluble corn co-products which can improve the bioavailability of those commercial co-products. Protein hydrolysates can be produced by acid treatment, microbial fermentation or enzymatic hydrolysis (Li et al., 2019). The conditions of acid hydrolysis of proteins are difficult to control, while the microbial fermentation is usually less efficient compared with other approaches. Enzymatic hydrolysis has been the predominant approach to produce bioactive protein hydrolysates, and the bioactivity of food proteins can be improved significantly (Lu, Chen, & Tang, 2000).

The objectives of this study were to investigate the effects of various factors (types of proteases, enzyme-to-substrate concentration, and reaction time) on the production of CGM protein hydrolysate and the antioxidative properties of produced hydrolysates. The fractionated hydrolysates with promising antioxidant activities were used in two model food systems including O/W emulsion and ground meat to evaluate their antioxidant performance. Anticancer effect of the hydrolysate in human hepatocarcinoma (HepG2) cells was also investigated. In addition, peptide compositions of the promising hydrolysate fraction were identified using MALDI-TOF/TOF MS.

2.3 Experimental Section

2.3.1 Materials

The corn gluten meal (CGM) contains 61.3% crude protein (dry base), and was provided by Grain Processing Corporation (Muscatine, IA, USA). Neutrase, Flavourzyme, Alcalase, Everlase, Protamex, papain, and trypsin were purchased from Sigma-Aldrich (St.

Louis, Mo, USA). Ficin was purchased from Tokyo Chemical Industry Co. (Kita-ku, Tokyo, Japan), and bromelain was purchased from Acros Organics (Fairlawn, NJ, USA). All other chemicals, solvents, and reagents used were of at least analytical grade and purchased from Sigma-Aldrich (St. Louis, Mo, USA) or Fisher Scientific (Fairlawn, NJ, USA).

2.3.2 Preparation of corn gluten meal hydrolysates

CGM was pretreated with deionized (DI) water (1:6, w/v) and stirred for 1 h at room temperature, and then filtrated. Water washing was repeated for twice. The washed CGM was dried in oven at 45 ℃ for 48 h. Fat was then removed by mixing dried CGM with hexane (1:6, w/v) and stirring for 0.5 h. Following three times defat, the treated CGM was dried in fume hood for 24 h. Before adding enzymes, thermal denaturation process was performed to increase the efficiency of hydrolysis. The 4% CGM suspension (w/v, protein base) was prepared by mixing 16.3 g CGM with 250 mL of DI water and the suspension was heated in 100℃ water bath for 10 min. After cooling down to room temperature, pH was adjusted to optimum level according to enzymes' properties (Table 2.1). The enzymatic hydrolysis was conducted in water bath shaker with optimum temperature for each enzyme depending on their characteristics (Table 2.1).

2.3.3 Determination of antioxidant yield

The antioxidant yield was calculated to estimate the ratio of soluble protein or peptides after hydrolysis. It was calculated by equation as follow: Antioxidant yield = (Wp / Wi) * 100%, where Wi was the weight of initial protein, and Wp was the weight of soluble proteins that lyophilized from hydrolysate supernatant.

2.3.4 Determination of degree of hydrolysis (DH)

Degree of hydrolysis (DH) was defined as percentage of cleaved peptide bonds after hydrolysis (Bougatef et al., 2010). It was determined based on the reaction of primary amino groups with o-phthaldialdehyde (OPA) reagent according to the method of Nielsen, Petersen, & Dambmann (2001). OPA reagent was prepared as follow: (1) 7.62 g di-Na-tetraborate decahydrate ($Na_2B_4O_7$) and 200 mg Na-dodecyl-sulfate (SDS) were dissolved completely in 150 mL DI water (solvent A); (2) 160 mg OPA was dissolved in 4 mL ethanol and then transferred to solvent A (solvent B). (3) 176 mg dithiothreitol (DTT) was added to the solvent B and total volume was adjusted to 200 mL with DI water. To measure the DH, 400 uL sample or standard solution was mixed with 3 mL of OPA reagent and stored at room temperature for exactly 2 min. The absorbance of the mixture was then read immediately at 340 nm on a spectrophotometer (VWR UV-6300PC, Radnor, PA, USA). All hydrolysate was prepared at 1.2 mg/mL. The DI water was used as control, and serine standard solution (0.9515 mM) was prepared as standard. Triplicates of each sample were measured.

According to Adler-Nissen (1986), DH was calculated as follows:

$$\mathrm{DH} = \frac{\mathrm{h}}{\mathrm{h_{tot}}} \times 100\%$$

Where h is the number of hydrolyzed bonds and the h_{tot} is the total number of peptide bonds per protein equivalent which depends on the amino acid composition of the raw material. In this study, h_{tot} = 8.3 mmol/g.

Determination of h:

$$\text{Serine-NH}_2 = \frac{\mathrm{A_{sample}} - \mathrm{A_{blank}}}{\mathrm{A_{standard}} - \mathrm{A_{blank}}} \times 0.9516 \,/\, (\mathrm{X} \times \mathrm{P})$$

Where X is the sample concentration, and P is protein purity of sample. Hence,

$$\mathrm{h} = \frac{\mathrm{SerineNH_2} - \beta}{\alpha}$$

Where $\alpha = 1$, $\beta = 0.4$.

2.3.5 Ultrafiltration fractionation

Hydrolysates treated by different enzymes were ultrafiltrated by an Amicon® Stirred Cell device (EMD Millipore Corporation, Billerica, MA, USA) with Ultracel® Ultrafiltration Discs (EMD Millipore Corporation, Billerica, MA, USA). Four different cut-off size (1, 3, 5, and 10 kDa) of ultrafiltration discs was used. Hydrolysate sample was prepared at 5 mg/mL and loaded onto the stirred cell. Separation was conducted with continuously stirring (60 rpm) under compressed nitrogen (60 psi). The eluent fractions were lyophilized and then stored in freezer for further analysis.

2.3.6 Measurement of total phenolic content

The total phenolic content (TPC) of CGM hydrolysates was evaluated by Folin-Ciocalteu method (Thamnarathip et al., 2016). Briefly, 2 mL of protein sample at 1 mg/mL was mixed with 2 mL of diluted Folin-Ciocalteu reagent (1:10, v: v), and 6 mL of 7% sodium bicarbonate solution. The mixture was incubated at room temperature for 30 min and then read absorbance at 760 nm. Gallic acid (0-0.06 mg/mL) was used as standard, and the TPC was calculated as mg gallic acid equivalents (GAE)/ g of protein.

2.3.7 Determination of antioxidant activities

DPPH radical scavenging activity

The 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of protein hydrolysates was measured following a previous method reported by Li et al. (2008) with slightly modifications. Briefly, 5 mL of each samples solution at 5 mg/mL was added to 5 mL DPPH solution (2.88 mg DPPH in 100 ml 95% ethanol). The mixture was vortexed for 30 sec and rest in dark for 30 min, and then absorbance was read at 517 nm. The DPPH radical scavenging activity was expressed as follows:

DPPH scavenging activity (%) = $[(A_{blank} - A_{sample})/ A_{blank}] \times 100$.

ABTS radical scavenging activity

The ABTS radical scavenging activity was determined according to the modified method of Thaipong et al. (2006). The ABTS radical cation stock solution contained 7.4 mM ABTS and 2.6 mM potassium persulfate solution was prepared and stored for 12-16 h in dark before use. The stock solution was then diluted using ethanol to achieve an absorbance of 1.1 ± 0.02 at 734 nm. To measure ABTS of hydrolysate solution, 0.15 mL of sample solution at 1 mg/mL was mixed with 2.85 mL of diluted ABTS radical solution. Following 60 min storage in dark at room temperature, absorbance of the mixture was read at 734 nm. The radical scavenging activity was calculated using follow equation:

ABTS scavenging activity (%) = $[(A_{blank} - A_{sample})/ A_{blank}] \times 100$.

Metal ion (Fe^{2+}) chelating activity

The ability of protein hydrolysates or peptides to chelate Fe^{2+} irons were measured following a precious published protocol with some modifications (Elias, Kellerby, & Devker, 2008). Briefly, 25 μL of hydrolysate samples at 1 mg/mL was loaded into microcell plate, and then 150 μL DI water and 25 μL of 0.2 mM $FeCl_2$ solution were added. The mixture was incubated at room temperature for 30 sec. After adding 50 μL of 1mM ferrozine solution, absorbance of plate was then read at 562 nm. The chelating ability of sample was calculated as follows:

Fe^{2+} chelating ability (%) = $[(A_{blank} - A_{sample})/ A_{blank}] \times 100$.

2.3.8 Identification of antioxidant peptide sequences

Peptide fractions with promising antioxidant capacities was further analyzed using an Ultraflex Ⅲ Matrix-assisted Laser Desorption Ionization-Time of Flight/Time of Flight Mass Spectrometry (MALDI-TOF/TOF MS) (Bruker Daltonik GmbH, Bremen, Germany) with Flex Analysis version 3.3 (Bruker Daltonik GmbH, Bremen, Germany) to determined peptide sequences. The selected lyophilized samples were first analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC) with a C8 column, and the significant peaks from RP-HPLC were then analyzed using MALDI-TOF/TOF MS.

2.3.9 Determining the effects of selected CGM hydrolysates on lipid oxidation

Determination of lipid oxidation in O/W emulsion

Oil-in-water emulsion samples were prepared with CGM hydrolysates according to the method reported by Cheng, Xiong, & Chen (2010). Briefly, 25 mg of selected antioxidative peptides were dissolved in 45 mL of 0.1 M phosphate buffer (pH = 7.0), and sequentially added with 5 mL of soy oil and 0.45 ml of Tween 20 to obtain final hydrolysate concentrations (5 mg per mL of soy oil, respectively). The mixtures were blended using a homogenizer (PowerGen 700, Fisher Scientific Inc., Ottawa ON, USA) for 2 mins followed by passing through a high-pressure microfluidizer (Microfluidics Corp., MA, USA) twice at 30,000 psi to uniform dispersion. Finally, 3mM sodium azide was added into all emulsions to prevent microbial growth. An emulsion without addition of hydrolysates was prepared as a negative control. All prepared emulsion samples were incubated in dark at 37 °C for autoxidation. Emulsion oxidative stability was estimated by measuring hydroperoxide values (PV) and thiobarbituric acid reactive substances (TBARS) at each two days during 14 days' incubation. Standard curves were prepared with 1,1,3,3 tetramethoxypropane (for TBARS) and cumene hydroperoxide (for PV) (Cheng, Xiong, & Chen, 2010; Faraji, Mcclements, & Decker, 2004; Zhao et al., 2012).

Determination of lipid peroxidation in meat system

Lipid oxidation in meat system was measured by thiobarbituric acid reactive substance (TBARS) assay following a previously reported protocol with modification (Zhang, Li, and Zhou, 2010). Meat samples were prepared by mixing hydrolysates solution at 5 mg/mL and three drops of 0.2% sodium azide with 50 g of ground pork. Prepared meat samples were stored at 4 °C until analysis. To measure oil oxidation in meat samples, 5 g of prepared meat was homogenized with 50 mL DI water, 10 mL of reducing agent (0.01% propyl gallate, 0.02% EDTA), and 0.1 mL of sodium dodecyl sulfate (SDS,10%,) for 2 minutes. The homogenate (1 mL) was immediately transferred into a 15 mL centrifuge tube, and 4.0 mL of TBA solution (0.4% TBA, 0.5% SDS, and 9.3% acetic acid) was added. The mixture was heated in a 95 ˚C water bath for 1 h. After cooling down to room temperature, 5 mL of pyridine/butanol (1:15, v/v) was added. Following centrifuge at 3500 xg and 4 °C for 15 min, the organic layer was collected and measured at 532 nm. The 1, 1, 3, 3-tetramethoxypropane (TMP) solution (0 to 10 μM) were prepared as standard. Final TBARS value was expressed as mg malonaldehyde (MDA) equivalents per kilogram of meat (mg MDA equiv. /kg).

2.3.10 Anticancer effect on HepG2 cell growth

The HepG2 cells (5×10^4 cells/well) were cultured and attached in 24-well plate at 37 °C for 24 h with media of DMEM (10% FBS, 100 μg/mL streptomycin, and 100 units/mL penicillin). The media was removed after 24 h incubation, and 1 mL of corn hydrolysates solution (50 and 200 μg/mL) was treated after another 24, 48, and 72 h. The HepG2 cells were harvested every 24 h. To harvest the cells, media was removed, and the plate was then rinsing with Dulbecco's Phosphate Buffered Saline (DPBS, 1 mL/well). Cells were then

enzymatic detached by 0.05% trypsin-EDTA solution (1 mL/well) at 37 °C for 8 min. The fresh media was then added (1 mL/well) and the detached cell suspension was transferred into a centrifuge tube. By centrifuging at 600g for 6 min (Eppendorf 5702, New York City), the cell pellet in the bottom was collected and counted with a cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA) using AOPI staining method.

2.3.11 Statistic analysis

Experiments were carried out at least in triplicate, and all results were illustrated as mean ± standard deviation and were analyzed with SAS 9.3 software (SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was performed with Tukey's post-hoc test to determine significant differences between the means which considered significant at $p < 0.05$.

2. 4 Results and Discussion

2.4.1 Evaluation of CGM hydrolysates prepared by different enzymes from various sources

Eight enzymes from microbial (Alcalase, Flavourzyme, Everlase, Protamex), plant (papain, bromelain, ficin) and animal sources (trypsin) were used in this study. Enzyme-to-substrate ratio used for hydrolysis were shown in Table 2.1 and same reaction time was performed (5 h). The antioxidant yield, DH and DPPH radical scavenging activity of CPH were presented in Fig.1.

As shown in Fig. 1A, hydrolysates prepared by Protamex had the highest antioxidant recovery (64.18%). Hydrolysates prepared by Alcalase, bromelain and ficin also showed good recovery which was over 50%. DH of CGM hydrolysates was listed in Fig.1B. Papain was the most efficient enzyme with the highest DH value of 17.97%, while trypsin was the least efficient with the DH reaching only about 6.25%. It may be because that trypsin prefers

to cleave peptide chains at the carboxyl side of lysine or arginine but the level of those amino acids in CGM is very low (Gao, et al., 2015). Figure 1.C showed that CGM hydrolysate generated by Alcalase exhibited the highest DPPH radical-scavenging activity with the value as high as 82.06% at 5 mg/ml concentration. Hydrolysate produced by Everlase showed the weakest radical scavenging capacity. Several researches (Moosman and Behl, 2002; Klompong et al., 2007) reported that hydrolysates with higher DH had better antioxidant activities due to lower MW peptides, while hydrolysates prepared by some enzymes showed opposite results in this study. It may be caused by the different hydrolysis specificity of different types of enzyme. For examples, Alcalase is an endopeptidase produced from *bacillus licheniforms* and has performance for large uncharged amino acid residues (Li et al., 2015), while Flavourzyme is from *Aspergillus oryzae* and contains both endoprotease and exopeptidase activities (Kou et al., 2013).

Considering accessibility and cost-effectiveness, Neutrase with 41.3% antioxidant yield, 6.25% DH and 80.38% DPPH scavenging activity was selected for preparation of antioxidant peptides used in further analysis.

2.4.2 Optimization of hydrolysis conditions for Neutrase

To optimize hydrolysis conditions for Neutrase, hydrolysis of CGM was conducted with three Neutrase-to-substrate ratios (0.2, 0.4, and 0.8 Au/g) and different hydrolysis times (0.5-20 h). Antioxidant recovery, DH, total phenolic content as well as antioxidant activity of those hydrolysates were measured to determine the optimum hydrolysis conditions.

Protein recovery and DH

The reaction conditions including E/S ratio (0.2, 0.4, 0.8 Au/g) and hydrolysis time (0.5 h to 20 h) were examined and optimized for Neutrase to produce CGM peptides with

desired antioxidant activity and consistency. As shown in Fig. 2.2A, protein recovery consistently increased as reaction time was prolonged but further hydrolysis after 4 h showed no more effects on antioxidant yield. Although E/S was increased from 0.2 to 0.4 and then to 0.8 Au/g, there was no obvious difference between antioxidant recovery under the three treatments. Similarly, DH values at all E/S were remarkably increased until reaching a steady stage at around 3 h. However, a decrease trend was observed in DH when the E/S increased (Fig. 2.2B). That is because substrate concentration gradually decreases during hydrolysis progress and the reaction site was saturated. The reaction rate would not increase anymore when reaching the peak (Shu et al., 2016). However, a decrease trend was observed in DH when the E/S increased (Fig. 2B). According to the research reported by Liu et al. (2015), higher substrate concentration would conduct better productivity in an enzymatic reaction. Increasing E/S meant the substrate concentration was decreased relatively since CGM is the same amount in each reaction which formed a strong competitive inhibition during reaction which caused lower hydrolysis efficiency (Shu et al., 2016).

Total phenolic content (TPC)

Phenolic compounds could be released during enzymatic hydrolysis. The TPC indicated the content of total phenolic compounds and phenolic amino acids in the hydrolysates. TPC of hydrolysates under each treatment was shown in Fig. 2.2C in the form of mg GAE/g of protein. TPC consistently increased from 0 to 4 h and reached at 153.38 mg GAE/g of corn protein (with Neutrase at 0.8 Au/g) but further hydrolysis did not increase the TPC anymore, and there was no obvious difference of TPC produced with different E/S, which was in agreement with the results of protein recovery. Thamnarathip et al. (2016) reported similar results that increasing reaction time from 2 to 6 h by Neutrase showed no significant difference between rice bran protein hydrolysate in TPC. Furthermore, optimum

enzyme to substrate ratio is more efficient in disrupting protein polyphenol complexes and releasing more phenolic compounds and peptides with phenol groups such as tyrosine. In this study, three was no obvious difference in TPC of hydrolysates prepared with different Neutrase-to-protein ratios.

DPPH radical scavenging activity

Enzymatic hydrolysis could unfold and break the structure of proteins and release antioxidative peptides (Elias et al., 2008; Wang et al., 2014). DPPH assay was used to further analyze and confirm the antioxidant activities of the hydrolysates produced by Neutrase. Fig. 2.2D demonstrated that all hydrolysates exhibited high DPPH scavenging capacities but there was no obvious relationship between hydrolysis time and scavenging values of DPPH radical. Hydrolysates showed high DPPH% value (88.05% at 0.2 Au/g, 79.17% at 0.4 Au/g, 64.13% at 0.8 Au/g) even only hydrolysis for 0.5 h, while longer reaction time did not increase the DPPH value. In addition, higher ratio of Neutrase did not benefit the DPPH radical scavenging value.

Overall, extended hydrolysis beyond 4 h and increase of Neutrase ratio from 0.2 Au/g to 0.4 Au/g and 0.8 Au/g were unnecessary to improve protein recovery, DH and DPPH scavenging activity of hydrolysates. Since higher enzyme concentration may cause stronger competitive inhibition during hydrolysis reaction, it is not suggested to use extremely high E/S ratio. Furthermore, extensive hydrolysis may cause loss of essential structures of small peptides and generate excess free amino acids which may retard antioxidant properties. To obtain desirable antioxidant recovery, DH and antioxidant activity, and considering the cost factor, the center point for E/S ratio at 0.4 Au/g and hydrolysis time of 4 h were selected as preferred reaction conditions for Neutrase hydrolysis.

2.4.3 Purification and evaluation of antioxidant peptides produced by Neutrase

Hydrolysates produced by Neutrase with E/S ratio of 0.4 Au/g and hydrolysis time of 4 h was ultrafiltrated into five molecular ranges with F1 being the fraction with smallest M_w (below 1 kDa), and F5 being the fraction with largest M_w (above 10 kDa). The yield amount of each fraction was measured. As shown in Fig. 2.3, Neutrase efficiently hydrolyzed corn protein into small peptides and the fraction with M_w below 1 kDa showed highest yield with 40.64% in total protein recovery.

TPC of the fractions was illustrated in Fig 2.4A. F3 showed the highest TPC among all fractions with 29.75 μg GAE/mg, followed by F4 (28.80 μg GAE/mg) and F2 (28.33 μg GAE/mg) with no significant difference, and TPC of F1 was the lowest (24.44 μg GAE/mg). Overall, peptides with medium MW demonstrated better TPC. Antioxidant activities were further analyzed using various assays including DPPH radical scavenging activity (Fig. 2.4B), ABTS scavenging activity (Fig. 2.4C), and metal chelating ability (Fig. 2.4D). The results of each assays were not in the same trend due to the different mechanisms.

Only F4 exhibited higher DPPH radical scavenging activity than the crude hydrolysate mixture with DPPH inhibitory value of 74.64%. F1 exhibited the lowest DPPH radical scavenging activity (39.42%). In the study of Pena-Raos, Xiong and Arteaga (2004), antioxidant activities of protein hydrolysates were reported to depend on their molecular size. A number of researches have shown that protein hydrolysate with low M_w interacted more effectively with radical scavenging than higher M_w fractions (Bougatef et al., 2009; Chi et al, 2015; He et al., 2013; Liu, et al., 2010; Raghaven & Kritinsson, 2008; Ranathunga et al., 2005). On the other side, Sabeena Farvinet al. (2010) found that the higher M_w fraction of yoghurt hydrolysates exhibited significantly higher DPPH radical scavenging activity. In addition, according to Wang et al. (2015), zein hydrolysate showed good antioxidant

activities not only for smaller fraction (M_w < 1kDa), but also for the fraction between 1 kDa and 3 kDa.

ABTS scavenging activity was also performed (Fig. 2.4C). Comparing with crude hydrolysate, F3 showed significantly higher ABTS scavenging capacity with value reaching 39.55%, and there was no obvious difference between mixture and F2. Other three fractions with no obvious difference showed lower ABTS scavenging activity than crude hydrolysate

In addition, the Fe^{2+} chelating activity of different fractions was evaluated (Fig. 2.4D). At a concentration of 1mg/mL, F3 and F4 showed significantly higher Fe^{2+} chelating capacity than crude hydrolysate, and F3 demonstrated highest Fe^{2+} chelating capacity (17.21%). Some studies have reported the medium-sized peptides with relatively higher M_w may related to the Fe^{2+} chelating (Bamdad, Wu, & Chen, 2011). Proteins and peptides formed metal chelating activity may either through interacting with charges amino acid residues, or through metal ion entrapment by peptide (Zhang et al., 2009). High activity of F3 may be due to the exposure of amino acid residues, such as histidine, which bind metal efficiently through electrostatic interactions. Another possible reason may be the higher amount of metal binding free amino acids in the fraction, such as histidine and cysteine (Klompong et al., 2008).

In summary, the F3 (3-5 kDa) ultrafiltrated from Neutrase-hydrolyzed CGM was the fraction with promising yield and antioxidant capacities relatively and selected for further analysis.

2.4.4 Identification of peptide sequences in F3

The peptide fraction F3 (Neutrase-hydrolyzed with optimum conditions) from ultrafiltration with desired antioxidant activities was separated by RP-HPLC and the peaks collected were further analyzed using MALDI-TOF/TOF MS. Several peaks were analyzed

due to the complex protein composition in CGM, and the peptide sequences of those identified peptides were shown in Table 2.2.

According to Jin et al. (2016), bioactivity of corn peptides was dependent on peptide sequence, amino acid composition and structure. The study of Jin et al. (2016) discussed a corn peptide containing a repetitive sequence of Leu-Leu showing higher antioxidant activities, and this Leu-Leu presented in several synthesized peptides in F3 (Table 3.2). Besides, Rajapakse et al. (2005) reported that peptide Tyr-Phe-Cys-Leu-Thr exhibited high antioxidant activity. Zhang, Tang and Yuan (2013) reported another sequence of Leu-Pro-Phe possessing promising antioxidant properties. It was widely accepted that the presence of certain amino acids, such as His, Leu, Lys, Met, Trp, and Tyr contributed to the antioxidant properties of corn peptides (Li et al., 2019). Rajapakse et al. (2015) reported that the presence of hydrophobic amino acid residues as well as aromatic residues was important to radical scavenging. Hydrophobic amino acids could increase the solubility of peptides in oil which enhanced the reaction between peptides and radicals, and the amino acids could work as an electron donator to convert radicals into more stable molecules (Jin et al., 2016; Rajapakse et al, 2015). The presence of amino acids in certain positions is also critical to antioxidant properties of peptides. Tang and Zhuang (2014) identified two antioxidant peptides from zein contained the Phe at the C-terminal end. Chen et al (2012) also reported the same results. In addition, Cys in Cys-Ser-Gln-Ala-Pro-Leu-Ala might contribute to its antioxidant activity (Jin et al., 2016).

2.4.5 Antioxidant performance of selected CGM hydrolysate in model systems

O/W emulsion system

Oxidative stability of emulsion samples was evaluated by a combination of two methods to measure both primary (by PV) and secondary (by TBARS) lipid oxidation

products (Wang & Xiong, 2005). During storage, obviously higher lipid hydroperoxides and TBARS were formed in the negative control emulsion. Both POV and TBARS of control showed a gradually increasing trend, and the highest values were observed on day 15 which were 19.53 mM MDA equiv. (Fig. 2.5A) and 2.01 mM hydroperoxide eqv. (Fig. 2.5B). At 5 mg/mL application, F3 hydrolysate showed high prevention of lipid oxidation with reduction of 82.17% for TBARS and 90.71% for PV on day 15, respectively. Kong and Xiong (2006) reported that emulsions with zein hydrolysate generated by Alcalase had a high oxidative stability, which was related to the composition of specific amino acids or peptides presented in zein hydrolysates. The study of Li et al. (2019) also reported that Alcalase-hydrolyzed zein significantly reduced the oxidation of O/W emulsion at 5 mg/ml. Li explained that zein hydrolysate was an amphiphilic molecular and could distribute both at the surface and in the aqueous phase of emulsion to inhibit lipid oxidation.

Ground pork system

Several studies have indicated that specific peptides or hydrolysates from different food protein sources, such as milk protein, soy protein, and rice protein, can be used as natural antioxidant in food system to improve both food quality and shelf stability (Hogan et al., 2009; Nikousaleh, & Prakash, 2016; Oliveira et al., 2014; Zhou, Canning, & Sun, 2013). Ground pork is a lipid-rich food product and commonly used as food model to evaluate effects of additives in lipid oxidation. In this study, selective peptides with higher antioxidant potential (F3 fraction, hydrolyzed by Neutrase with 4 h at 0.4 Au/g)) were added into raw ground pork to investigate their inhibition efficiency of lipid oxidation. It was observed that during 5 days storage at 4 °C, meat with F3 at 200 mg /kg showed no obvious reduction for oil oxidation compared with the control. But increasing the amount of F3 up to 1000 mg/kg showed obvious prevention of lipid oxidation with reduction of 49.2% (Fig. 2.6). Zhou,

Canning and Sun (2013) found that rice protein hydrolysate presented antioxidant capacity in fresh beef. Beef with 500 μg/g of 1-3 kDa fraction from Validase-hydrolyzed rice protein showed 14.7% reduction of oil oxidation on day 15. Cooked beef with addition of Validase-hydrolyzed milk protein hydrolysate (200 μg/g) also prevented lipid oxidation by 35.5% (Hogan et al., 2009). One possible mechanism of the prevention in lipid oxidation is the acidic and basic peptides can reduce the production of aldehydes which are main factor for deterioration of lipid sensory quality (Part et al., 2019). The inhibition of lipid oxidation may also attribute to the chelating effect of the pro-oxidative metal ions and scavenging free radicals. There are heme and non-heme irons in pork which can catalyze the oxidation of unsaturated fatty acids. Therefore, the metal chelating ability of hydrolysates can inhibit lipid oxidation in pork meat (Jadhav et al., 1996).

Anticancer performance of F3 from Neutrase-hydrolyzed CGM

The peptide F3 (3-5 kDa) ultrafiltrated fraction from Neutrase-hydrolyszed CGM (0.4 Au/g, 4 h) with promising antioxidant properties was also evaluated in a liver cancer HepG2 cell model to determine its anticancer performance. As shown in Fig 2.7A, there was no obvious inhibition of cell growth with treatment of F3 peptide within 48 h incubation. However, HepG2 cells treated with 50 and 200 μg/mL both showed significantly ($p < 0.05$) slower growth compared with the control (no antioxidant treatment) after 72 h incubation, although no significant difference was found between these two treatments. The cell viability was measured to determine the cytotoxicity of HepG2 cells (Fig. 2.7B). It was observed that treatment of F3 peptide did not affect the cellular viability ($p < 0.05$) at both 50 and 200 μg/mL, which indicated that F3 peptides were nontoxic to the HepG2 cells. After 72 h treatment, the F3 peptide was removed but the cell was continually incubated for another 72 h with fresh media. According to Fig 3.7C, the number of control cells without treatment

gradually increased and reached up to 925% growth with 144 h incubation. The growth of HepG2 cells treated with F3 peptide was significantly ($p < 0.05$) inhibited, with only 331% and 263% growth after 144 h at 50 and 200 μg/mL, respectively, which were reduced by 64.2% and 71.6% compared with the control. The cellular viability ($p < 0.05$) was also not affected by F3 peptide at both 50 and 200 μg/mL during incubation from 72 to 144 h. The results showed that the selected corn peptide fraction had the potential to prevent the growth of HepG2 cells without toxicity. Xu et al. (2019) reported inhibitory effect of kafrin peptides in cell model. They found that treatments with 50 and 200 μg/mL kafirin peptide (1-3 kDa) demonstrated significantly reduced HepG2 cell growth, which may be caused by the inducement of S-phase arrest of cancer cells. Some researchers have explained the phenolic compounds with antioxidant potentials could directly affect the cell cycle progression (Jafari, Saridia, & Abdollahi, 2014) and thus perform anti-cancer activity. Protein peptides may perform in a similar way but there lacks study about corn peptides in cell models, and further study is needed to explain the anticancer mechanisms.

2.5 Conclusion

Enzymatic hydrolysis of CGM produced hydrolysates with antioxidant properties. The type of proteases used, enzyme-to-substrate ratio and reaction time are key factors affecting the protein recovery, DH as well as antioxidant activities. Hydrolysis of CGM by Neutrase was prepared under various reaction conditions, and the evaluation of hydrolysates was performed by measuring antioxidant recovery, DH and DPPH scavenging activity. The optimum conditions for hydrolysis were obtained with 4 h reaction at a ratio of 0.4 Au/g. Among all fractions generated by membrane ultrafiltration, the fraction with M_w of 3-5 kDa exhibited desirable antioxidant penitential according to DPPH scavenging activity, ABTS activity as well as metal chelating ability. It was also demonstrated that the antioxidative

hydrolysates appeared to be effective in improving oxidative stability of O/W emulsion and ground pork model systems. In addition, it efficiently prevented the growth of HepG2 cells with no toxicity. Overall, Neutrase-hydrolyzed CGM hydrolysates showed potential to be used as a natural antioxidant adding into food and food products to improve quality and shelf stability.

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Table 2.1 Conditions for enzymatic hydrolysis

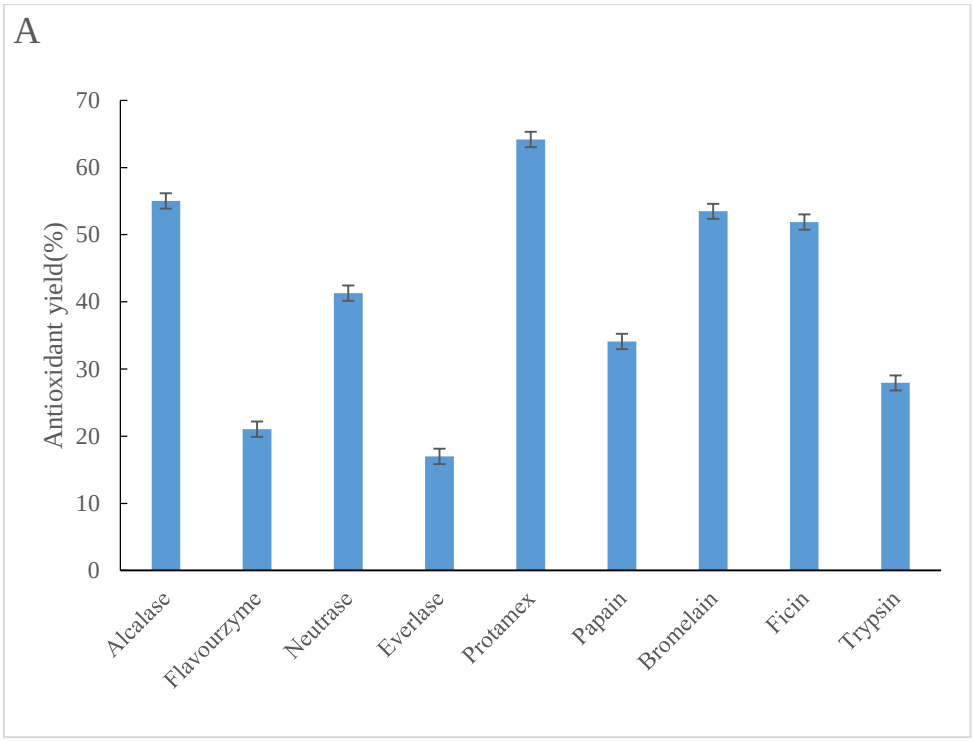
Enzyme Type	**Enzyme unit**	**Enzyme-to-substrate ratio**		**pH**	**Temperature (°C)**
Neutrase	0.8 Au/g	0.4 Au/g	0.397 ml/g	6.0	45
Flavourzyme	500 U/g	10 Au/g	0.016 ml/g	7.0	50
Alcalase	2.4 U/g	0.4 AU/g	0.141 ml/g	8.0	50
Everlase	16 U/g	0.4 Au/g	19.685 µl/g	8.0	50
Protamex	1.5 U/g	0.4 Au/g	267 mg/g	7.0	50
Papain	1.5-10U/mg	180 U/g	120 mg/g	6.5	50
Bromelain	1200 GDU/g	180k GDU/g	56.514 mg/g	5.0	50
Ficin	680 MCU/mg	60k GDU/g	225.5 mg/g	6.0	50
Trypsin	2500 USP/mg	25k U/g	10mg/g	8.0	37

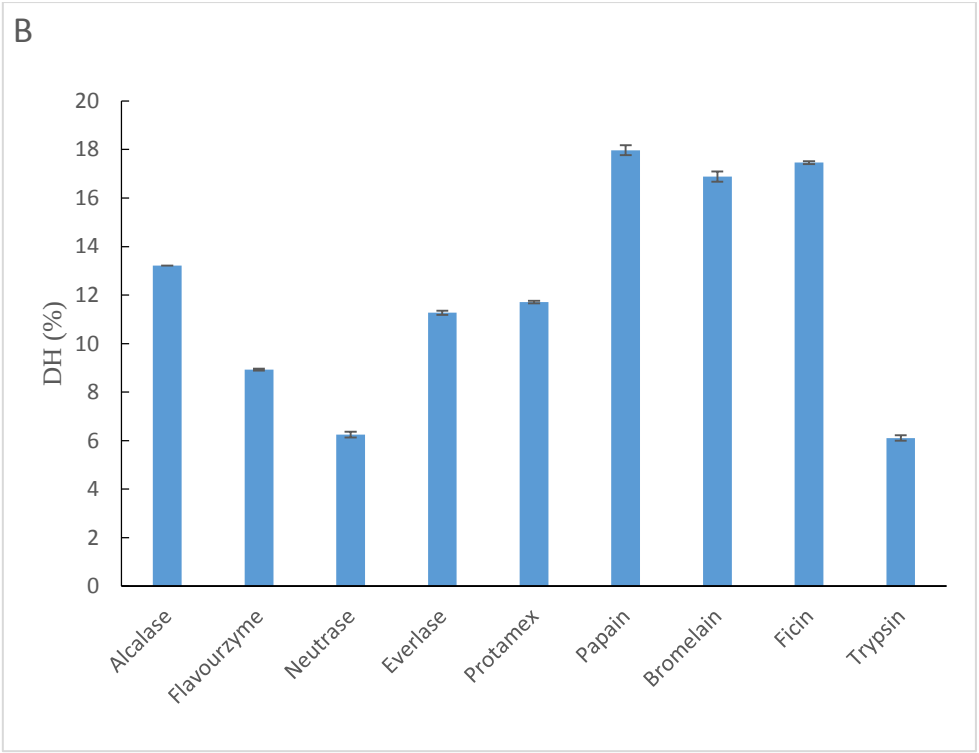
Table 2.2 Peptide sequences identified from 3-5 kDa fraction from Neutrase-hydrolyzed CGM

Compared peptides	RP-HPLC peak retention time	5.05 min	6.8 min	13.3 min	16.5 min	18.3 min	19.5 min	21.5 min	24.5 min	42.7 min	52.9 min
Zein-16	Area%	11.1%	8.9%	17.6%	20.7%	2.3%	8.3%	37.1%	19.0%	20.5%	34.0%
	Coverage%	47.5%	62.3%	43.2%	89.1%	42.6%	92.9%	98.4%	84.2%	18.6%	84.2%
	Sequences	MKVLIVA	VALAL	KVLIVALAL	MKVLIVALAL	MKVLIVALAL	VLIVALAL	MKVLIVALAL	KVLIVALAL	VEFL	KVLIVALAL
		PPFYM	LALA	LALAASAA	LALAASAASS	HLPPPFYM	LALAASAASS	LAL	LALAASAASS	PLHRY	LALAASAASS
		PPPFYLPPQQ	GGCGCQTP	TSGGCGCQ	TSGGCGCQTP	PPPFYLPPQQ	TSGGCGCQTP	SAASS	TSGGCGCQTP	FLQQ	TSGGCGCQTP
		QP	PF	PPPFYLPPQ	PF	QPQPW	PFHLPPPFYM	TSGGCGCQTP	PFHLP	QPQGELAA	PFHLPPPFY
		PPQLSPCQQF	PPPFYM	FLGQCVEFL	PPPFYLPPQQ	PTQ	PPPFYLPPQQ	PFHLPPPFYM	QPWQYPTQ	QVAQQL	PQQ
		GSCG	PPP	RYQATY	QYPTQ	PPQLSPCQQF	QPQPWQYPTQ	PPPFYLPPQQ	PPQLSPCQQF	GLQLQQ	QPQPW
		PFLGQCVEFL	YLPPQQ	GVV	PPQLSPCQQF	GSCG	PPQLSPCQQF	QPQPWQYPTQ	GSCGVGSVGS		LSPCQQF
		RHQC	PQLSPCQQF	QPQGELAALM	GSCGVGSVGS	PFLGQCVE	GSCGVG	PPQLSPCQQF	PFLGQCVEFL		GSCGVGSVGS
		EPLHRYQATY	GSCGVGSVGS	QLTA	PFLGQCVEF	TY	GQCVEFL	GSCGVGSVGS	RHQCSPAATP		PFL
		GVVLQSFL	CSPAATP	LQQPG	HQCSPA	GVVLQSFL	RHQCSPAATP	PFLGQCVEFL	YGSPQCQALQ		CVEFL
		QLTA	YGSPQCQALQ	PCPCN	YGSPQCQALQ	PCPCNA	YGSPQCQALQ	RHQCSPAATP	QQCCHQI		CSPAATO
		MCGLQLQQPG	QQCCHQIRQV		QQCC	PG	QQCCHQIRQV	YGSPQCQALQ	HRYQATY		YGSPQCQAL
		TQ	EPLHRYQATY		EPLHRYQATY		EPLHRYQATY	QQCCHQIRQV	GVVLQSFLQQ		QCCHQIRQV
			AQVAQQLTA		GVVLQSFLQQ		GVVLQSFLQQ	EPLHRYQATY	QPQGELAALM		EPLHRYQATY
			MCGLQLQQPG		QPQGELAALM		QPQGELAALM	GVVLQSFLQQ	AAQVAQQLTA		GVVLQSFLQQ
			PCN		AAQVAQQLTA		AAQVAQQLTA	QPQGELAALM	MCGLQLQQPG		QPQGELAALM
					MCGLQLQQPG		MCGLQLQQPG	AAQVAQQLTA	PCPCNAAAG		AAQVAQQL
					PCPCNAAAGG		PCPCNAAAG	MCGLQLQQPG			GLQLQQPG
					QV			PCPCNAAAG			PCPCNAAAG
					VY			VY			
Zein-19	Area%	11.3%	13.0%	25.5%	17.7%	1.2%	4.8%	13.6%	3.5%	31.3%	15.3%
	Coverage%	27.9%	44.2%	34.6%	67.5%	11.2%	67.9%	93.3%	87.1%	17.5%	44.6%
	Sequences	ATIFPQCSQA	KIFSLLM	KIFSLLM	MATKIFSLLM	IPLS	LLALSTCVAN	MATKIFSLLM	ATIFPQCSQA	MATKIFSLL	LLALSTCVAN
		PIAS	LLALSTC	LLALSTC	LLALSTCVAN	PLLF	ATIFPQCSQA	LLALSTCVAN	PIASLLPPYL	IAASNIPL	ATIFPQCSQA
		CEN	IASLLPPYL	PIASLLPPYL	ATIFP	LLPFYQ	PIASLLPPYL	ATIFPQCSQA	PSIIASICEN	ALAN	PIASL
		PALQPYRLQ	PSTTA	PSIIAS	PIASLLPPYL	QFA	PSIIASICEN	PIASLLPPYL	PALQPYRLQQ	LSP	RLQQ
		NQLSTLN	LS	AL	PSIIASCICEN	PATLL	PALQPYRLQQ	PSIIASICEN	AIAASNIPLS	QQQF	AIAA
		PAAYLQ	PLLFQQ	SLVQSLVQTI	PALQPYRLQQ	QLQQL	AIAASNIPLS	PALQPYRLQQ	PLLFQQSPAL	FNQ	NIPLS
		LLPFYQ	SLVQSLVQTI	RAQQL	AIAASNIPLS		PLLFQQS	AIAASNIPLS	SLVQSLVQTI	LAA	PLL

		QFAANPATLL QLQQL LALT FYQ	PLINQVA QQQLL PFSQL QQQLLPFNQ LAAL QQILLPFSQ QQQLLPFYQ QFAA QLLPF VL	QLVL QQILLPF LLPFYQ QLQQLL HIIGGAL PL QF LL	VQTI RAQQLQ QVALAN LSPYSQQQ LQQQLL FPSQLATA AYL QQQILLPFS NRASFL TQQQLLPFYQ QFAANP QQLLPFVQ LALTDPAASY QQHIIG		RAQQLQQLVL PLINQ SPYSQQQQF LPFNQLSTLN PAAYLQQQLL QQQQLLPENQ LAALNPAAYL ANRASFL TQQQLLP ALLTDPAASY QQHIIG	PLLFQQSPAL SLVQSLVQTI RAQQLQQLVL PLINQVALAN LSP QQQF LPFNQLSTLN PAAYLQQQLL FPSQLATAYS QQQQLLPENQ LAALNPAAYL QQQILLPFSQ LAALNPAAYL TQ LPFYQ QFAANPATLL QLQQL LALTDPAASY QQ	RAQQLQQLVL PLINQVALAN LSPYSQQQQF LPFNQLSTLN PA PFSQLATAYS QQQQLLPENQ PAAYL QQQILLPFSQ LAALNPAAYL TQQQLLPFYQ QFAANPATLL QLQQLLPFVQ LALTDPAASY QQHIIGGALF	LAAANRA	VQSLVQTI RA PLINOVA QQQF QLLPENQ QQQILL AANRASFL TQQ LLPFVQ LALTD LAA LVL YL
Glutenin	Area%	23.4%	7.9%	27.4%	34.3%	10.9%	15.1%	32.2%	20.4%	10.5%	31.7%
	Coverage%	51.6%	89.2%	34.1%	80.0%	47.5%	69.1%	96.9%	76.7%	5.4%	77.1%
	Sequences	MRVLLVA HTSGGCGCQP PPPVHL HP CPCQQPHPSP CQLQGTCGVG STPILGQCVE FLRHQCSPTA TPYSCPQCQC LRQQCCQQLR QQQPQSGQ GLQQP TPCPYAAAGG IL	RVLLVALAL LALAASATST HTSG CQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL HVPPPVHLPP PPCHYPT RPQPHPQPHP CPCQQPHPSP STPILGOCVE FLRHQCSPTA LRQQCCQQLR	MRVLLVALAL LALAA TSGGCGCQ HVPPPV TQPP RPQPHPQPHP PILGQC VLQSIL QQQ VA GLLAAQIAQQ LTAMC	MAAKIFSILM LLALSACVLD ATIFPQYSQA PIAALL PSMTASV EN PTLQPYRLQQ NLPLS PLLFQQSPAL SLVQ VQTI RAQQLQQLVL QVALAN LSPYSQQQ	AASATST HTSGGCGCQP PP VHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHLPPPV HVPPPVHL PCHYPTQPP CQQPHPSP CSPTA TPYCSP QQQPQSGQ RP	RVLLVALAL LALAASATST HTSGGCGCQP PPPVHLPPPV CHYPTQPP RPQPHPQPHP CPCQQPHPSP TCGVG STPILGQCVE FLRHQCSPTA TPYCSPQCQS LRQQCCQQLR QVEPQHRYQA IFGLVLQ	RVLLVALAL LALAASATST HTSGGCGCQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHLPPPV HVPPPVHLPP PPCHYPTQPP RPQPHPQPHP CPCQQPHPSP CQLQGTCGVG STPILGOCVE FLRHQCSPTA	MAAKIFSILM LLALSACVLD HTSGGCGCQP PPPVHLPPPV HLPPPVHLPP VPPPVHLPP PPCHYPTQPP RPQPHPQPHP CPCQQPHPSP CQLQGTCGVG STPILGOCVE TPYCSPQCQS LRQQCCQQLR HRYQA	VE FLRH PQSGQVA	VLLVALAL LALAASATST HTSGGCGCQP PP VHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHLPPPV HVPPPVHLPP PPCHYPTQPP RPQPHPQP HPSP CQLQGTCGVG CVE

QVEPQHRYOA	LQQQLL	CQ	GLLAAQTAQQ	TPYCSPQCQS	GLVLQSI	TPYCSPQCQS
IFGLVLQSIL	PFSQLATA	IL	LTAMCGLQQP	LRQQCCQQLR	QQQPQSGQVA	LRQQCCQQL
QQQPQSGQV	FLPENQ		TPCP	QVEPQHRYQA	LTAMCGLQQP	PQSGQVA
LAAQIAQQ	LAALNPAAYL			IFGLVLQSIL		GLLAAQIAQQ
LTAMCGLQQP	QQQILLPFGQ			QQQPQSGQVA		LTAMCGLQQP
TPC	LATTNRASFL			GLLAAQIAQQ		TPCPYAAA
TPYC	TQQQLLPFYQ			LTAMCGLQQP		ST
	PATLL			TPCPYAA		PLR
	QLQQLL					
	NPAAFY					
	QQHIIGGAI					
	QF					


A
Antioxidant yield(%)
70
60
50
40
30
20
10
0
Alcalase
Flavourzyme
Neutrase
Everlase
Protamex
Papain
Bromelain
Ficin
Trypsin


B
DH (%)
20
18
16
14
12
10
8
6
4
2
0
Alcalase
Flavourzyme
Neutrase
Everlase
Protamex
Papain
Bromelain
Ficin
Trypsin

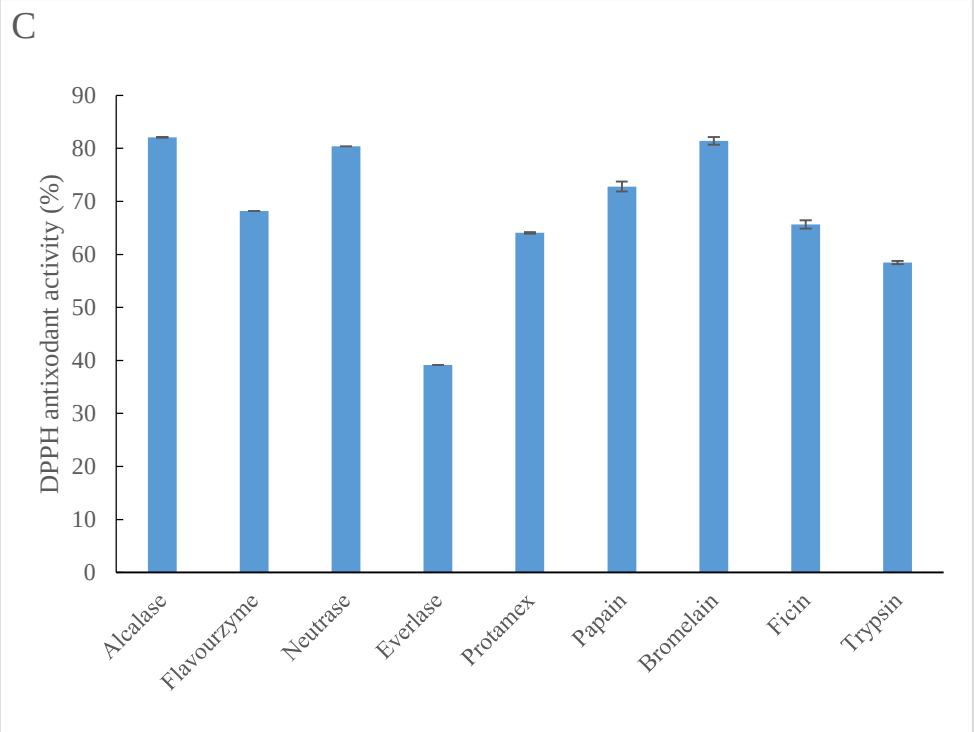
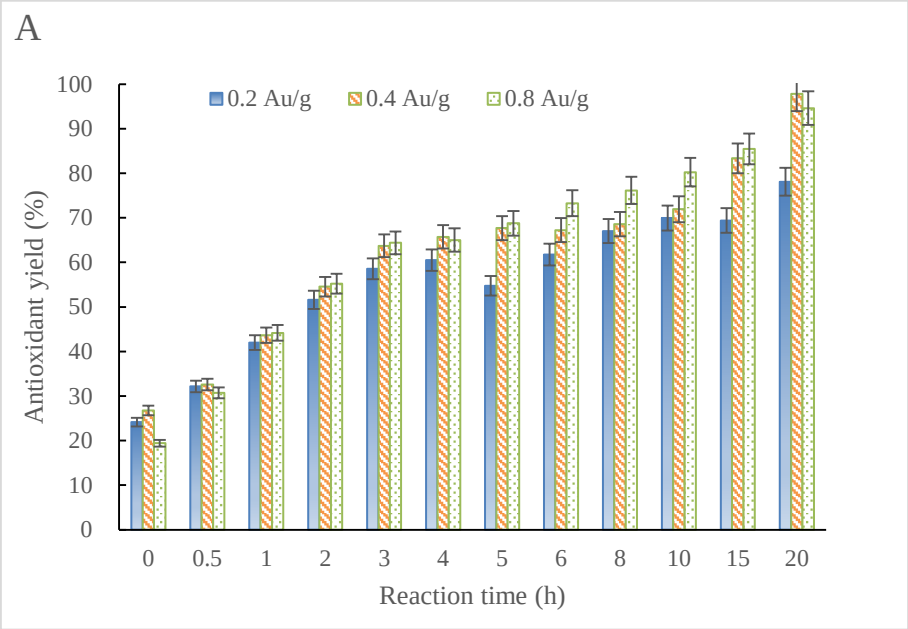
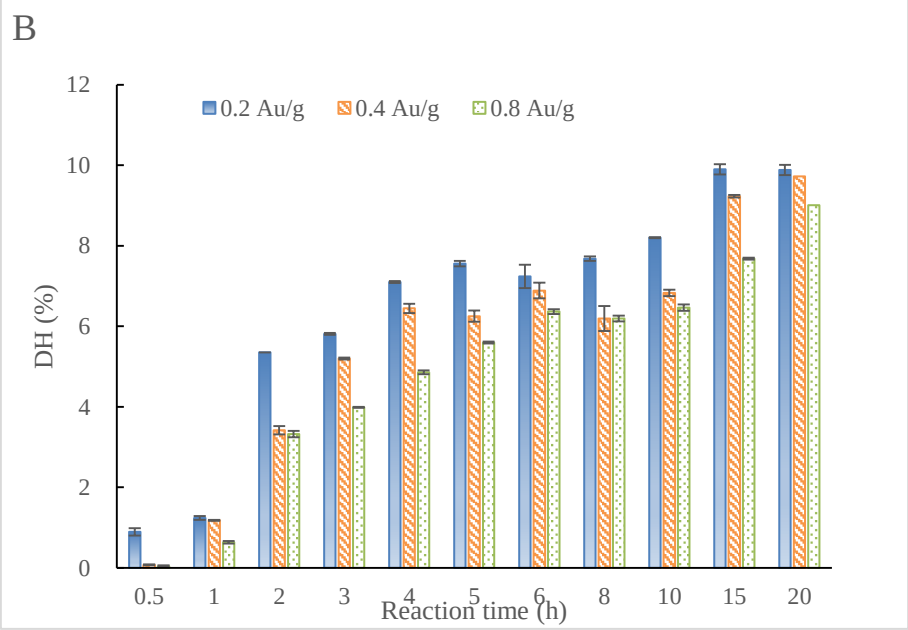
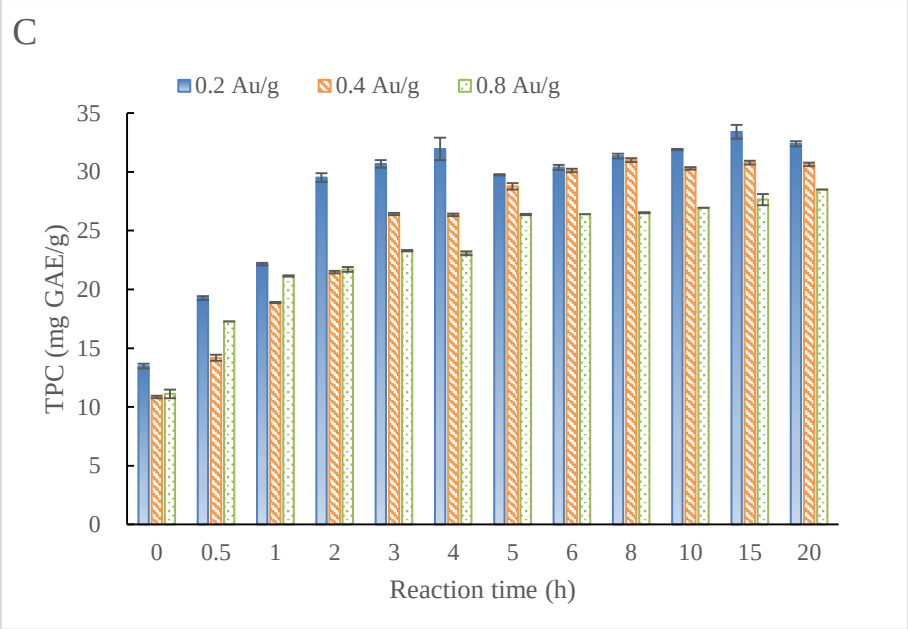


Fig. 2.1 - Effects of different types of enzymes on antioxidant yield, DH, and antioxidant activity of CGM hydrolysates. (**A**) Antioxidant yield; (B) DH; and (C) DPPH scavenging activity.


A
0.2 Au/g
0.4 Au/g
0.8 Au/g
Antioxidant yield (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
2
3
4
5
6
8
10
15
20
Reaction time (h)


B
0.2 Au/g
0.4 Au/g
0.8 Au/g
DH (%)
12
10
8
6
4
2
0
0.5
1
2
3
4
5
6
8
10
15
20
Reaction time (h)



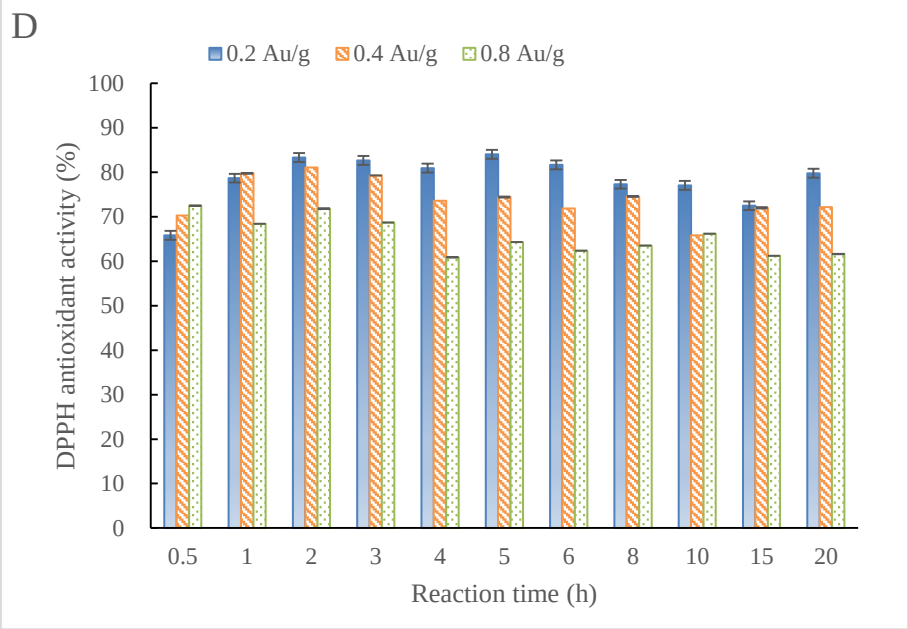


Fig. 2.2 - Neutrase- hydrolyzed CGM prepared with different reaction time (0.5-20 h) and enzyme-to-substrate ratio (0.2, 0.4, 0.8 Au/g). (A) Antioxidant yield; (B) Degree of hydrolysis; (C) Total phenolic content; and (D) DPPH radical scavenging activity.

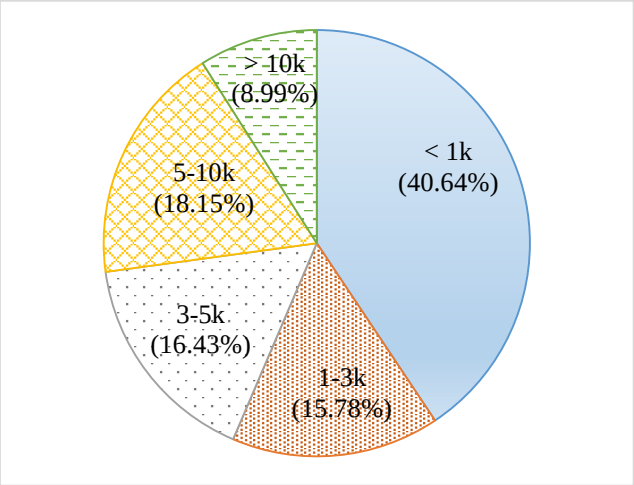
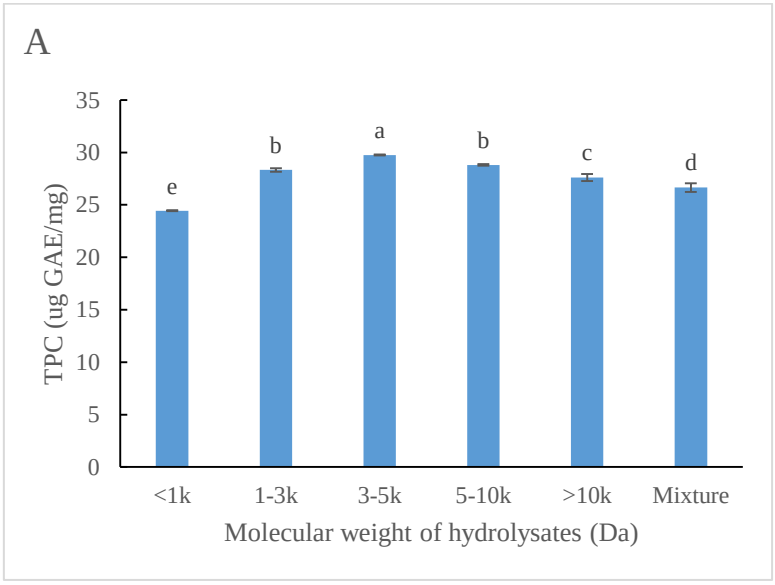
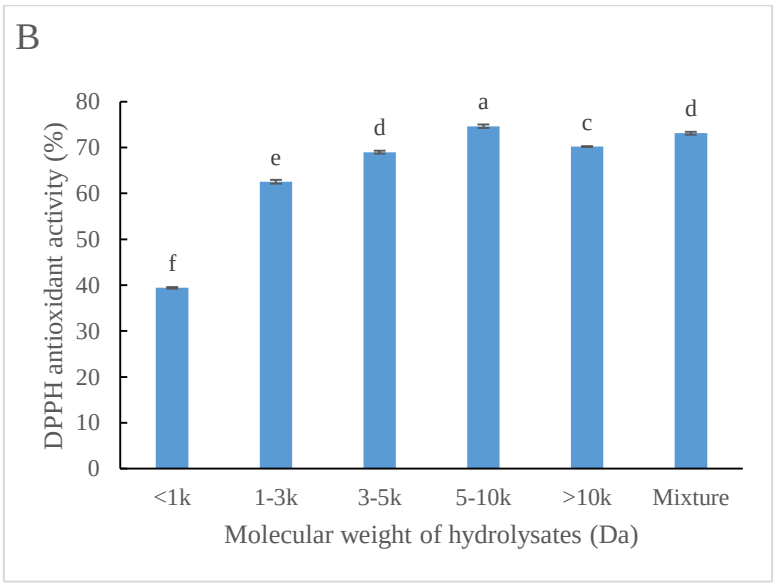
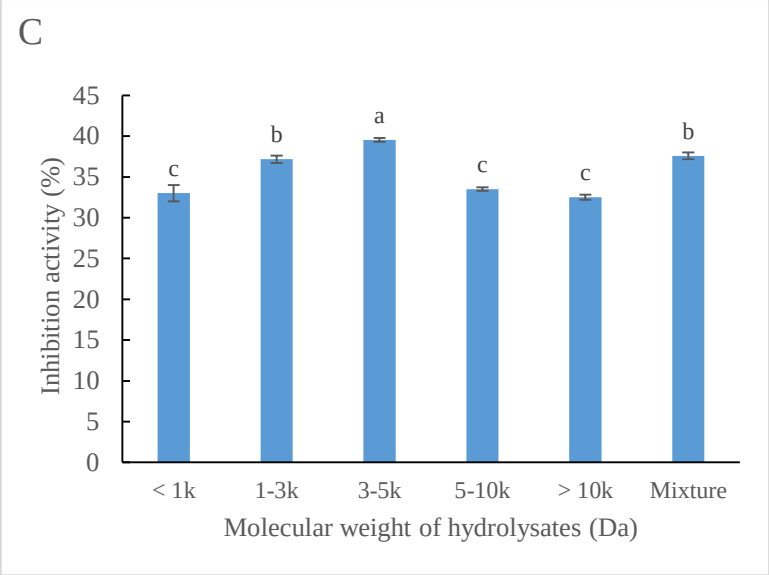


Fig. 2.3- Weight distribution of ultrafiltrated fractions from Neutrase-hydrolyzed CGM (0.4 Au/g, 4 h).


A
35
30
25
20
15
10
5
0
TPC (ug GAE/mg)
e
b
a
b
c
d
<1k
1-3k
3-5k
5-10k
>10k
Mixture
Molecular weight of hydrolysates (Da)


B
80
70
60
50
40
30
20
10
0
DPPH antioxidant activity (%)
f
e
d
a
c
d
<1k
1-3k
3-5k
5-10k
>10k
Mixture
Molecular weight of hydrolysates (Da)



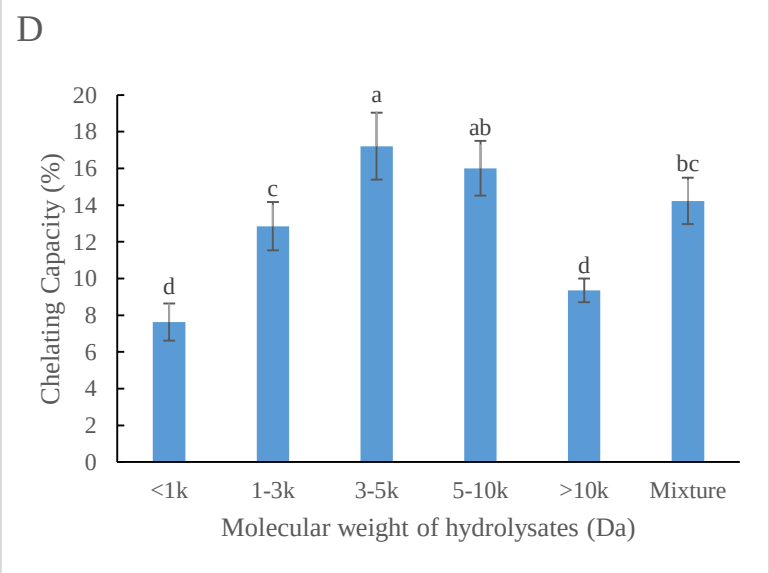
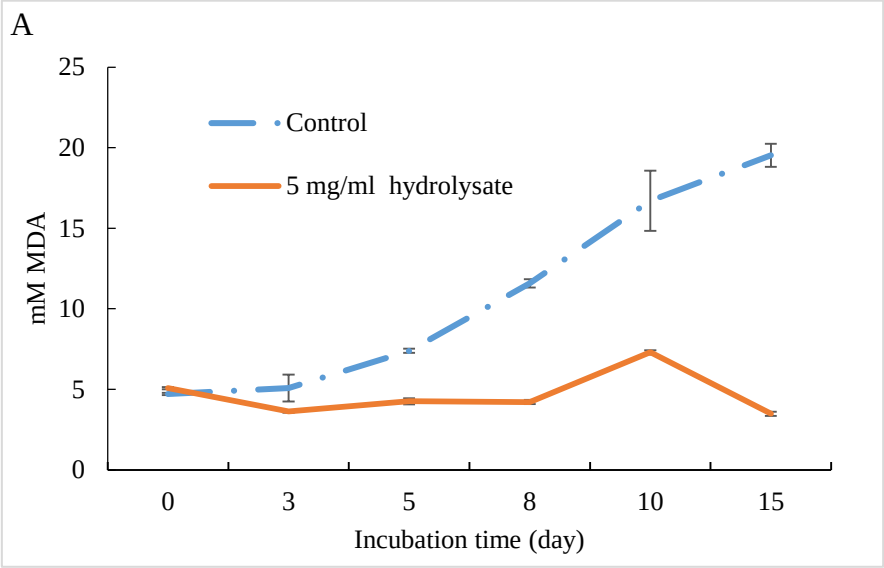


Fig. 2.4 - Antioxidant activities of different peptide fractions ultrafiltrated from Neutrase-hydrolyzed CGM (0.4 Au/g, 4 h). (A) Total phenolic content; (B) DPPH radical scavenging; (C) ABTS radical scavenging activity; and (D) Fe^{2+} chelating activity.



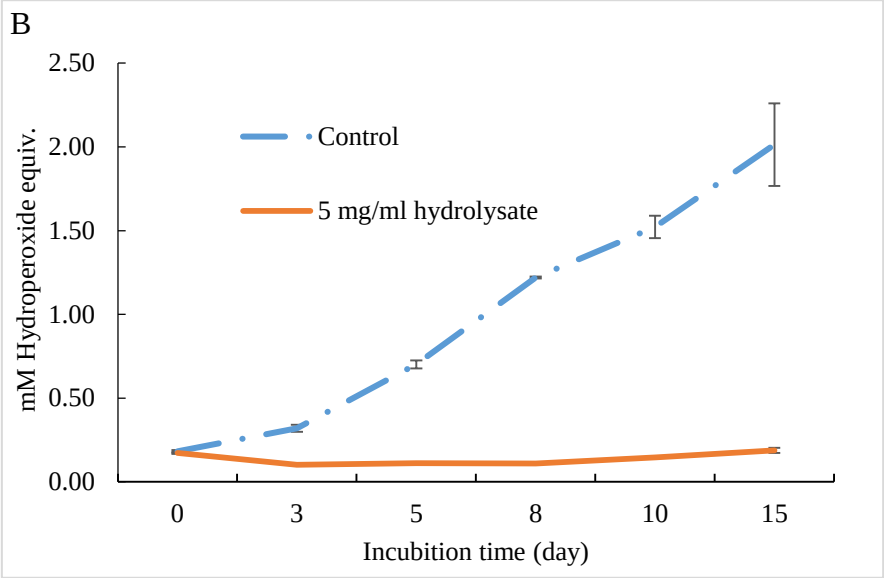


Fig. 2.5 - Inhibition of lipid oxidation in emulsion system with Neutrase-hydrolyzed CGM (0.4 Au/g, 4h). (A) TBARS values; and (B) Lipid hydroperoxidation.

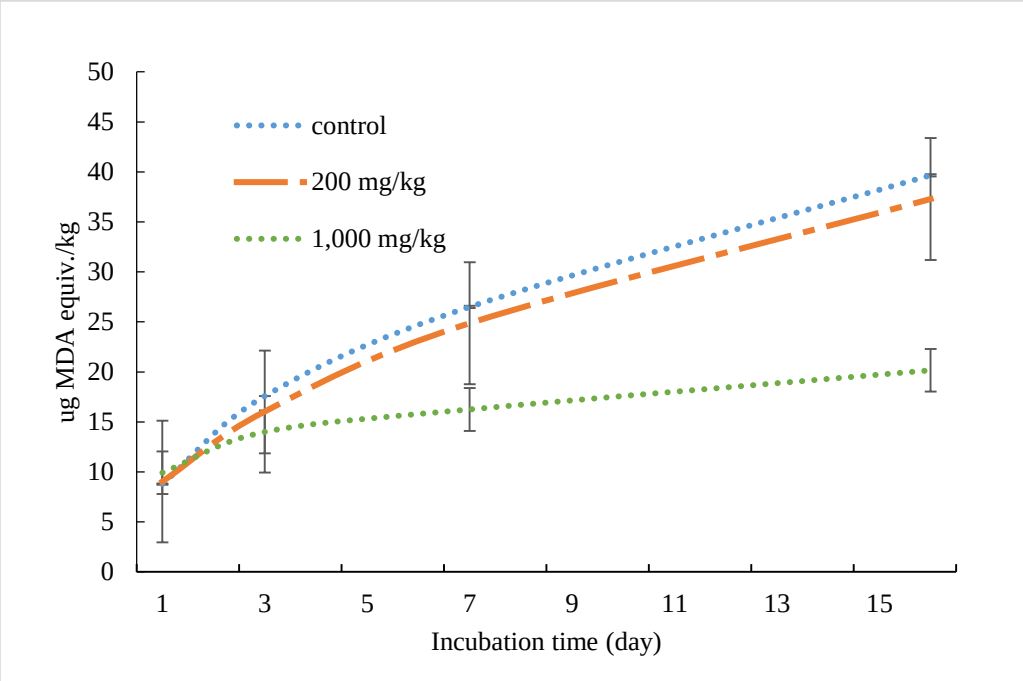
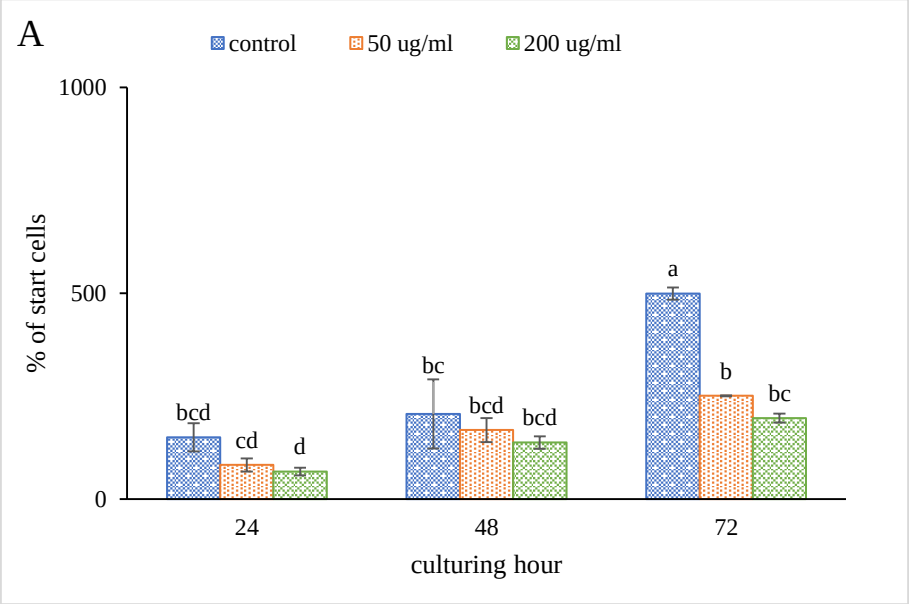
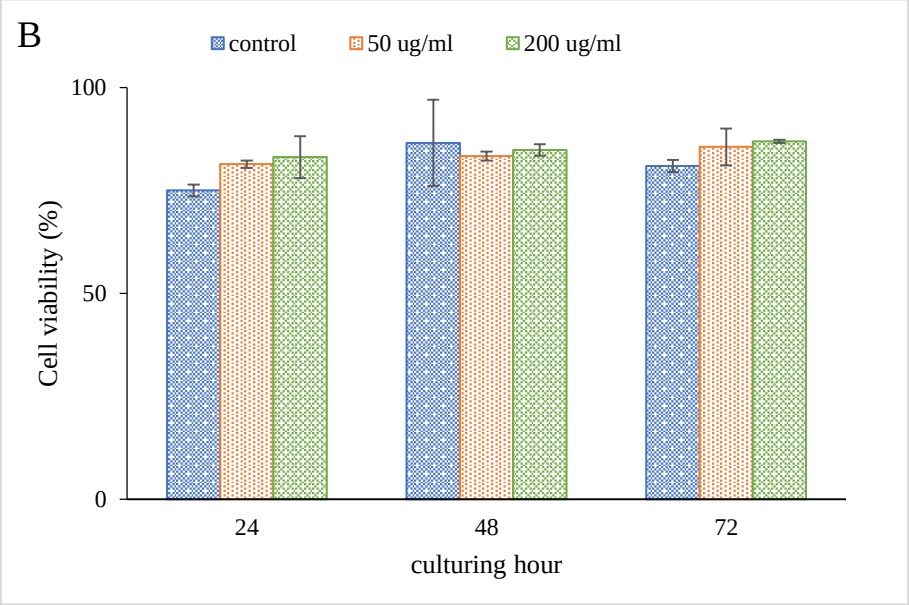
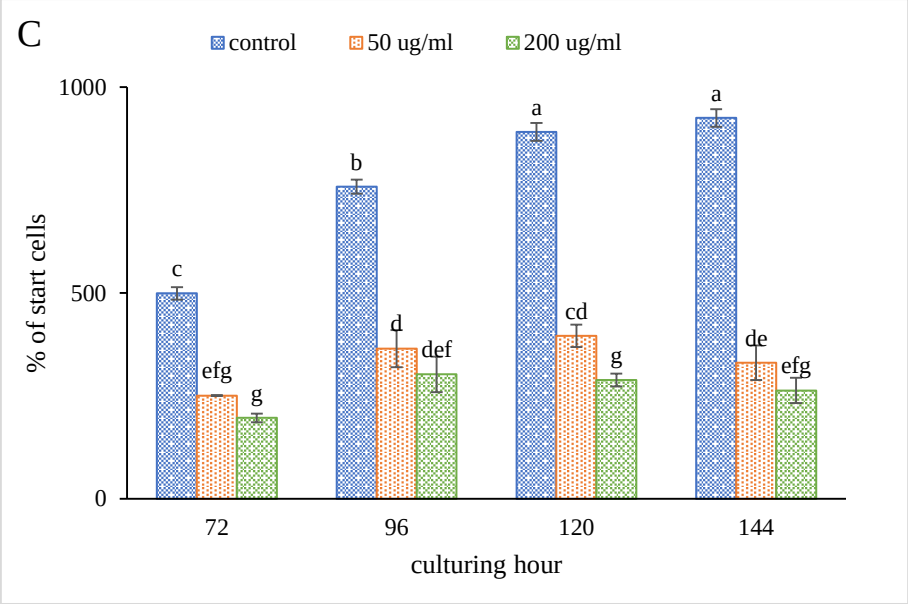


Fig. 2.6 - TBARS of ground meat system with 3-5 kDa fraction ultrafiltrated from Neutrase-hydrolyzed CGM (0.4 Au/g, 4h) at 200 mg/kg and 1,000 mg/kg.


A
control
50 ug/ml
200 ug/ml
1000
500
0
% of start cells
bcd
cd
d
bc
bcd
bcd
a
b
bc
24
48
72
culturing hour


B
control
50 ug/ml
200 ug/ml
100
50
0
Cell viability (%)
24
48
72
culturing hour



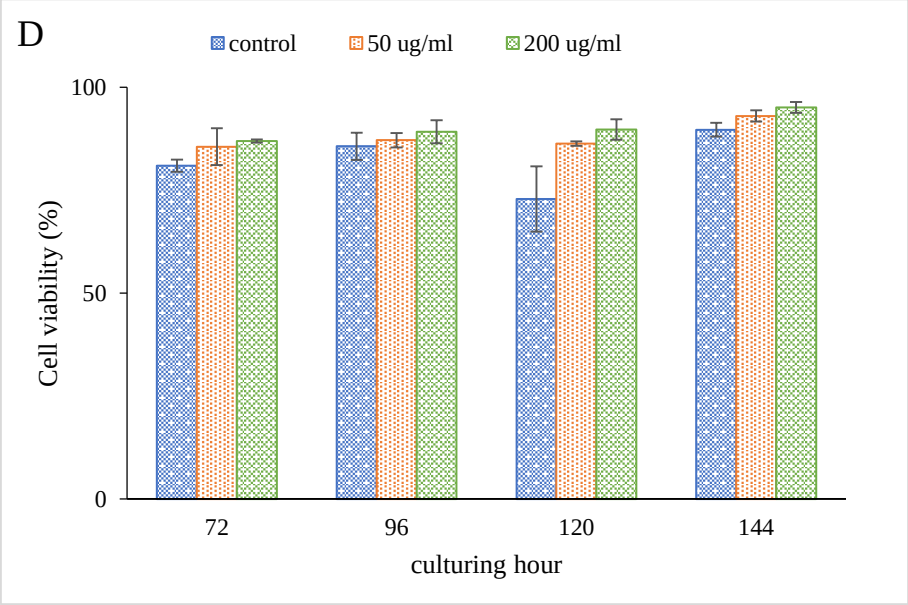


Fig. 2.7 - Effects of F3 peptide (3-5 kDa) from Neutrase-hydrolyzed CGM (0.4 Au/g, 4h) on (A) HepG2 cell growth, and (B) Cell viability. Post effects of F3 peptide (3-5 kDa) from Neutrase-hydrolyzed CGM (0.4 Au/g, 4h) on (C) HepG2 cell growth, and (D) Cell viability. Significant differences at $p < 0.05$.

Chapter 3 - Antioxidative Hydrolysates from Corn Gluten Meal via Enzymatic Hydrolysis Using Plant Proteases

3.1 Abstract

Corn gluten meal was hydrolyzed by three plant proteases: papain (60 U/g), ficin (90 kGDU/g), and bromelain (180 kGDU/g), and degree of hydrolysis (DH), antioxidant yield, total phenolic content (TPC) as well as DPPH radical scavenging activity of hydrolysates were determined. The optimum hydrolysis time for papain was 3 h, and for ficin and bromelain was 4 h. The hydrolysates were further separated by sequential ultra-filtration to 5 hydrolysate fractions named F1 to F5 from low MW (< 1 kDa) to high MW (> 10 kDa), which were further characterized for TPC, free radical scavenging capacity against DPPH and ABTS, and metal chelating activity. The fraction F4 produced by papain (CH-P4), F1 produced by ficin (CH-F1), and F3 produced by bromelain (CH-B3) showed the strongest antioxidant activity and yield, respectively. These three fractions were incorporated into ground pork to determine their inhibition effects on lipid oxidation for 16 days storage period. The inhibition effect was enhanced with the addition of higher amount of hydrolysate (e.g., 500 vs. 1,000 mg/kg). The CH-P4 reduced lipid oxidation in ground meat by as much as 30.45%, and CH-B3 reduced oxidation by 27.2% at the same level, but the inhibition was only 13.83% with 1000 mg/kg of CH-F1.

3. 2 Introduction

Corn is one of the main crops cultivated worldwide with a production exceeding 1074 million tons in 2017/2018, and about 34.5% is grown in the U.S. (Zhu, He, & Hou, 2019). It is also one of the most important food and industrial crops in U.S. Corn usually contains 10-15% of proteins, while corn gluten meal (CGM), a major coproduct generated during corn

wet-milling process, contains high level of proteins (e.g., 60-70%) (Wang et al., 2016). Corn proteins can be classified into four fractions depending on their solubility: prolamins (zein), glutelins, albumins, and globulins. The major seed proteins in corn are zein (68%) and glutelin (28%), but both are insolubly in water (Margarita et al., 2017). Comparing to legumes, protein quality in corn is poor due to lack of certain essential amino acids, such as Lys and Trp (Li et al., 2019; Margarita et al., 2017). Although CGM is a protein- rich source, it is traditionally used as feed materials or otherwise under-utilized due to low solubility and poor protein quality. Modification of proteins in CGM will increase its market value and application in food industries.

In recent years, researches have reported that various proteins from low value sources such as poultry industry residues (Rossi et a., 2009), fish byproducts (Bougatef et al., 2008), and algae waste (Shei, Wu, & Fang, 2009) could produce bioactive peptides through enzymatic hydrolysis. Generally, there are several pathways to produced protein hydrolysates including enzymatic or chemical hydrolysis, and microbial fermentation. Chemical hydrolysis is conducted using chemical reagents (acid or alkaline), but it is more difficult to be controlled than enzymatic hydrolysis, and the final products contain more salt (Kim et al., 2004; Samaraweera et al., 2013). Microbial fermentation was also found to be a promising method for the production of bioactive peptides, while it is still in early stage and less efficient (Zhu, He, & Hou, 2019). Comparing to other methods, enzymatic hydrolysis is considered to be the predominate pathway to produce protein hydrolysates with high efficiency and low safety concerns (Wang et al., 2014). Some researchers have already reported corn peptides or domains have high bioactive functions, such as antioxidant, antihypertensive, and anti-obesity (Huang et al., 2011; Li et al., 2010; Sun, Tian, & Shi, 2017). Alcalase, Protamex, and Flavorzyme are common enzymes used for CGM hydrolysis (Jin et al., 2016; Lu, et al., 2015; Wang et al., 2014). Plant enzymes such as papain, are also

can be used for efficient hydrolysis of proteins. For example, papain had used for hydrolysis of atlantic salmon skin collagen (Gu et al., 2011) and sea urchin (Qin et al., 2015) and were reported could generated antioxidant peptides. However, it lacks information about plant enzymes to produce corn bioactive hydrolysates.

The primary objective of this study was to optimize hydrolysis time for three plant enzymes (papain, ficin, and bromelain) in the production of CGM hydrolysates. The second objective was to evaluate the antioxidant activities of ultra-filtrated fractions with different MW, and to identified peptide sequences of the fraction with promising antioxidant properties. Finally, promising peptide fraction from each hydrolysate was applied into ground meat system to evaluate their antioxidant performance in prevention lipid oxidation.

3.3 Experimental Section

3.3.1 Materials

The corn gluten meal (CGM, 61.3% crude protein) was provided Grain Processing Corporation (Muscatine, IA, USA). Papain (from papaya latex, crude powder) was purchased from Sigma-Aldrich (St. Louis, Mo, USA). Ficin (from figs latex, lyophilized powder) was purchased from Tokyo Chemical Industry Co. (Kita-ku, Tokoyo, Japan). Bromelain (from stem, lyophilized powder) was purchased from Acros Organics (Fairlawn, NJ, USA). All other chemicals, solvents, and reagents used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, Mo, USA) or Fisher Scientific (Fairlawn, NJ, USA).

3.3.2 Preparation of corn gluten meal hydrolysates

CGM was pretreated with water washing and fat removal for efficient hydrolysis. CGM was mixed with deionized (DI) water (1:6, w/v) at room temperature for two times with 1h stirring for each time. The mixture was then filtrated and dried in an oven at 45 °C for 48

h. Fat was removed by stirring dried CGM with hexane (1:6, w/v) for total three time with 0.5 h stirring for each time. Defatted CGM was placed in a fume hood for at least 24 h to completely volatilize the hexane. CGM suspension (4%, w/v, protein base) was prepared by dispersing 16.3 g of CGM in 250 mL DI water. The CGM suspension was heated in 95 °C water bath for 10 min to denature proteins and enhance hydrolysis efficiency. The pH of the suspension was then adjusted to optimum level (based on manufacturer recommendation) when it was cooled down to room temperature. Enzymatic hydrolysis was conducted in water bath shaker with optimum temperature for each enzyme. The enzyme-to-substrate ratio, pH, temperature and reaction time used for the three enzymes were shown in Table 3.1.

3.3.3 Determination of antioxidant yield

The yield of antioxidant was calculated as the ratio of soluble protein after hydrolysis using the equation as follow: Antioxidant yield = $(W_2 / W_1) * 100\%$, where W_1 was the amount of protein in CGM used for hydrolysis, and W_2 was the amount of lyophilized hydrolysate supernatant.

3.3.4 Determination of degree of hydrolysis (DH)

The DH of CGM hydrolysates was determined by o-phthaldialdehyde (OPA) assay according to a previously established protocol (Nielsen, Petersen, & Dambmann, 2001). Serine (0.9515 mM) was used as standard. Hydrolysate samples were measure at a concentration of 1.2 mg/ml, and triplicates were measured for each sample.

3.3.5 Fractionation of CGM hydrolysate by ultrafiltration

CGM hydrolysates were fractionated by an Amicon® Stirred Cell device (EMD Millipore Corporation, Billerica, MA, USA) under pressure of nitrogen (60 psi) with

continually stirring on a magnetic stirrer (60 rpm). Separation of hydrolysates was conducted based on molecular weight using ultrafiltration membranes (EMD Millipore Corporation, Billerica, MA, USA) with different cut-off sizes (1, 3, 5, and 10 kDa). The eluent fractions were lyophilized and stored at -20 °C until further analysis.

3.3.6 Determination of total phenolic content (TPC)

TPC of CGM hydrolysates at 1 mg/mL was evaluated based on Folin-Ciocalteu method according to the method of Thamnarathip et al. (2016). Gallic acid (0-0.06 mg/mL) was used as a standard. Total phenolic content of hydrolysates was expressed as mg gallic acid equivalents (GAE) per gram of sample.

3.3.7 Determination of antioxidant activity

Determination of DPPH radical scavenging activity

The scavenging activity of CGM hydrolysates on 1.1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured according to the modified method of Li, Han & Chen (2008). Briefly, 5 mL of DPPH solution (0.2 mM) in 95% ethanol was added into 5 mL hydrolysate solution (5 mg/mL). The mixture was vortexed for 1 min and rested in dark for 30 min, and the absorbance was measured at 517 nm. DI water was used as control. The DPPH radical scavenging activity was expressed as follows:

$$\text{DPPH scavenging rate (\%)} = [(A_{blank} - A_{sample})/ A_{blank}] \times 100.$$

Determination of ABTS radical scavenging activity

ABTS radical scavenging activity of hydrolysate solution at 1 mg/mL was determined following a previous method reported by Thaipong et al. (2006). DI water was used as control. The ABTS radical scavenging activity was calculated using the equation as follows:

ABTS scavenging rate (%) = $[(A_{blank} - A_{sample})/ A_{blank}] \times 100$.

Determination of ferrous ion (Fe^{2+}) chelating activity

Fe^{2+} chelating activity was assessed according to a previously reported protocol with slight modifications (Elias, Kellerby, & Devker, 2008). Briefly, 25 μL of hydrolysate (1 mg/mL), 150 μL of DI water and 25 μL of $FeCl_2$ solution (0.2 mM) were loaded into microcell plate. After incubating at room temperature for 30 sec, 50 μL of ferrozine solution (1 mM) was then added into the mixture, and the absorbance was read at 562 nm. DI water was used as control. The chelating ability was calculated as follows:

Fe^{2+} chelating ability (%) = $[(A_{blank} - A_{sample})/ A_{blank}] \times 100$.

3.3.8 Identification of peptide sequences of selected antioxidant peptide

Peptide fractions with promising antioxidant properties as well as desirable yield and DH were selected for peptide sequence analysis using an Ultraflex Ⅲ Matrix-assisted Laser Desorption Ionization-Time of Flight/Time of Flight Mass Spectrometry (MALDI-TPF/TOF MS) (Bruker Daltonik GmbH, Bremen, Germany).

3.3.9 Antioxidant activity of selected hydrolysates in ground pork system

The inhibition effects of selected CGM hydrolysates on lipid oxidation in ground pork system was determined based on thiobarbituric acid reactive substance (TBARS) assay following a previously reported protocol of Zhang, Li, and Zhou (2010) with some modifications. Meat sample was prepared by mixing 50 g ground pork with 5 ml hydrolysate solutions (1 mg/mL) and three drops of 0.2% sodium azide, and then stored at 4 °C. To extract the oxidation products, 5 g of prepared meat was homogenized with 50 mL DI water, 10 mL of reducing agent (0.01% propyl gallate, 0.02% EDTA), and 0.1 mL of sodium

dodecyl sulfate (SDS,10%,) for 2 minutes. The homogenate (1 mL) was transferred into a 15 mL tube and mixed with 4.0 mL of TBA solution (0.4% TBA, 0.5% SDS, and 9.3% acetic acid), and then reacted in a 95 ˚C water bath for 1 h. The mixture was cooled down in cold water for 10 min, and 5 mL of pyridine/butanol (1:15, v/v) was added. Following centrifuge at 3500 xg and 4 ˚C for 15 min, the first layer was collected, and the absorbance was measured at 532 nm. The 1, 1, 3, 3-tetramethoxypropane (TMP) solutions (0 to 10 μM) were used as standard, and result was expressed as mg malonaldehyde (MDA) equivalents per kilogram of meat (mg MDA equiv. /kg).

3.3.10 Statistic analysis

Results were analyzed with SAS 9.3 software (SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was performed, and Tukey's post-hoc test was used to determine significant differences between the means which considered significant at $p < 0.05$.

3. 4 Results and Discussion

3.4.1 Optimization of hydrolysis conditions for papain, ficin, and bromelain

Time of hydrolysis for enzymes was critical to prepare antioxidant peptides from CGM. In this study, papain, ficin and bromelain were used at 60 U/g, 60k GDU/g, and 180k GDU/g. To determine optimum reaction time, hydrolysis of CGM was conducted with different reaction times from 0.5 to 5 h. Antioxidant yield, degree of hydrolysis, total phenolic content as well as DPPH radical scavenging activity of prepared hydrolysates were measured. The hydrolysis time which leads to most promising antioxidant peptides with regards to antioxidant activities and yield would be determined as the optimum one.

Hydrolysis of CGM was performed only up to 5 h in this study considering economic efficiency. As shown in Fig. 3.1, an increase trend of antioxidant yield was observed as the

hydrolysis time prolonged for CGM hydrolysates prepared by papain (CH-P), ficin (CH-F), and bromelain (CH-B). Overall, CH-P had lower yield compared with CH-F and CH-B with the same reaction time. For CH-F and CH-B, yield of antioxidant hydrolysates increased rapidly from 0.5 h to 3 h, and then became relatively stable until 5 h. The total increase of yield from 0.5 to 5 h were up to 43.20% and 41.04% for CH-F and CH-B, respectively. However, the yield from 3 h to 5 h only increased by 6.03% and 10.71% for CH-F and CH-B, respectively. Degree of hydrolysis (DH) is defined as the percentage of cleaved peptides bonds after hydrolysis, and it is a critical factor which contributes to the composition and functional properties of corn protein peptides (Bougatef et al., 2009; Zhuang et al., 2013). DH of each hydrolysate was shown in Fig. 3.2. Papain was more efficient than other two types of enzymes to cleave corn peptide bonds; however, no clear trend was observed of DH with time prolonged. The DH of CH-P was decreased a little bit from 0.5 to 2 h and then increased again, and the highest value was observed at 4 h with DH of 16.7%. DH of CH-F and CH-B gently increased with time prolonged. The highest DH of CH-F was obtained at 5 h (12.1%). After 4 h, DH of CH-B was no longer affected. The difference of efficiency between the three enzymes may be caused by their different specificity. Papain can be used for the complete proteolytic cleavage of proteins and prefers to cleave the amino acids in hydrophobic side chain. Ficin cleaves proteins at the carboxyl side of amino acids, such as Gly, Ser, Thr, and Met, and bromelain is a cysteine endopeptidase (Zhu, He, & Hou, 2019).

TPC of hydrolysates under different reaction times was measured and shown in Fig 3.3. For CH-P, extension of hydrolysis time increased the TPC slightly, and 3 h hydrolysis resulted in significantly higher TPC with concentration of 44.62 mg GAE/g. CH-B showed the highest TPC at 4 h hydrolysis with TPC value of 48.29 mg GAE/g, while CH-F had the highest TPC (41.3 mg GAE/g) at both 2 and 5 h hydrolysis. Overall, ficin-hydrolyzed CGM had relatively lower TPC.

DPPH radical scavenging activity of CGM hydrolysates was shown in Fig 3.4. All hydrolysates exhibited high DPPH scavenging activity with inhibitory rate over 60% even only with 0.5 h hydrolysis. The best antioxidant capacity was observed for CH-B under 4 h hydrolysis with DPPH inhibitory as high as 81.6%, followed by CH-P with 1 h reaction (80.1%). Enzymatic hydrolysis was reported to benefit DPPH scavenging activity of several food proteins, such as milk protein (Mao et al., 2011), wheat herm protein (Zhu et al., 2006), and rice protein (Zhou, Canning, & Sun, 2013). Hidalgo et al. (2003) found that DPPH scavenging of bovine sodium caseinate hydrolysates tended to increase with hydrolysis time, while this study showed no clear correlation between hydrolysis time and DPPH scavenging activity for all three enzymes since hydrolysates exhibited high DPPH scavenging activity even only with 0.5 h hydrolysis. However, it is difficult to directly compare the antioxidant activities of hydrolysates prepared by different enzymes due to their different specificity in hydrolysis which could produce different peptides (Oliveira et al., 2014).

Considering hydrolysis efficiency, antioxidant activities as well as economic cost, optimum hydrolysis time for papain was 3 h, and for ficin and bromelain were 4h. CGM hydrolysates were prepared with optimum hydrolysis conditions for each enzyme and used for further analysis.

3.4.2 Antioxidant properties of ultrafiltrated hydrolysate fractions

Antioxidant activities of protein peptides was reported to be related with their MW (Chi et al., 2015; Liu et al., 2017; Zhuang, Tang, & Yuan, 2013). Prepared CGM hydrolysates were separated into five fractions named F1 to F5 from lowest MW (below 1 kDa) to highest MW (above 10 kDa) and evaluated for their antioxidant properties. Fig. 3.5 showed the weight distribution of each peptide fraction. F5 (>10 kDa) took up most of the CH-P (57.21%), and the second largest fraction was F4 (5-10 kDa, 26.37%), followed by F2

(1-3 kDa), F3 (3-5 kDa) and F1 (<1 kDa). The largest fraction of CH-F was also F5 (30.38%), following by F1, F3, F4, and F2. F4 was the largest fraction (26.97%) in CH-B, and the second largest fraction was F5 (24.67%) followed by F1, F3, and F2.

Total phenolic content of each fraction as well as crude hydrolysate mixture were measured (Fig 3.6). Peptide fraction with higher MW exhibited lower TPC, relatively. It was found that F1 of CH-P had significantly higher TPC of 51.49 mg GAE/g than other fractions. The F2 of both CH-F (41.39 mg GAE/g) and CH-B (40.87 mg GAE/g) possessed higher TPC among all fractions. For all three types of hydrolysate, F5 exhibited the lowest TPC relatively, with the TPC value as low as 33.03 mg GAE/g (CH-P), 26.16 mg GAE/g (CH-F), and 22.87 mg GAE/g (CH-B).

Proteins and peptides can perform antioxidant activity through different mechanisms (Elias, Kellerby, & Decker, 2008). Hence, the antioxidant capacity of each fractions was measured using various assays including DPPH radical scavenging activity, ABTS scanning activity and metal chelating capacity. DPPH radical scavenging of peptide fractions was illustrated in Fig 3.7A. Lower MW peptides from CH-P exhibited significant higher DPPH% value, and the highest DPPH radical scavenging was observed from F1 (90.1%). Medium sized peptides of CH-F showed better scavenging capacity, and highest value was observed for F4 (76.0%), followed by F3 (74.9%). The F2 of CH-B existed the highest scavenging capacity (72.1%) among all fractions, and there was no significant difference between F1, F3 and F4 but the DPPH% was higher than the crude hydrolysate mixture. Overall, peptide fractions of CH-P shown relatively higher scavenging capacity against DPPH than CH-F and CH-B under the same reaction time, and low or medium MW peptides exhibited better antioxidant activities in DPPH scavenging.

ABTS scavenging activity of ultrafiltrated fractions was also measured (Fig. 3.7B). For CH-P, F1 revealed significantly higher inhibition (64.0%) than other fractions. No

significant difference was observed between F2 and F3, as well as F4 and mixture. Lowest inhibition of CH-P was found in F5 (42.1%). There was no significant of ABTS inhibition between all fractions of CH-F, except for F5 with lowest inhibition rate (35.6%). The F1 of CH-B indicated highest inhibition with value of 67.3%, followed by F3 (58.9%) and the mixture (55.8%). The results of ABTS and DPPH scavenging were not in agreement which may due to the distinct solubility of ABTS radicals (water-soluble) and DPPH radicals (oil-soluble), and different stereoselectivity of the radicals (Zhu et al., 2008). Over all, small-sized peptides were considered exhibiting better antioxidant activity against DPPH and ABTS. Peptides perform their antioxidant activity by serving as a protein donor to free radicals (Li et al., 2010). Previous study has shown that the lower their molecular weights, the higher their chances of accessibility adsorbed to the oxidative agents (Roberts et al., 1999; Zhou et al., 2015).

According to Fig 3.7, higher metal chelation capacity was observed for medium sized MW fractions for all three types of hydrolysates. The F4 (36.2%) and hydrolysates mixture (37.8%) exhibited highest chelating capacity for CH-P with no significant differences. For CH-B, both F3 (24.6%) and F4 (24.5%) showed obvious higher chelating activity with no significant differences. The highest chelating activity of CH-B was observed at F4 with inhibition of 36.2%. As previously reported, the MW of peptides was found to be related to their antioxidant performance (Agrawal, Joshi, & Gupta, 2017; Hogan et al., 2009; Wang et al., 2014; Xu et al, 2019a). Zhou et al. (2015) reported that the chelation capacity of CGM hydrolysates was highly correlated to small MW as well as high content of bioactive amino acids. Peptides with lower MW were more active as metal ion binder. Besides, the presences of some amino acids could generate extra electrons which improve electrostatic and ionic interaction between themselves and metal ions, such as Asp and Glu which contributed to

strong metal chelation activity especially when they are at the end terminal of peptide chain (Sonklin, Laohakunjit, & Kerdchoechuen, 2018; Zhu et al., 2008).

3.4.3 Identification of peptide sequences

The F4 from CH-P, F1 from CH-F, and F3 from CH-B with promising antioxidant activities were identified by RP-HPLC and MALDI-TOF/TOF MS for peptide compositions (Table 3.2, 3.3, and 3.4). Numerous peptide sequences were observed for each peptide fraction due to the complex protein composition in CGM, and there were high levels of Glu, Pro, Ala, Leu, Phe and Tyr in all peptides which was in agreement with Li et al. (2007). Many studies have shown that antioxidant properties of protein hydrolysates were related to their structure. Zhuang et al. (2013) reported that Leu-Pro-Phe, Leu-Leu-Pro-Phe, and Phe-Leu-Pro-Phe from CGM had high radical-scavenging capacities for ABTS, hydroxyl, DPPH and superoxide radicals. Besides, peptide Tyr-Phe-Cys-Leu-Thr also exhibited excellent antioxidant activities (Rajapakse et al., 2005). One possible explanation was the present of specific amino acids. The aromatic residues, such as Tyr and Phe, could donate protons to electro-deficient radicals and were usually observed in antioxidant peptides (Li et al., 2019; Rajapakse et al., 2005). The position of specific amino acids was also critical. For example, Cys residues play an important role as free-radical scavengers when it was in the center of peptide because the thiol group could interact with radicals directly (Harman, Mottley, & Mason, 1984).

3.4.4 Inhibition of lipid oxidation in ground pork system

The F4 from CH-P (Fig. 3.7A), F1 from CH-F (Fig. 3.7B), and F3 from CH-B (Fig. 3.7C) was applied in ground pork systems to evaluate their antioxidative performance. The fresh ground meat samples with antioxidant peptides were incubated at 4 °C, and TBARS

was measured during 16 days storage. As shown in Fig 3.7, TBARS value was gradually increased from day 0 until the end of storage, and the value for control was increased from 29.68 to 64.59 mg MDA equiv./kg. For all the three fractions, meat with addition of 1,000 mg/kg demonstrated better protection of lipid oxidation than 500 mg/kg. F4 from CH-P showed best protection with reduction of lipid oxidation as high as 41.9% on day 16 compared with control, following with F3 from CH-B with 34.6%v reduction, relatively. F1 from CH-F was the weakest in protection of lipid oxidation with only 6.47% inhibition. Zhou, Sun and Canning also verified the antioxidant performance of corn peptides in meat system. They added corn hydrolysates prepared by both Neutrase and Alcalase into fresh beef and found the 1-3 kDa fraction from Neutrase hydrolyzed corn protein exhibited high protection at both 250 mg and 500 mg/kg. Not only corn protein, researches have also showed that several other plants or animal protein could be potential source of bioactive peptides, such as sorghum kafirin (Xu et al., 2019a; 2019b), soy protein (Moure et al., 2006), fish protein (Klompong et al., 2007), and milk protein (Hogan et al. 2009). Those bioactive peptides could be used as alternative antioxidant in food systems to prevent lipid oxidation due to the chelating effect of the pro-oxidative metal ions as well as scavenging free radicals (Jadhav et al., 1996). In addition, they could effectively exhibit antioxidant activity in meat system by forming a physical barrier to prevent pro-oxidants approaching the lipid (Cheetangdee & Benjakul, 2015).

3.5 Conclusion

The hydrolysates prepared from CGM using papain, ficin and bromelain showed different yield, hydrolysis degree and antioxidant properties which indicates the antioxidant activity of CGM hydrolysate is highly depending on enzyme type, as well as hydrolysis time. The antioxidant activities of CGM hydrolysates were MW dependent. Low or medium size peptide fractions exhibited higher antioxidant activities. Application of antioxidant peptides in ground pork system was efficiently inhibited lipid oxidation at 1,000 mg/kg. This study suggested that CGM could be a potential source of bioactive proteins or hydrolysates, and papain, ficin and bromelain could serve as efficient enzymes to hydrolyze CGM and then improve its bioactivity.

3.6 References

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Table 3.1 - Parameters for enzymatic hydrolysis

Enzyme Type	**Enzyme-to-protein substrate ratio**	**Enzyme amount, mg/g of protein**	**pH**	**Temperature, °C**	**Time, h**
Papain	60 U/g	40	6.5	50	0.5-5
Ficin	90 kGDU/g	225.5	6.0	50	0.5-5
Bromelain	180 kGDU/g	150	5.0	50	0.5-5

Table 3.2 - Peptide sequences of 5-10 kDa peptide fraction unltrafiltrated from papain-hydrolyzed CGM prepared at 60 U/g with 3 h hydrolysis

	RP-HPLC peak retention time	4.68 min	8.8 min	13.23 min	15.7 min	17.3 min	18.4 min	19.4 min	20.5 min	21.5 min	37.05 min	49.5 min
Zein-16	Area%	7.3%	4.2%	12.4%	34.5%	12.0%	4.3%	7.6%	4.7%	1.1%	7.9%	14.2%
	Coverage%	24.6%	37.7%	57.4%	27.3%	78.7	66.7%	75.4%	44.8%	42.1%	7.1%	25.7%
	Sequences	QTP PFHLPPP PFLGQCV SPAATP YGSPQCQALQ QQ QATY GVVLQ	PPPFYLPPQQ QP PQLSPCQ QIRQV EPLHRYQATY LQSFLQQ QPQGELAALM AAQVAQQLTA MCGLQLQQ	MKVLIVAL TSGGCGCQTP YQ PPQLSPCQQF GSCGVGSCGS PFLGQCVE AATP YGSPQCQALQ QQCCHQIRQV EPL VVLQSFLQQ QPQ CGLQLQQPG PCPCNA	VLIVALAL LALAAS YM PPPFYLP QYPTQ PPQLSPCQ GS PFLGQ SPAATP	LAL LALAASAASS TSGGCGCQTP PFHLPPPFYM PPPFYLPPQ PQLSPCQQF GSCGVGSVGS PFLGQCVEFL RHQCSPAATP YGSPQCQALQ QIRQV EPLHRYQATY GVVLQSFLQQ QP QVAQQLTA MCGLQLQQPG PCPCNAA	CQTP PFHLPPPFYM PPPFYLPPQQ QPQPWQ SPCQQF GSCGVGSVGS PFLGQCVEFL RHQCSPAATP YGSPQCQALQ QQCCHQIRQV EPLHRYQA QPQGELAALM AQQLTA MCGLQLQQPG	GCQTP PFHLPPPFYM PPPFYLPPQQ QPQP SPCQQF GSCGV GSCGV PFLGQCVEFL RHQCSPAATP YGSPQCQALQ QQCCHQIR EPLHRYQATY GVVLQSFLQQ QPQGELAALM AAQVAQQLTA MCGLQLQQPG PCPCNAA	IVALAL LALAASAASS TSGG CQTP PFHLPPPFYM PPPFYLPPQ FLQQ QPQGELAALM AAQVAQQLTA MCGLQLQQPG PCP	MKVLIVALAL LALAASAASS TSGGCGCQTP PFHLPPPFY SPCQQF GSCGVGSVGS QALQ QQCCHQIRQV EPLHRYQA	MKVLI AASS TSGG	ALAL LALAASAASS TSGGCGCQTP PFHLPPP PPFYLPPQQ MCGLQ
Zein-19	Area%	68.9%	17.2%	10.9%	28.2%	14.5%	2.5%	7.6%	4.0%	11.5%	10.1%	14.9%
	Coverage%	35.4%	37.5%	50.0%	13.3%	65.4%	29.6%	49.2%	52.5%	62.5%	8.3%	10.4%
	Sequences	KIFSLLM LLALSTC QCSQA PIASLL PYRLQQ AIAA AL SLVQ YSQQQ PFNQLSTLN YS QQQ QQILL LLPFY	MATKIFSLLM LLALSTCVAN ATIFP PIASLLPP ASICEN PALQPYRLQQ SQQQQF LPFN PAAYLQQQLL PFSQLA QQLLPFYQ QEAAN	TKIFSLLM LLALSTCVAN ATIFPQCS LQPYRLQQ AIAASNIPLS PLLFQQSP LAALNPAA QQQILLP ANRASFL TQQQLLPFYQ QFAANPATLL QLQQLLP LALTDPAASY QQHI	LLPPYL PSIIASICEN YQ QFAA	KIFSLLM LLALSTCV LPPYL PSIIASICEN PALQPYRL TI RAQQLQQLVL PLINQVALAN PYSQQQQF LPFNQLSTLN PAAYLQQQLL FPSQLATAYS QLLPENQ LAALNP	PSIIASICEN PALQPYRL LVQSLVQTI RAQQLQQLVL PLI QFAANPATLL QLQQLLPFVQ LALTDPA	TCVAN ATIFPQCSQA PIASLLPPYL PSIIASICEN PALQPYRLQQ AIA SPYSQQQQF LPF LLPFNQ LAALNPAAYL QQQILLPF AAANRASFL TQQQILLPFYQ QFAAN	STCVAN ATIFPQCSQA PIASLLPPYL PSIIAS CEN PALQPYRLQQ AIAASNIPLS PLLFQQSPAL SLVQSLVQTI RAQQ LINQVALAN LSPYSQ PENQ LAALNPAAYL	QCSQA PIASLLPPYL PSIIASICEN PALQPYRLQQ AIAASNIPLS PLLFQQSPAL SLVQSLVQTI RAQQ SQQQQF LPFNQLSTLN PAA FSQLATAYS QQQQ QQQILLPFSQ	KIFSLLM LIALSTC DPAASY	MATKIFSLLM LIALSTC PIASLLPP

		LL QLQQLL HIIGGAL				QQQILLPFSQ LAALNPAAYL TQQQLLPFYQ VQ LALTDPAASY QQ		ALTDPAASY	QQQILLPFVQ LALTDPAAS	LAA QFAANPATLL QLQQLLPFVQ LALTDPAAS QQHI		
Glutenin	Area% Coverage% Sequences	43.6% 19.7% TST HTSGGC GVG STPILGQCV QQCCQQL VA GLLAA TPCPYAAAG	7.9% 46.2% GGCGCQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHL HVPPPV CPCQQP PSP CQLQGTCGVG ILGQCVE FLRHQCSPTA SGQVA GLL IAQQ LTA	10.0% 67.3% AL LALAASATST HTSGGCGCQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHLPPPV HVPPPVHLPP YPTQPP RPQPHPQPHP CPCQQPHPSP CQLQGTCGV TPYCSPQCQS PQHRYQA IFGLVLQSIL QQQ GLLAAQIAQQ LT	42.6% 35.9% VLLVALAL LALAASATST HTSGGCGCQP PPPVHLP QLQGTCG RHQCSP IFGLVLQSIL QQ GLLAAQTAQQ PYAAAGG VP	17.1% 76.2% LALAASATST HTSGGCGCQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHLPPPV HVPPPVHLP QPHPQPHP CPCQQPHPSP CQLQGTCGVG GQCVE FLRHQCSPTA TPYCSPQCQS LRQQCCQQL EPQHRYQA IFGLVLQSIL QQPQSG AMCGLQQP TPCP	4.8% 64.1% LALAASATST HTSGGCGCQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHLPPPV HVPPPVHLP PPCHYPT PHPQPHP CQ LVLQSIL QQQPQSGQVA GLLAAQIAQQ LTAMCGLQQP TPCPYAAAGG	7.4% 65.9% GCGCQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHLPPPV HVPPPVHLP QPHPQPHP CPCQQPHPSP CQLQGTCGVG STPILGQCVE FLRHQCSPTA LRQQCCQQL QVEP QQQPQSGQVA GLQIAQQ LTAMCGLQQP	5.2% 26.5% LALAASATST HTSGGCGCQP LPP PPCHYPTQPP CQLQGTCGVG STPILGQCVE FLRHQ	1.8% 39.9% LLVALAL LALAASATS TSGGCGCQP PPPVHLPPPV HLPPPVHLPP PVHLPPPVHL PPPVHLPPPV QQQPQSGQVA GLLAAQIAQQ LTAMC	17.0% 15.7% SATST HT HVPPPV QVEPH SGQVA GLL IAQQ LTA	37.8% 35.4% TST HTSGGCGCQP PPPVHLPPPV HLPPPV PILGQC QA IFGLVLQSIL QQQPQSGQVA GLLAAQIAQ AMCGLQ CPYAAAG

Table 3.3 - Peptide sequences of <1 kDa peptide fraction unltrafiltrated from ficin-hydrolyzed CGM prepared at 60 kGDU/g with 4 h hydrolysis

	RP-HPLC peak retention time	4.1 min	6.4 min	12.9 min	14.2 min	15.9 min	17.5 min	19.3 min	19.9 min	21.7 min	23.03 min	41.9 min	51.3 min
Zein-16	Area%	23.5%	44.5%	6.0%	9.2%	11.8%	3.9%	19.9%	20.7%	18.0%	13.4%	2.0%	14.0%
	Coverage%	43.7%	18.6%	8.7%	51.9%	23.0%	67.2%	79.8%	65.0%	30.1%	31.7%	13.1%	60.7%
	Sequences	KVLIVALAL	GCQTP	LTA	MKVLI	VLIVALAL	MKVLIVALAL	MKVLIVALAL	KVLIVALAL	KVLIVALAL	AASAASS	SVGS	ALAASAASS
		LALAA	GQCVEF	MCGL	LPPPFYM	AASS	LALAASAASS	LALAAS	LALAASAASS	LALAASAASS	TS	PFLG	TSGGCGC
		GGCGCQTP	RHQCS	PG	PPPFYLPP	TSGGCGC	TSGGCG	TSGGCGCQTP	TSGGCGCQTP	TSG	GCQTP	QCSPAATP	LPPPFYM
		PF	HQIRQ	PCP	GSCGVGSVGS	CQQF	PFYM	PFHLPPPFYM	PFH	QPQPWQYPTQ	LPPQQ	YGSPQC	CGVGSVGS
		PPFYM	GVVLQS		PFLG	GSCGVGSCGS	PPPF	PPPF	FYM	PPQLS	PW		PFLGQCVEFL
		PPFYM	QPQGELA		FL	PAATP	LPPQQ	QLSPCQQF	PPPFYLPP	GSPQCQ	YPTQ		RH
		LPPQQ			PHQCSPA	YG	QPQPWQYPTQ	GSCGVGSCGS	PW	HRY	PP		CSPAATP
		GSCGVGSVG			YGSP		PPQLSPCQQF	PFLGQCVEFL	YPT	PCNAAAG	VGSVGS		YGSP
		GQCVEF			AL		GSCGVGSVGS	RHQCSPAATP	VGSVGS		HRYQ		AL
		LQ			QCCHQIRQV		PFLGQCV	YGSPQCQ	PFLG		GVVL		QQCCH
		QQCC			EPLHRYQATY		GSPQCQAL	QQCCHQTRQV	CVE		PCNAAAG		PLHRY
		QV			GVVLQSFLQ		QQCCHQIR	EPLHRYQATY	GSP				GELAA
		EPLH			ALM		QSFLQ	GVVLQSFLQQ	AL				AQQLTA
		QATY			AA		QPQGELAA	QPQG	QQC				MCG
		GVVLQSF			PCPCNA		CGLQLQQPG	TA	QV				PCNAAAG
		LM					PCPCNAA	MCGLQLQQPG	EPLHRYQATY				VY
		AAQV						PCPCNAAAGG	GVVL				
								VYY	SF				
									LAALM				
									QVA				
									QLTA				
									AA				
Zein-19	Area%	22.8%	52.0%	23.6%	9.6%	2.3%	2.8%	4.0%	3.2%	2.6%	8.6%	11.2%	12.2%
	Coverage%	21.7%	20.0%	28.8%	55.8%	7.9%	61.7%	67.1%	52.9%	69.2%	20.0%	10.8%	29.6%
	Sequences	MATKIFSLLM	KIFSLLM	MATKIFSLLM	KIFSLLM	KIFSLLM	IFSLLM	FSLLM	SLLPPYL	TKIFSLLM	QSPAL	MATKIFSLL	SLLM
		LLALSTC	LLALSTC	LLALSTC	LLALSTCVAN	LLALS	LLALSTCVAN	LLALSTCVAN	PSIIASICEN	LLALSTCV	ALAN	LLALSTC	LLALS
		IFPQCS	AL	TIFPQC	ATIFPQC	AAANRAS	ATIFPQCSQA	ATIFPQCSQA	PALQP	PPYL	LSPYSQQQQF	ATIFPQCSQA	ATIFPQCSQA
		PIASLLPP	SLVQ	PIASLLPP	SIIASI		PIASLLPPYL	PIASLLPPYL	SNIPLS	PSIIASICEN	FNQLSTLN		YRLQQ
		QLVL	QLVL	QLVL	AIAASNIPLS		PSIIASICEN	PSIIASI	PLLFQQSPQL	PALQPYRLQQ	PAAYLLQQ		AIAASNIPL
		PLLN	PL	QQQLL	PLLFQQSPAL		PALQPYRLQQ	AIAASNIPLS	SLVQSLVQTI	AIAASNIPLS	QQQQL		ALAN
		LLPFYQ	QQILLPF	QQQLL	SLVQS		AIAASNIPLS	PL	RAQQLQQLVL	PLLFQQSPAL	TQQQL		LSPYSQQ
		HIIGGAL	LLALSTC	LAALNP	NQLSTLN		PLLFQQSPAL	PAL	PLINQVALAN	SLVQSLVQTI			FNQ
			QLQQLL	QQQLLP	PAAYL		SLVQSLV	SLVQSLVQTI	LSPYSQQQQF	RAQQLQ			LAALNPA

			HIIGGAL	HIIGGAL	PFSQLATAYS QQ LLPEN ALNPAAYL QILLPFSQ RASFL TQQQLLPFYQ QFAA TLL QLQQLLPFVQ		PLINQVALAN LSPYSQQQF LPPQQ PFSQLATAYS QQQQLLPEN NPATLL QLQ PFVQ LALTDPAASY	RAQQL QF LPFNQLSTLN PAAYLQQQLL PFSQLATAYS QQQQLLPFNQ LAALNPAAYL QQQILLPFSQ AANPATLL QLQQLLPFVQ LALTDPAA	LPFNQLST QQQILLPFSQ LAAANR LQQLLPFVQ LALTDPAASY QQHIIG	LVL PLINQVALAN LSPYSQQQQF LPFNQLST QQQQLLPENQ LAAL QILLPFSQ LAAANRA ATLL QLQQLLPFVQ LALTDPAASY QQHIIG			QILLPFSQ ASFL TQQQ
Glutenin	Area%	35.4%	25.0%	28.2%	5.2%	3.0%	3.8%	24.8%	26.6%	7.0%	18.0%	10.0%	23.0%
	Coverage%	27.8%	5.8%	19.3%	19.3%	12.6%	71.3%	67.3%	84.8%	65.0%	27.4%	9.4%	28.3%
	Sequences	ALAL LALAA QPP QRPQ PQPHP CPC PILGQCVE PYCSPQ QCCQQL QVA GLLAA QPP TPCPYAAA	PILGQC QVA GLLAA	AASATST HTSGGCGCQP PILGQC IL QVA GLLAA LTAMCGL	SATST HTSGGCGCQP PTA TPYCSPQCQS LR QLR QVEP QVEPQHRY	VLLVALAL LA TST HTSGG QP PPPVHLPP	VLLVALAL LA HLPPV HLPPVHLPP PVHLPPPVHL PPPVHLPPPV VHLPP PPCHYPTQPP PC QPHPSP GTCGVG STPILG CVE FLRHQCSPTA TPYCSPQCQS QQLR QVEPQ QA IFGLVLQSIL QQQPQSG AQIAQQ LTAMCGLQQP **TPCP**	VLLVALAL LALAASATST HTSGGCG PPVHLPPPV HVPPPVHLPP PSP CQLQGTCGVG STPILGQCVE FLRHQCSPTA TPYCSPQCQS LRQQCCQQLR QVEPQH QA IFGLVLQSIL QQQPQSGQVA GLLAAQIAQQ LTAMCGLQQP TPCPYAA	LLVALAL LALAASATST HTSGGCGCQP PPVHLPPPV HLPPVHLPP PVHLPPPVHL PPPVHLPPPV HVPPVHLPP PPCHYPTQPP PQPHPQPHP CPCQQPHPSP CQLQGTCGVG STPILGQCVE FLR PTA TPYCSPQCQS LRQQCCQQLR QQQPQSGQVA GLLAAQIAQQ LTAM GLQQP TPCPY **AAAG**	MRVLLALAL LALAASATST HTSGGCGCQP PPVHLPPPV HLPPVHLPP PVHLPPPVHL PPPVHLPPP ILGQCVE FLR CSPQCQS LRQQCC QHRYQA IFGLVL AQIAQQ LTAMC TPCPYAAGG VP	MRVLLALAL LAASATST HTSGG CHYPT RP PHP QGTCGVG STP QHRY PYAAAGG VP	VHLPPPV HVPPPVHL VCVE FLR	MRVLLV VHLPPPV HVPPPVHLPP PPCH SP GTCGVG ST CVE FLR GLLAA IA LTAMCG

Table 3.3 - Peptide sequences of 3-5 kDa peptide fraction unltrafiltrated from bromelain-hydrolyzed CGM prepared at 180 kGDU/g with 4 h hydrolysis.

	RP-HPLC peak retention time	4.0 min	5.55 min	6.5 min	8.1 min	12.3 min	13.1 min	14.5 min	15.6 min	19.1 min	23.03 min	24.9 min	41.9 min
Zein-16	Area%	21.1%	48.6%	10.6%	9.6%	80.2%	11.2%	23.3%	6.4%	45.0%	4.1%	8.5%	45.8
	Coverage%	15.8%	16.4%	8.7%	8.2%	14.2%	19.1%	15.3%	12.6%	71.0%	69.9%	43.2%	22.4
	Sequences	VALAL	GC	KVLIVALAL	MKVLI	RH	KVLIVALAL	VALAL	QQCC	MKVLI	KVLIVALAL	IVALAL	ASAASS
		LALAASA	TP	FYLPP	QQ	CS	LALAA	QQF	ATY	SGGCGC	LALAASAASS	LALAASAASS	TS
		FYM	VGSVGS		QP	LQ	GGCGC	GSCGVGSVGS	GVVL	TP	TSGGCGC	TSGGCGC	LPP
		PPPFYL	RH		GELAA	QPQGELA	TP	PFLG	QQCC	PFHLPPPFYM	TP	PFHLPPPFYM	HRY
		CH	CS			AQQLTA	QQCC	IR	QP	PPPFY	PFH	PPPFY	LTA
		IR	GVVL				ATY		GELAA	SPC	YM	SVGS	PCNAAAG
			GELA				GVVL			GSCGVGSVGS	PPPFYLPP	PFLG	
										PFLG	PW	CSPAATP	
										CVEFL	YP	YGSP	
										RH	PP	GL	
										PAATP	LSPC	PG	
										YGSP	GSCGVG	PCPCNA	
										CCH	IR		
										EPLHRY	EPLHRY		
										ATY	ATY		
										GVVL	GVVL		
										SFL	SFL		
										VA	ALM		
										LTA	AA		
										MCGL	VA		
										CPCNAAAGG	LTA		
										VYY			
Zein-19	Area%	26.3%	27.6%	17.8%	21.5%	75.2%	24.3%	56.6%	16.2%	35.4%	5.4%	5.9%	14.2
	Coverage%	23.3%	8.8%	16.2%	26.7%	22.1%	13.1%	30.8%	12.5%	61.2%	76.5%	39.6%	10
	Sequences	LLM	KIFSLLM	KIFSLLM	MATKIFSLLM	RLQQ	KIFSLLM	KIFSLL	KIFSLLM	CVAN	KVLIVALAL	IFSLLM	MATKIFSLL
		LLM	LLALSTC	LLALSTC	LLALSTC	ASNIPLS	LLALSTC	LLALSTCVAN	LLALSTC	ATIFPQCSQA	LALAASAASS	LLALSTC	LLALSTC
		LLPPYL	HIIGGAL	IFPQCS	PIASLLPP	AL	QLVL	ATIFPQC	AIAASNIPL	PIASLLPPY	TSGGCGCQTP	ATIFPQCSQA	PIASLLPP
		PSII		QLVL	SIIASI	SLVQ	LLPFY	AIAASNIPLS	ALAN	SIIACICEN	PFH	PIASLLPPY	
		QQ		PL	IAASNIPL	RAQQL	HIIGGAL	AL	LSP	PALQPYR	YM	PSIIA	
		AIAASNIP		FL	ALAN	NQVALAN		SLVQ		AIAASNIPLS	PPPFYLPPQQ	LVQTI	
		FNQLSTLN		TQQQ	LLPFN	QQILL		LSPYS		PL	QPQPWQYP	RAQQLQQLV	

		AYS QQQQLLP SFL TQQLLP		HIIGGAL	LAALNPAA LPFVQ	LAAANR LL QLQQLL		QQILL LLPFY TLL QLQQLLP HIIGGAL		LVQTI RAQQL NQVALAN LSPYSQQQQF LPFNQLSTLN PAAYLQQQLL PFSQLATAYS QQQQLLPFNQ LAAANRASFL TQQ TLL QLQQLLPFVQ	PPQLSPCQQF GSCGVGSVGS PFLGQCV IRQV EPLHRYQATY GVVLQSFLQQ QP ALM AAQVAQQLTA MCGLQLQQPG PCPCNAAAGG VY	AAYLQQQLL PFSQLATA PAAYL QQQILLP PAASY QQQHIIGGAL	
Glutenin	Area%	52.9%	46.6%	5.3%	20.2%	100.0%	11.2%	42.1%	5.9%	29.0%	5.8%	7.1%	12.1%
	Coverage%	39.9%	8.5%	4.0%	15.2%	24.2%	12.1%	20.2%	15.7%	63.2%	68.6%	29.6%	12.6%
	Sequences	VALAL LALAASATST HTSG PILG LRH CSPTA PYCSP LRH HRY GLVL VALAL GLLAA IA MCGL TPCPYAAAG	GVG STPILG QVA GLLAA	VLLVALAK	AL LALAASATST PHP CP GLLAA GLLAA TP	HP PH CQQPH GVG STPILG RH CS QS LR QQQPQSG VA GLLAA LTA	LAL LALAA QPP RP QQCCQQL TPCPYAA	VLLVALA GTCGVG STPILG PTA TPYCSP IFG VA GLLAA	HVPPPV QCC QL SG VA GLL TAQ LTA TPCPYAA	LAASATS HTSGGCG PPVHLPPPV HVPPPVHLPP PPCH RP PHP CPC PHPSP GTCGVG STPILG PTA TPYCSP LR CC IFGLVL SIL LLAA IA LTAMCGL TPCPYAA	LVALAL LALAASATST HTSGGCG CPC PHPSP GTCGVG STPILG CVE FIRH CSPT YA LTAMCGL TPCPYAAAG	AL LALAASATST HTSGGCGC VHLPPPV HVPPPVHLPP PPCHYP SP GTCG VL SIL SG	SATST HVPPPV SG VA GLL IA LTA

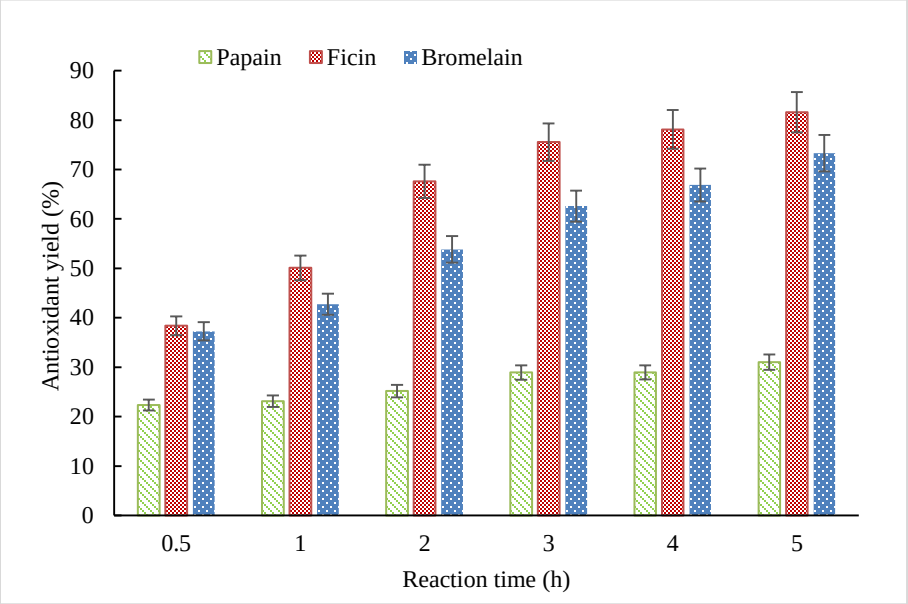


Figure 3.1 - Antioxidant yield of CGM hydrolysates with different reaction time prepared by papain at 60 U/g, ficin at 60 kGDU/g, and bromelain at 180 kGDU/g.

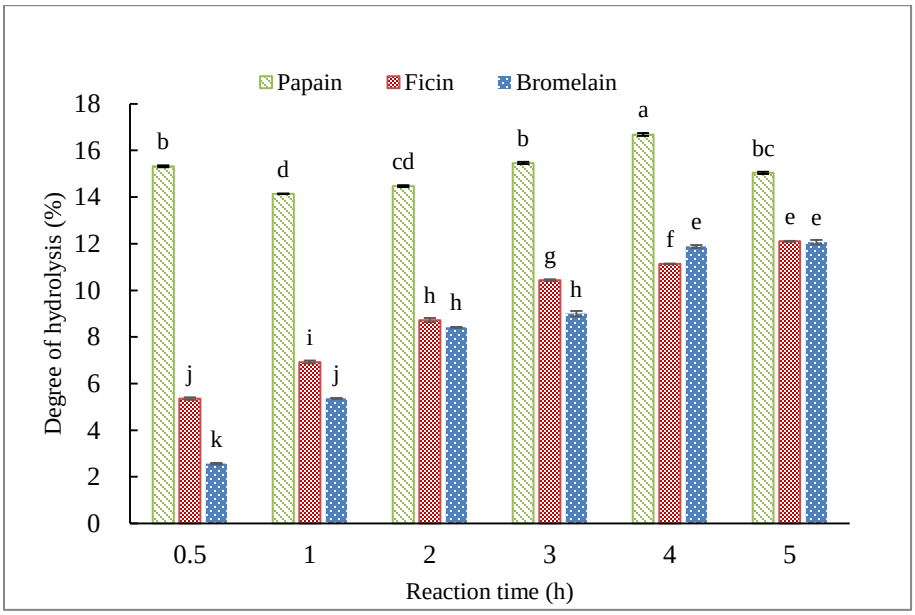


Figure 3.2 - Degree of hydrolysis of CGM hydrolysates under different reaction times prepared by papain at 60 U/g, ficin at 60 kGDU/g, and bromelain at 180 kGDU/g.

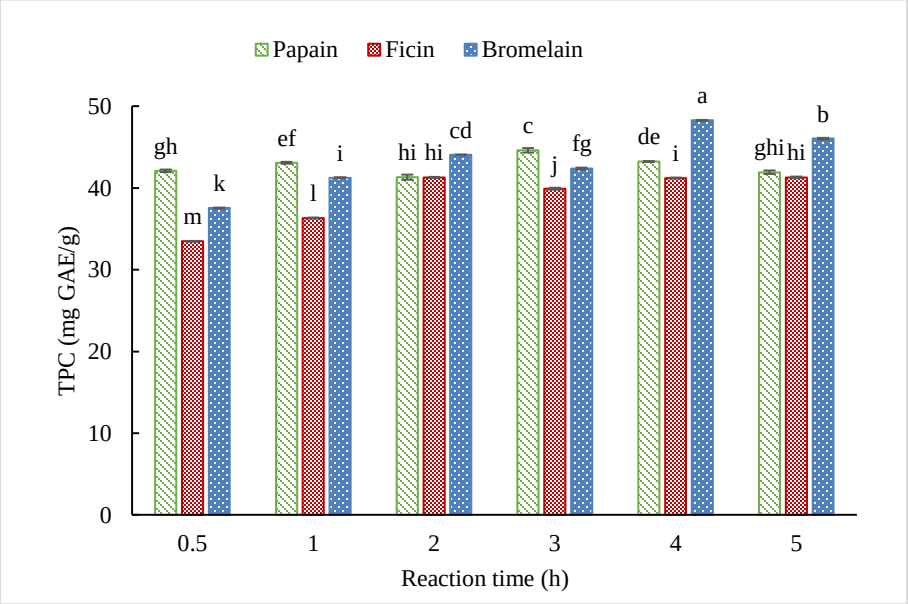


Figure 3.3 - Total pehnolic content of CGM hydrolysates at 1 mg/mL under different reaction times prepared by papain at 60 U/g, ficin at 60 kGDU/g, and bromelain at 180 kGDU/g.

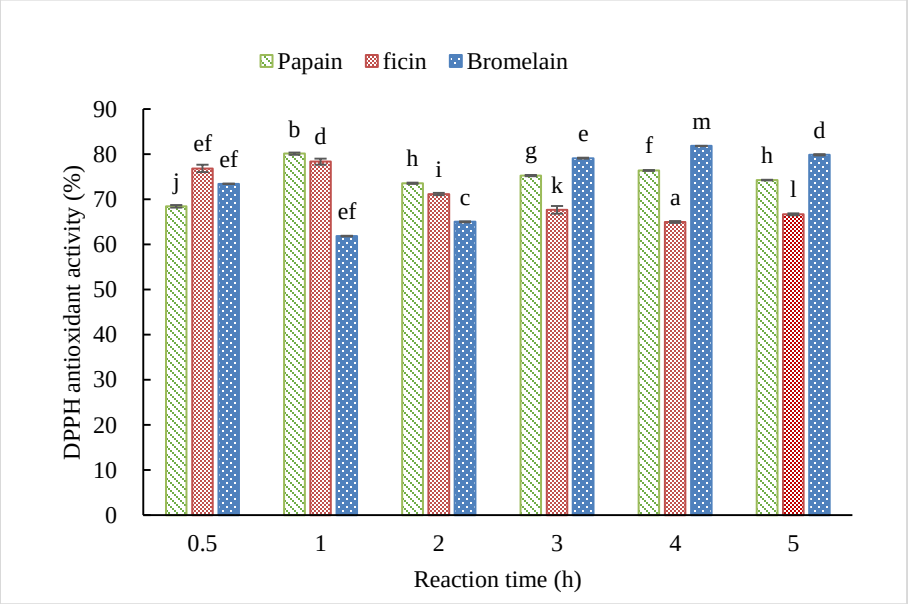


Figure 3.4 - DPPH radical scavenging activity of hydrolysates at 5 mg/ml under different reaction times prepared by papain at 60 U/g, ficin at 60 kGDU/g, and bromelain at 180 kGDU/g.

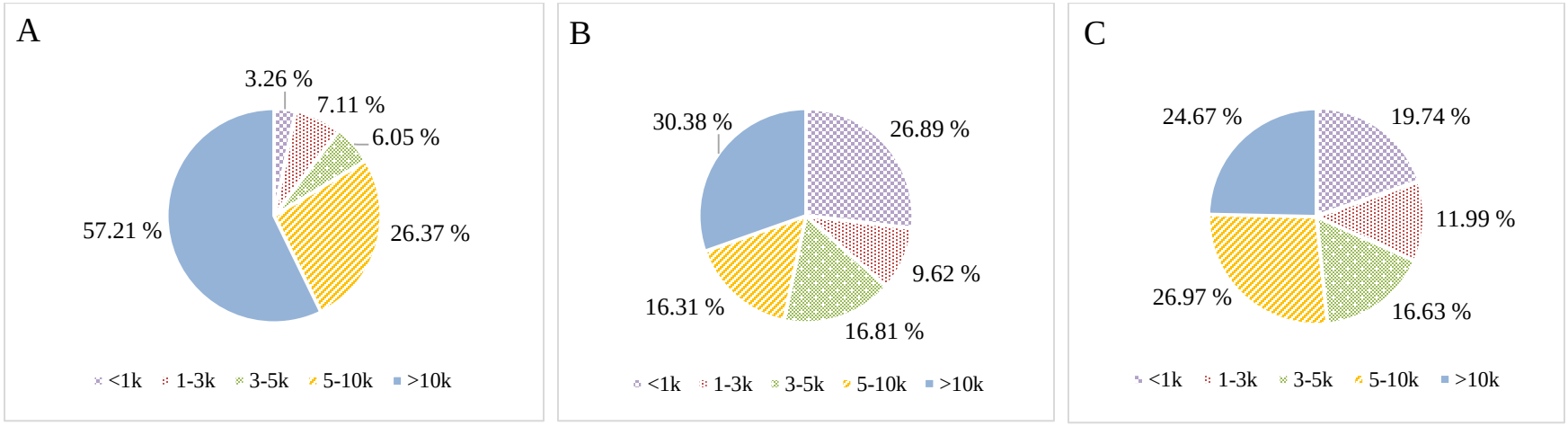


Figure 3.5 - Antioxidant yield of peptide fractions unltrifiltrated from CGM hydrolysates prepared by :(A) Papain at 60 U/g with 3 h reaction; (B) Ficin at 60k GDU/g with 4 h reaction; and (C) Bromelain at 180k GDU/g with 4 h reaction

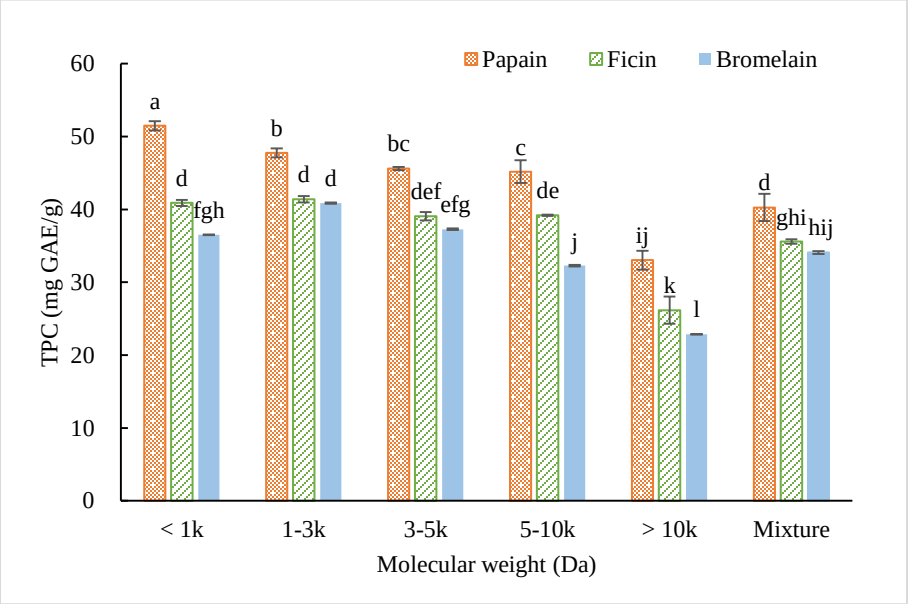
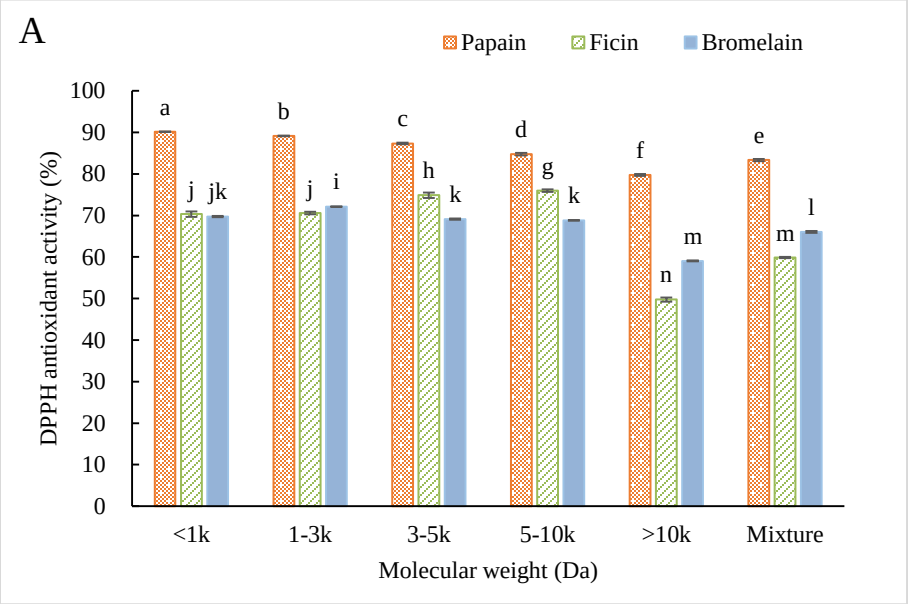
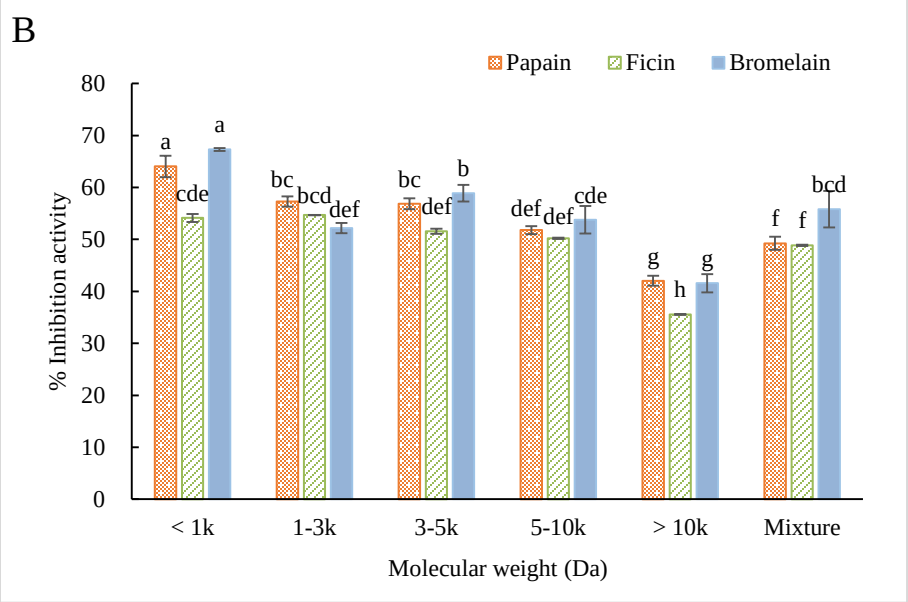


Fig. 3.6 - Total phenolic content of different peptide fractions (1mg/mL) ultrafiltrated from CGM hydrolysates prepared by papain (60 U/g, 3 h), ficin (60k GDU/g, 4 h), and bromelain (180k GDU/g, 4 h).


A
Papain
Ficin
Bromelain
DPPH antioxidant activity (%)
100
90
80
70
60
50
40
30
20
10
0
a
j
jk
b
j
i
c
h
k
d
g
k
f
n
m
e
m
l
<1k
1-3k
3-5k
5-10k
>10k
Mixture
Molecular weight (Da)


B
Papain
Ficin
Bromelain
% Inhibition activity
80
70
60
50
40
30
20
10
0
a
cde
a
bc
bcd
def
bc
def
b
def
def
cde
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3-5k
5-10k
> 10k
Mixture
Molecular weight (Da)

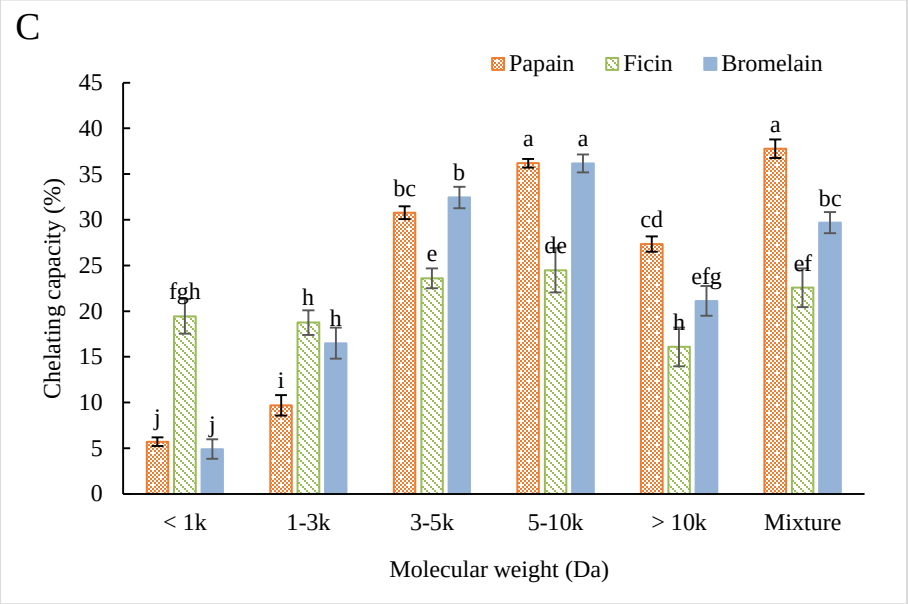
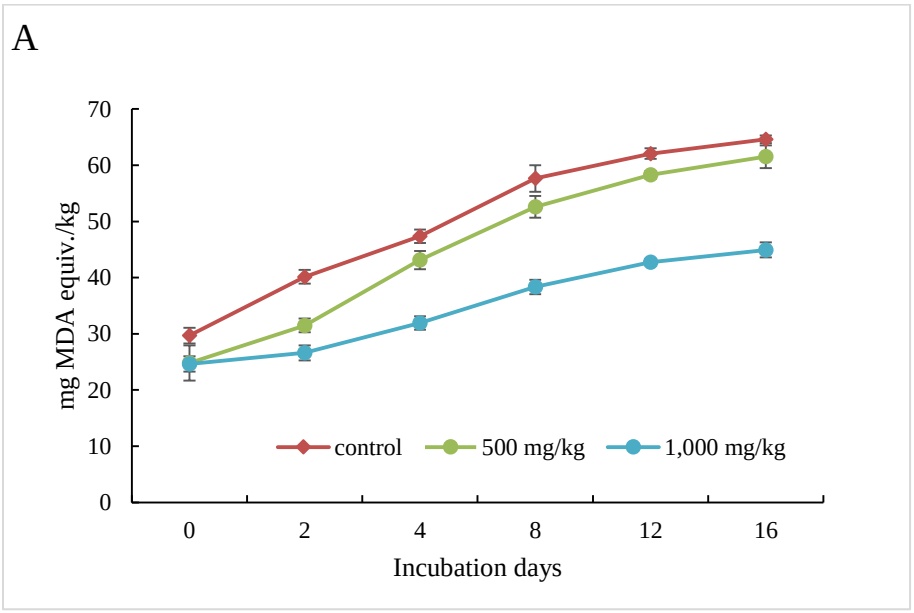
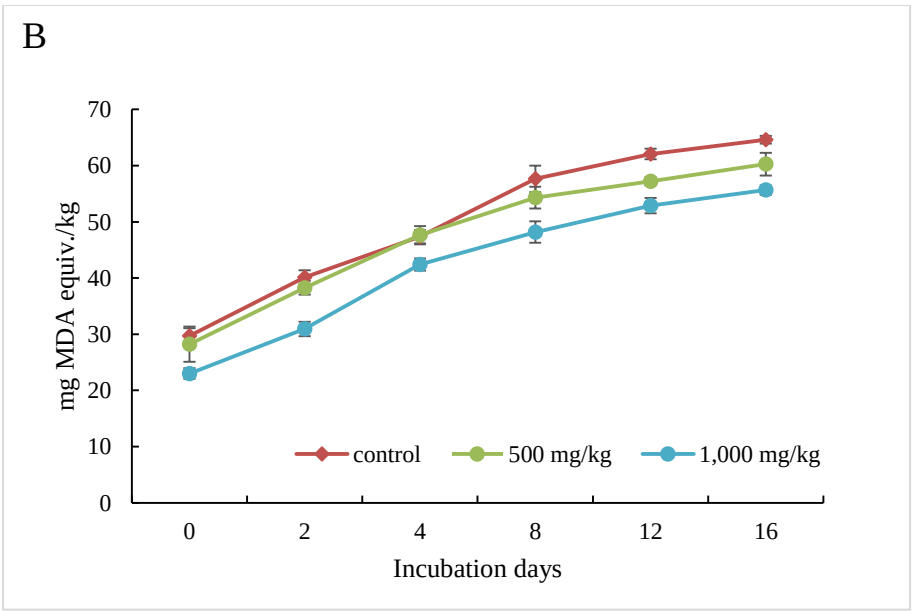


Figure 3.7- Antioxidant activities of different peptide fractions ultrafiltrated from CGM hydrolysates prepared by papain (60 U/g, 3 h), ficin (60k GDU/g, 4 h), and bromelain (180k GDU/g, 4 h). (A) DPPH radical scavenging activity at 5 mg/mL; (B) ABTS radical scavenging activity at 1 mg/mL; and (C) Fe^{2+} chelating activity at 1 mg/mL.


A
70
60
50
40
30
20
10
0
mg MDA equiv./kg
control
500 mg/kg
1,000 mg/kg
0
2
4
8
12
16
Incubation days


B
70
60
50
40
30
20
10
0
mg MDA equiv./kg
control
500 mg/kg
1,000 mg/kg
0
2
4
8
12
16
Incubation days

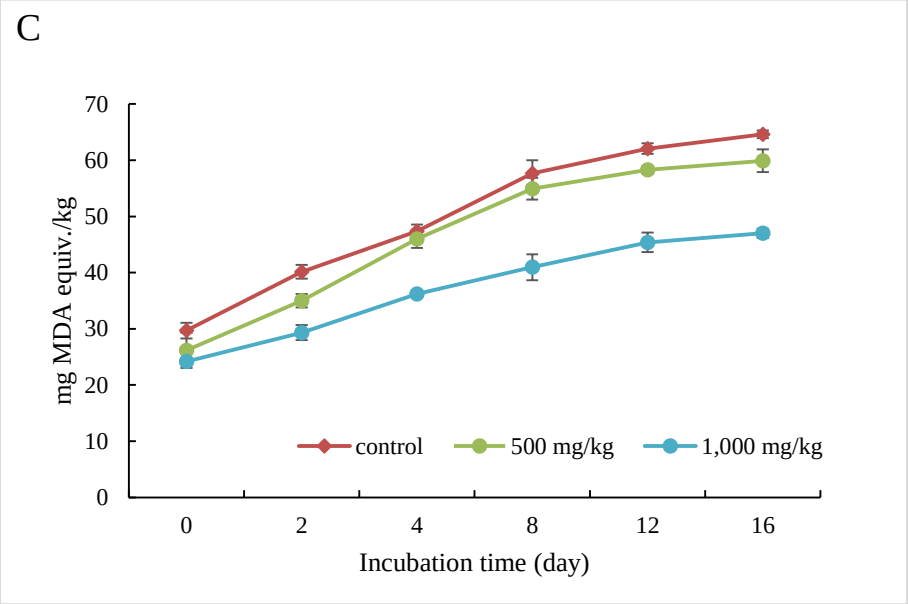


Figure 3.8 - TBARS value of selected peptide fractions at 500 mg/kg and 1,000 mg/kg in ground pork system. (A) 5-10 kDa fraction ultrafiltrated from CGM hydrolysates prepared by papain (60 U/g, 3 h); (B) < 1 kDa fraction untriafiltrated from CGM hydrolysates prepared by ficin (60 GDU/g, 4 h); and (C) 3-5 kDa fraction ultrafiltrated from CGM hydrolysates prepared by bromelain (180 kGDU/g, 4 h).

Chapter 4 - Antioxidant Performances of Corn Gluten Meal and DDGS Protein Hydrolysates in Food, Pet Food, and Feed Systems

4.1 Abstract

Protein hydrolysates from corn gluten meal (CGM) and distillers' dried grains with solubles (DDGS) were prepared with Neutrase and Alcalase, and the antioxidant activity of those hydrolysates in bulk oils, ground pork, canine pet food and pig feed were evaluated by measuring oxidation stability based on peroxide value (PV) and thiobarbituric reactive substances (TBARS) value. Alcalase-hydrolyzed CGM (CPH-A) and Neutrase-hydrolyzed CGM (CPH-N) had stronger DPPH radical scavenging activity than Alcalase-hydrolyzed DDGS (DPH-A) and Neutrase-hydrolyzed DDGS (DPH-N). CPH-N showed better prevention of lipid oxidation in both corn oil and fish oil compared with other corn antioxidants. The best oxidation prevention in ground meat was observed with 2 g/kg of CPH-N. Lipid oxidation in pet food containing 2% DPH-A was efficiently retarded by 37.8% reduction at the end of incubation, and TBARS value of pig feed containing 2% CPH-N was reduced the most compared with other treatments. Overall, CGM and DDGS protein hydrolysates could potentially be used as naturally derived antioxidant in food, pet food, and feed systems with good protection efficiency for lipid oxidation.

4. 2 Introduction

Lipid oxidation is a major cause of quality deterioration during processing, handling, and storage of high-fat/oil foods or ingredients (Mussinan & Morello, 1998). The formation of off-flavor and various oxidation products such as peroxides, hydroperoxides, aldehydes, and ketones

results in the loss of food texture, aroma, taste, nutrient, shelf stability as well as causing food safety concerns (Amaral, et al., 2018; Saiga et al., 2003; Zhou et al., 2013). To retard lipid oxidation, antioxidants have been widely used in these products. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), and ethoxyquin (EQ) are commonly used in various food and feed products. However, it has been reported that such antioxidants possibly increase health risks due to their toxicity and carcinogenicity (Oliveria et al., 2014; Shahidi & Zhong, 2008). In recent years, there is a growing interest in developing natural antioxidants with high efficiency and low cost. Some plant extracts have been reported to inhibit lipid oxidation in different food products, such as extracts from olive oil mill waste (Leonardis et al. (2007), broccoli sprout extract (Ahn, et al., 2008), ginger powder (Zhao et al. 2011), and garlic extract (Iqbal & Bhanger, 2007). Besides, bioactive protein hydrolysates could also be a potential source of natural and safer antioxidants. Numerous studies have shown that the hydrolysates or bioactive peptides produced from rice bran protein (Cheetangdee & Benjakul, 2014), milk casein (Blanca et al., 2007), soy protein (Oliveira et al., 2014), sorghum kafirin (Xu et al., 2019a; 2019b), and corn protein (Li et al., 2019; Liu, et al., 2015; Zhu, He, & Hou, 2018) exhibited high antioxidant activities in both *vitro* and *vivo* models.

Corn is one of the most cultivated crops worldwide and is an important food, feed and biofuel source in the U.S. Previous researches have shown that specific protein hydrolysates or peptides produced from corn protein exerted significant antioxidant properties in scavenging free radicals or chelating transitional metal ions (Li et al., 2019; Li, et al., 2008; Wang et al., 2015; Yang et al., 2007). Corn gluten meal (CGM) is one of the major byproducts from corn wet milling and a protein-rich source with about 60-70% crude protein (Wang et al., 2016).

Distillers' dried grains with soluble (DDGS) is also a high nutrient by-product during corn ethanol production but contains less protein (about 27-35%) (Belyea, Rausch, & Tumbleson, 2004). Low water solubility of CGM and DDGS proteins limits their performance and application in food industry, but enzymatic hydrolysis can release functional peptides and domains which significantly improved their functionality and bioactivity (Li et al., 2019; Li et al., 2011). Alcalase (from *bacillus licheniformsis*) and Neutrase (from *bacillus amyloliquefaciens*) are two enzymes with high efficiency and commonly used for food protein hydrolysis for antioxidant production (Apar & Ozbek, 2007; Zhou, Sun, & Canning, 2012; Zhu, He & Hou, 2018).

Although previous studies reported that corn protein hydrolysates or peptides presented high antioxidant activities through different chemical assays, there still lacks information about how those protein hydrolysates and peptides could perform in food and non-food systems, which is critical for the practical application and potential commercialization of such antioxidant products. This study aimed to evaluate the performances of CGM and DDGS protein hydrolysates produced with Alcalase and Neutrase in bulk oils (e.g., corn oil, fish oil), ground meat, pet food, and animal feed systems, in order to explore their potential applications. This study will benefit the development of a novel class of natural antioxidants from low-cost corn byproducts.

4.3 Experimental Section

4.3.1 Materials

Corn gluten meal (CGM, 61.3% crude protein) and distillers' dried grains with soluble (DDGS, 28.7% crude protein) were provided by Grain Processing Corporation (Muscatine, IA,

USA). Neutrase and Alcalase were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Corn oil (pure, un-stabilized) was purchased from Kroger Co. (Cincinnati, OH, USA). Un-stabilized Virginia prime menhaden fish oil was provided by Omega Protein Co. (Houston, TX, USA). Ground pork (20% fat) was purchased from local market. Canine pet food kibble and phase 1 nursery pig feed pellet were formulated and produced in the Department of Grain Science and Industry, Kansas State University, and the ingredients and formulations are listed in Table 4.1. All other chemicals, solvents, and reagents used were at least analytical grade and purchased from Sigma-Aldrich (St. Louis, Mo, USA) or Fisher Scientific (Fairlawn, NJ, USA).

4.3.2 Preparation of CGM and DDGS hydrolysates

To prepare hydrolysates, CGM and DDGS were first defatted by stirring the sample with hexane (1:6, w/v) for 0.5 h at room temperature and then filtrated. Defatting was repeated for three times. The defatted sample was dried in fume hood for at least 24 h to evaporate the residue solvent. Neutrase and Alcalase were used for enzymatic hydrolysis of the proteins. Hydrolysis was performed with 4% CGM or DDGS suspension in distilled water (w/v, protein basis). Before adding enzyme, the suspension was heated in 95 °C water bath for 10 min and allowed to cool down to room temperature, and then pH was adjusted to the optimum level (based on the manufacturer recommendation). Hydrolysis was conducted under optimum temperature in a water bath shaker with shaking speed at 150 rpm. The enzyme-to-substrate ratio, pH, temperature and reaction time used for Neutrase and Alcalase were summarized in table 4.2.

4.3.3 Determination of antioxidant yield

Antioxidant recovery yield is defined as the yield of water-soluble fractions after hydrolysis, and was calculated as follows:

$$\text{Antioxidant yield} = (W_2 / W_1) * 100\%$$

Where W_1 was the weight of initial protein in CGM or DDGS used for hydrolysis, and W_2 was the weight of lyophilized hydrolysates from the supernatant after reaction.

4.3.4 Determination of DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of hydrolysates was determined according to the method of Li et al. (2008) with some modifications. DPPH solution was prepared by dissolving 7.88 mg DPPH in 100 mL 95% ethanol. Five mL of DPPH solution was added into 5 mL hydrolysate solution at 5 mg/mL. The mixture was vortexed for 1 min and allowed to react in dark for 30 min, and then the absorbance was measured at 517 nm using a spectrophotometer (UV-6300PC, VWR International, LLC, Radnor PA, USA). The DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH scavenging rate (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

4.3.5 Antioxidant performance of hydrolysates in oil system

Preparation of bulk oil containing CGM or DDGS hydrolysates

Hydrolysate was added to corn oil or fish oil following a literature method with slight modification (Elias et al., 2006). Hydrolysate was added into corn oil at 5 g/L and 10 g/L, and butylated hydroxytoluene (BHT) was added at 10 g/L as a positive control. For fish oil, hydrolysate was added at 25 g/L and 50 g/L, respectively. The mixture was vortexed for 2 min and then shaked at 150 rpm for 30 min in the dark. Control oils with no antioxidant were

processed similarly. The corn oil samples were then stored at 50 °C, and the fish oil samples were stored at 25 °C. The samples were collected at different times for further analysis.

PV determination

Peroxide value (PV) of bulk oil was determined according to the method of Cheetangdee & Benjakul (2008). Briefly, 0.5 mL of oil sample was added into 2.5 mL of isooctane-propanol (3:1, v/v), the mixture was vortexed for 1 min and then centrifuged at 4,000 xg for 2 min. The upper layer (200 μL) was mixed with 2.8 mL methonal-1-butanol (2:1, v/v), 15 μL ammonium thiocyabate (3.97 mol/L), and 15 μL ferrous iron solution (0.132 mol/L barium chloride ($BaCl_2$), and 0.144 mol/L ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)). After incubating at room temperature for 20 min, absorbance of the mixture was read at 510 nm. Cumene hydroperoxide was used as a standard, and PV was quantified as milligram of hydroperoxide equivalent per liter of oil (mg hydroperoxide equiv./L).

TBARS value determination

Thiobarbituric reactive substances (TBARS) value was determined according to a literature method (Papastergiadis et. al, 2012) with slight modification. Firstly, secondary oxidation products were extracted. Corn oil sample (0.5 mL) and 2.5 mL deionized (DI) water were added into a 15 mL tube and vortexed for 2 min. The mixture was centrifuged at 500 xg for 10 min. Then, 2.5 mL of the aqueous layer was transferred to another 15 mL tube and mixed with 2.5 mL TBA reagent (3.75 g/L thiobarbituric acid, 150 g/L trichoroacetic acid (TCA), and 0.25 mol/L hydrogen chloride). The tube was capped and heated in boiling water for 30 min. After cooling down to room temperature, the absorbance was measured at 532 nm. For fish oil

sample, 0.1 mL oil and 2.9 mL DI water were used for extraction, and the other procedures were the same as for corn oil. Malonaldehyde (MDA) was used as a standard, and TBARS value was expressed as milligrams of MDA equivalent per liter of oil (mg MDA equiv./L).

4.3.6 Antioxidant performance of hydrolysates in ground pork system

Antioxidant activity of hydrolysates in ground pork was determined by measuring lipid oxidation based on TBARS value following the method of Zhang, Li, and Zhou (2010) with slight modification. Hydrolysate was mixed with ground pork at 1 and 2 g/kg, respectively. Three drops of 0.2% sodium azide was added into each sample to prevent mold growth. Prepared samples were stored at 4 °C and collected at different times for analysis. To extract the oxidation products, 5 g of prepared meat was homogenized with 50 mL DI water, 10 mL reducing agent (0.01% propyl gallate, 0.02% ethylenediaminetetraacetic acid (EDTA)), and 0.1 mL of sodium dodecyl sulfate (SDS,10%,) for 2 minutes. Homogenate (1.0 mL) was then mixed with 4.0 mL TBA solution (0.4% TCA, 0.5% SDS, and 9.3% acetic acid). The mixture was heated at 95 °C for 1 h, and then cooled in cold water for 10 min. Then, 5 mL of pyridine/butanol (1:15, v/v) was added into the mixture and centrifuged at 3500 xg for 15 min. The absorbance of the upper layer was measured at 532 nm. The 1, 1, 3, 3-tetramethoxypropane (TMP) solution (0 to 10 μM) was used as a standard. TBARS value in ground pork was expressed as milligrams malonaldehyde equivalents per kilogram of meat (mg MDA equiv. /kg).

4.3.7 Antioxidant performance of hydrolysates in pet food and feed systems

Pet food and pig feed were grounded into fine powder using a coffee grinder (Keenstone, Milpitas, CA, USA). Fish oil was pre-added with different amounts of hydrolysate by vortexing

for 2 min. The amount of hydrolysates added to fish oil for pet food evaluation was 1 and 2% (dry basis), respectively, and that for pig feed testing was 0.5 and 2% (dry basis), respectively. Pet food powder was then coated with 3% fish oil (dry basis) containing different amount of hydrolysates, and pig feed powder was coated with 6% (dry basis) fish oil containing the hydrolysates. Coating process was conducted by mixing the mixture for 1 min at 2nd speed and 2 min at 3rd speed using a KitchenAid with a wire whip (Whirlpool Corporation, St. Joseph, MI, USA). Prepared samples were stored at 55 °C for different times until analysis.

TBARS value was measured following a previously reported method by Glodde et al. (2018). Briefly, 0.25 g of the sample was mixed with 4 mL of 5% TCA and 2.5 mL of 5% BHT in hexane (w/v). The mixture was vortexed for 30 sec and centrifuged at 4500 xg for 15 min. The aqueous layer (2.5 mL) was added into 2.5 mL of 5% TCA and centrifuged at the same speed for 10 min. Then, 2.5 mL of the aqueous layer was pipetted into 15 mL tube containing 1.5 mL 0.8% thiobarbituric acid. The mixture was vortexed, heated in 70 °C water bath for 30 min, and then cooled in cold water for 10 min. Absorbance of the mixture was determined at 532 nm. The 1,1,3,3-tetraethyoxypropane (TEP) was used as a standard, and final results were expressed as milligrams malonaldehyde equivalents per kilogram of pet food or pig feed (mg MDA equiv. /kg).

4.3.8 Statistic analysis

Experiments were carried out at least in triplicate, and results were analyzed with SAS 9.3 software (SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was performed, and Tukey's *post-hoc* test was used to determine significant differences between the means ($p < 0.05$).

4. 4 Results and Discussion

4.4.1 Oxidative stability of bulk oils added with CGM and DDGS hydrolysates

The inhibition effect of antioxidant hydrolysates in corn oil was evaluated based on PV (Fig. 4.1) and TBARS value (Fig. 4.2). The incremental of PV for all the treatments was relatively slow at the first 5 days, but a sharp increase was observed from day 5 to day 7 (Fig. 4.1). The PV of the control (without antioxidant) was 2.22 ± 0.33 meq./L on day 0 and reached 48.79 ± 3.31 meq./L after 7 days incubation at 50 ˚C, while the best protection was observed for corn oil containing 10 g/L BHT, where the PV was only 15.84 ± 0.16 meq/L on day 7, which was 67.5% reduction compared with the control. The PV of corn oil containing 10 g/L of Neutrase-hydrolyzed CGM (CPH-N) was reduced by 63.4% compared with the control (Fig 4.1 B), followed by 10 g/L of Alcalase-hydrolyzed CGM (CPH-A) with 62.5% reduction (Fig. 4.1 A). Neutrase-hydrolyzed DDGS (DPH-N) showed relatively weak oxidation inhibition effect where the PV was reduced by only 29.9% and 38.7% at 5 g/L and 10 g/L on day 7, respectively. Taghvaei et al. (2014) added olive leaf extract into soybean oil in comparison with BHT. They found that soybean oil containing 200 mg/kg BHT had better oxidation stability than that with 364.6 mg/kg olive leaf extract. BHT is a lipophilic organic compound, but most hydrolysates from CGM and DDGS have low solubility in oil system, which limits their oxidation protection performances. The complex mixture of various peptide compounds of hydrolysates may be another factor causing the relatively lower efficiency in preventing oil oxidation. TBARS values of all the oil samples gradually increased during storage (Fig.4.2). Treatment with 10 g/L of CPH-N was also the most efficient hydrolysate with highest reduction of TBARS value (27.1%) after 7 days storage comparing with other hydrolysates, which agreed with the result of PV. Overall, all hydrolysates exhibited promising antioxidant properties in corn oil with good

inhibition rate in both PV and TBARS. Higher dosage of hydrolysates (10 g/L) in oils had better oxidation prevention performance than those with lower amount of hydrolysates (5 g/L).

The PV and TBARS value of fish oils containing two level of hydrolysates (25 and 50 g/L) were also evaluated (Fig 4.3, Fig. 4.4). Addition of 50 g/L CPH-N or DPH-N into fish oil led to about 29% reduction of PV value compared with the control after 7 days incubation (Fig 4.3 B, D). CPH-A was not as efficient as other three hydrolysates, and it reduced lipid oxidation only by 9.4% at 25g/L and 11.6% at 50 g/L (Fig 4.3 A). TBARS values of fish oil mostly increased with storage time (Fig 4.4), while the TBARS of fish oil containing 25 and 50 g/L DPH-A and 50 g/L DPH-N decreased slightly after day 5 (Fig 4.4 C, D). This may be due to the formation of carboxylic acids and oxidation of the secondary autoxidation products (Gordon, 1997; Akoh & Min, 2008). Both PV and TBARS results revealed that higher concentration of CGM and DDGS hydrolysates resulted in better prevention of lipid oxidation.

4.4.2 Inhibition of lipid oxidation in ground pork system

The inhibition performance of CGM and DDGS hydrolysates on lipid oxidation in ground pork was also evaluated based on TBARS value. As reflected by TBARS results (Fig. 4.5), oxidation of lipid in gourd pork gradually increased from day 0 to day 8, but sharply increased after day 8 until the end of incubation. TBARS value of the control (no antioxidant additive) was dramatically increased from 20.48 ± 2.76 mg MDA equiv./kg on day 0 to 60.44 ± 1.80 mg MDA equiv./kg on day 16. The treatment containing 1 and 2g/kg CPH-A greatly reduced lipid oxidation by 36.7% and 44.5% after 16 days storage, respectively. With 1 g/kg of CPH-N, lipid oxidation was inhibited by 23.0%, and the inhibitory rate reached 50.6% when increasing the dosage to 2 g/kg. Lipid oxidation was reduced by 39.6% and 46.6% with 1g/kg

and 2g/kg of DPH-A, respectively, and the reduction rate for DPH-N was 23.9% with 1 g/kg and 34.01% with 2 g/kg. The result indicated that the antioxidative hydrolysate was more efficient when added at higher level, and Neutrase-hydrolyzed CGM at 2 g/kg was the most efficient among all the hydrolysates. Hogan et al. (2009) also found that TBARS value of cooked ground beef with milk protein hydrolysate was much lower at 800 μg/g than at 200 μg/g after 15 days storage. In addition, Oliveria et al. (2014) reported that adding 10 mg/mL of soy protein hydrolysate was more efficient than 2 mg/mL to prevent oxidation in both fresh pork and salmon. Zhou, Sun & Canning (2012) selected 5 different fractions from corn protein hydrolysates produced by Validase, Alcalase and Neutrase and applied in fresh beef at different amounts, while only the 1-3 kDa fraction produced with Neutrase inhibited oxidation of ground beef at both 250 mg/kg and 500 mg/kg additions. Overall, all hydrolysates form CGM and DDGS showed antioxidant potential to retard lipid oxidation in ground pork.

4.4.3 Inhibition of lipid oxidation in canine pet food and pig feed systems

Pet food and animal feed are fat-containing products, and dietary lipids can vary from 5 to 40% in these diet (Glodde, et al., 2018). Prevention of lipid oxidation is necessary for animal performance, health, as well as maintaining the quality of animal products. Canine pet food and pig feed were selected as two model systems to evaluate the effects of CGM and DDGS hydrolysates on their oxidative stability based on TBARS. As shown in Fig 4.6, TBARS values sharply increased from day 0 to day 2 and then gradually decreased for pet food samples. The results showed that DPH-A had the highest efficiency on retarding lipid oxidation of pet food with the highest inhibition rate of 37.8% at 2% compared with the control after 5 days incubation (Fig 4.6 C). CPH-N was less efficient compared with other hydrolysates with reduction of

around 26% at both 1 and 2% additions (Fig 4.6 D). Higher dosages of all the hydrolysates did not obviously enhance the prevention of lipid oxidation in this pet food system. Other natural antioxidants also increased the oxidation stability of pet foods, such as cranberry extracts (Karthirvel, Gong, & Richards, 2009), curcumin (Wang et al., 2012), and pomegranate (Glodde et al., 2018; Kanatt, Chander, & Sharma, 2009), but studies using food protein hydrostats as an additive in pet food are very limited.

TBARS values of pig feed with 0.5 and 2% hydrolysates are shown in Fig 4.7. TBARS values increased with prolonged incubation time, and feed with 2% hydrolysates had lower TBARS values than that with 0.5% after 5 days storage. TBARS value of the control sharply increased and reached peak on day 3 with 1.46 mg MDA equiv./kg in the pig feed. Highest inhibition of lipid oxidation was observed with reduction of 43.3% on day 3 for pig feed with 2% CPH-N. Soladoye et al. (2015) also reported that DDGS inhibited the oxidation of poly-unsaturated fatty acids in pig feed. For meat animals, such as fish, pig, chicken and cattle, adding natural antioxidants to the feed not only enhances its oxidation stability, but also improves the nutritional value and quality of meat products (Jiang & Xiong, 2016; Kasapidou et al., 2012). Adding rosemary extract or thyme into the diet of lamp showed improved oxidation stability of the meat and reduction of meat color deterioration (Nieto et al., 2010; Serrano, Jordan, & Banon, 2014). According to Liu et. al (2015), mice treated with 1000 mg/kg CGM hydrolysate suspension daily for 10 days showed lower MDA equivalent in plasma and with value of 9.78 ±0.01 nmol /mL in plasma, while MDA equivalent in the control group without any treatment was 18.52±7.52 nmol/L. These results indicated that protein hydrolysates including CGM and DDGS hydrolysates could be used as a natural antioxidant in pet food and animal feed not only inhibiting lipid oxidation but also potentially improving the health of animals.

4.5 Conclusion

CGM and DDGS hydrolysates produced with Alcalase and Neutrase showed promising antioxidant performances. Our study provided evidences that the hydrolysates from corn by-products can enhance oxidation stability in multiple food models (i.e., bulk oils, ground meat) by effectively retarding the production oxidation products. The hydrolysates also demonstrated antioxidant performances in pet food and feed containing fish oil; however, their performances need to be further enhanced. Further study could focus on the purification, identification, and modification of the specific antioxidant peptides in those hydrolysates, and *in vivo* evaluation can also be conducted in order to better utilize enzymatically generated peptides and hydrolysates in more food, pet food and animal feed products.

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proteases and ultrafiltration on free radicals and meat lipid oxidation. *Food Science and Technology*, 50, 331-335.

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Table 4.1 – Ingredients and formulation of canine pet food kibble and phase 1 nursery pig feed pellet

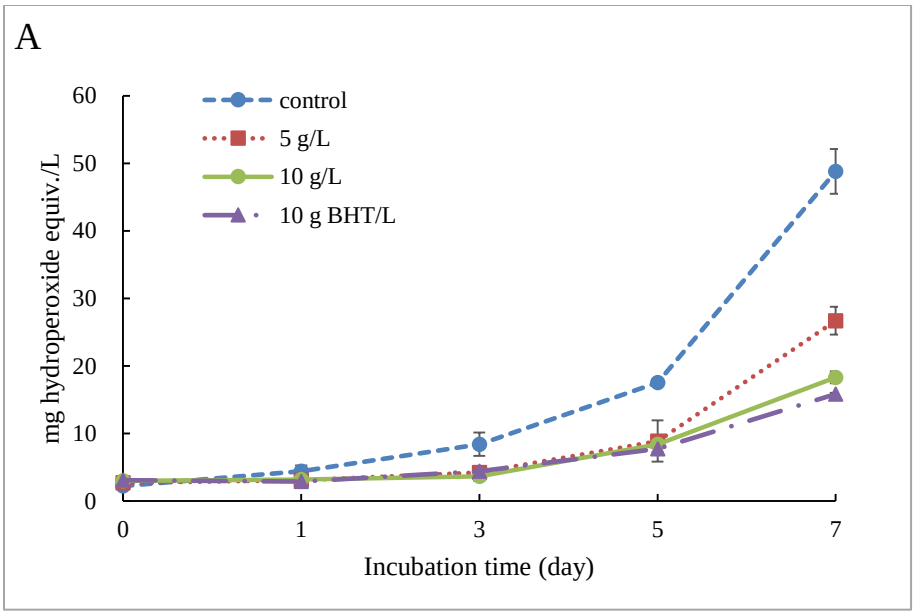
Canine pet food kibble	**Percentage**
Chicken meal (low ash)	41.9%
Brewer rice	36.8%
Corn (8.8% CP)	12.6%
Beet pulp	5.3%
Dicakcium phosphate (feed grade)	1.6%
Sodium chloride	0.6%
Potassium chloride	0.6%
TN Dog & Cat TM sulfate premix	0.2%
TN Dog & Cat VIT permix	0.2%
Choline chloride (60%, dry)	0.1%
Nursey pig feed pellet	
Corn	44.0%
Soyben meal	18.1%
Fish meal combined	6.0%
milk whey powder	12.5%
White crease	3.0%
Calcium phosphate	0.8%
Limestone (ground)	0.4%
Sodium chloride	0.4%
L-Lys-Hcl	0.4%
DL-Met	0.2%
L-Thr	0.2%
L-Trp	0.1%
L-Val	0.1%
Trace mineral premix	0.2%
Vitamin prtemix (no phytate)	0.3%
Chloride (60%）	0.0%
Ronozyme HiPhos 2700	0.0%
HP 300 loading	6.0%
Dlac 80	7.5%

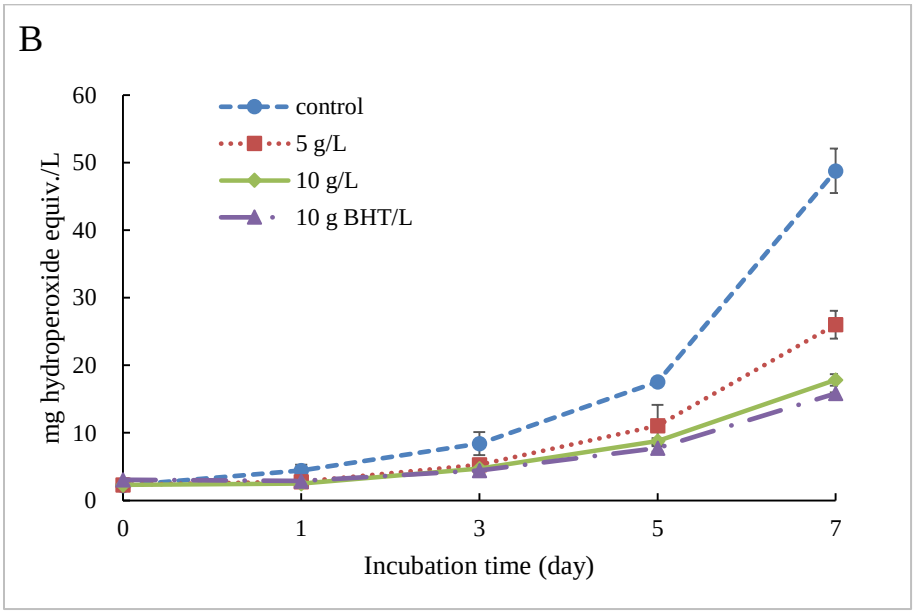
Table 4.2 – Hydrolysis conditions, antioxidant yield, and DPPH scavenging activity of CGM and DDGS hydrolysates.

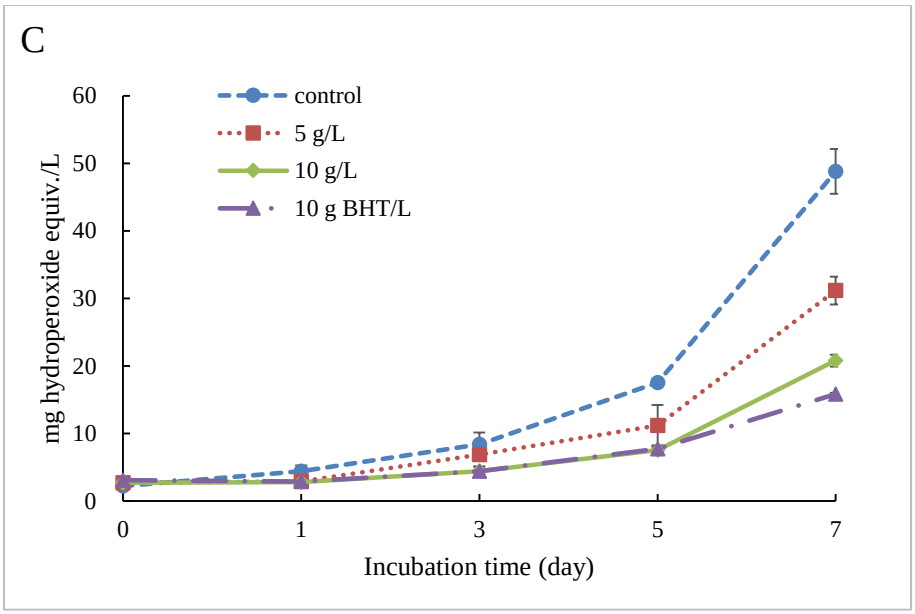
Antioxidant*	Substrates	Enzyme	Enzyme unit	Enzyme ratio	pH	Temperature (°C)	Time (h)	Yield (%)	DPPH (%)
CPH-A	CGM	Alcalase	2.4 U/g	0.4 Au/g	8.0	50	4	55.0 ± 1.0^{b}	81.4 ± 1.7^{a}
CPH-N	CGM	Neutrase	0.8 U/g	0.4 Au/g	6.0	45	4	62.9 ± 1.1^{a}	73.6 ± 1.1^{b}
DPH-A	DDGS	Alcalase	2.4 U/g	0.8 Au/g	8.0	50	4	54.4 ± 0.1^{b}	37.4 ± 1.0^{c}
DPH-N	DDGS	Neutrase	0.8 U/g	0.8 Au/g	6.0	45	4	48.4 ± 0.5^{c}	32.0 ± 0.7^{d}

Different lowercase letters indicate significant difference at $p < 0.05$.

*Alcalase-hydrolyzed CGM (CPH-A); Neutrase-hydrolyzed CGM (CPH-N); Alcalase-hydrolyzed DDGS (DPH-A); Neutrase-hydrolyzed DDGS (DPH-N).


A
control
5 g/L
10 g/L
10 g BHT/L
mg hydroperoxide equiv./L
60
50
40
30
20
10
0
0
1
3
5
7
Incubation time (day)


B
control
5 g/L
10 g/L
10 g BHT/L
mg hydroperoxide equiv./L
60
50
40
30
20
10
0
0
1
3
5
7
Incubation time (day)



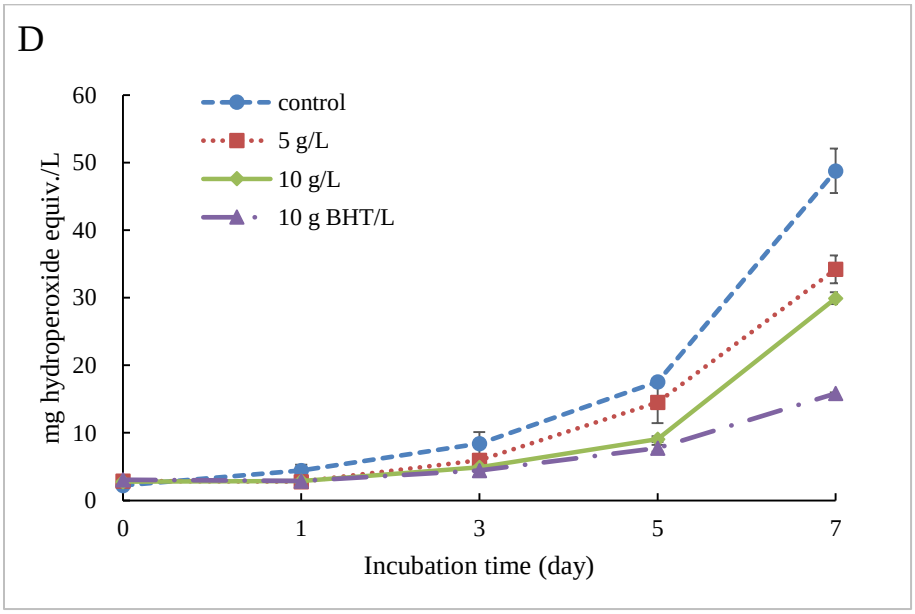
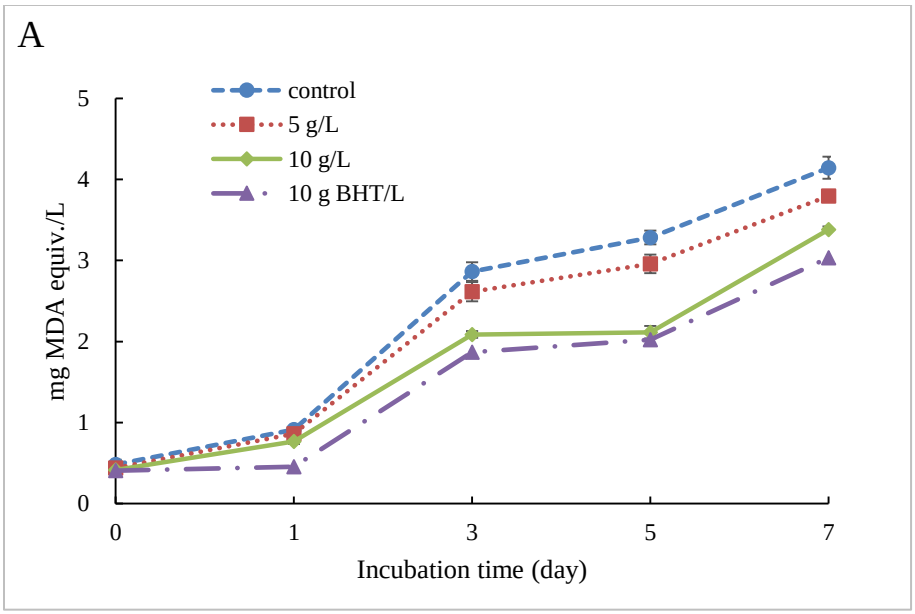
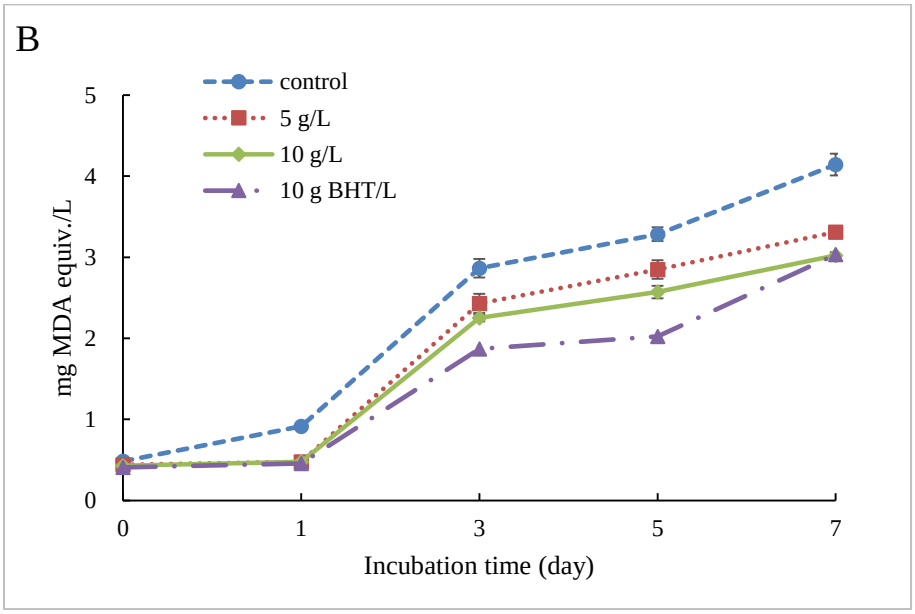
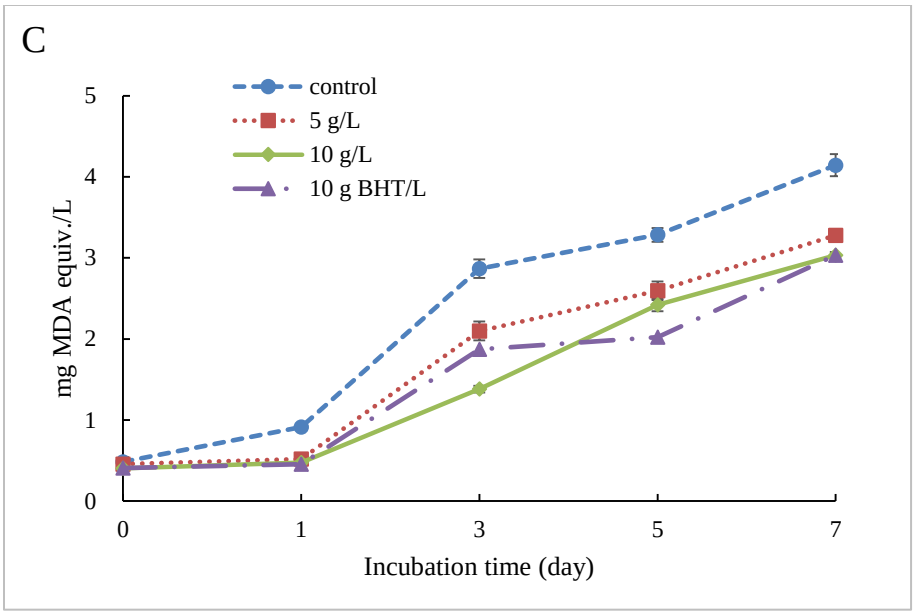


Figure 4.1 – PV of corn oil with hydrolysates at 5 and 10 g/L, and BHT at 10 g/L. (A) Alcalase-hydrolyzed CGM; (B) Neutrase-hydrolyzed CGM; (C) Alcalase-hydrolyzed DDGS; and (D) Neutrase-hydrolyzed DDGS.


A
control
5 g/L
10 g/L
10 g BHT/L
mg MDA equiv./L
0
1
2
3
4
5
0
1
3
5
7
Incubation time (day)


B
control
5 g/L
10 g/L
10 g BHT/L
mg MDA equiv./L
0
1
2
3
4
5
0
1
3
5
7
Incubation time (day)



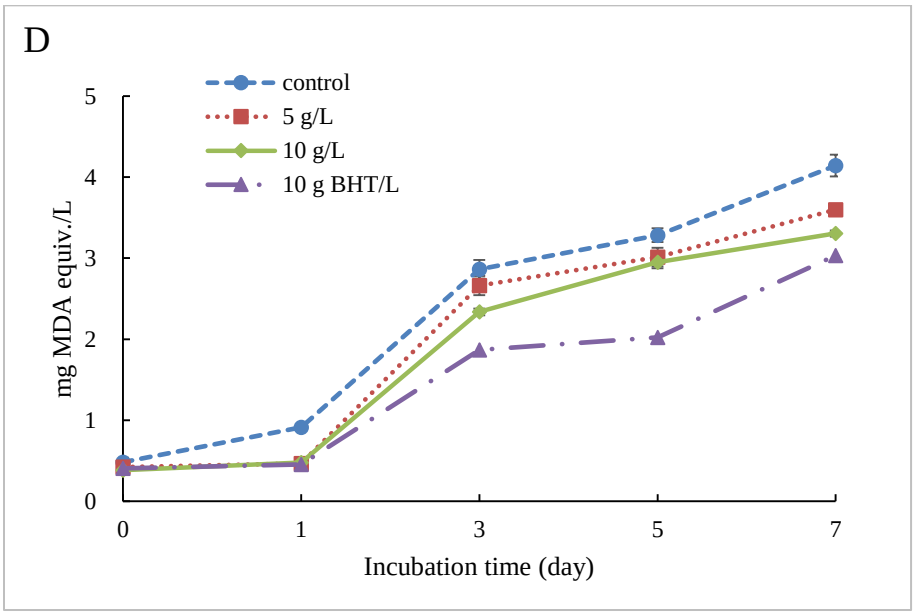
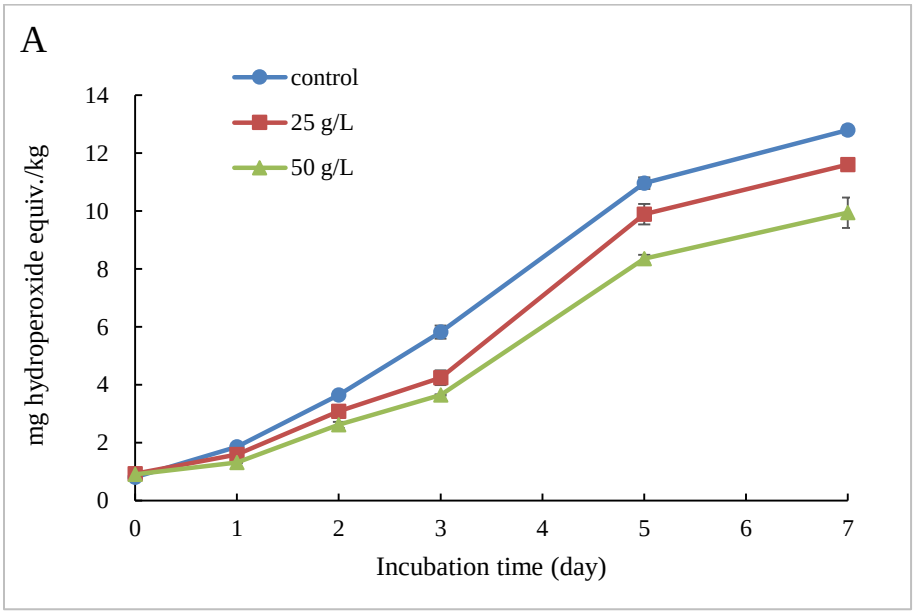
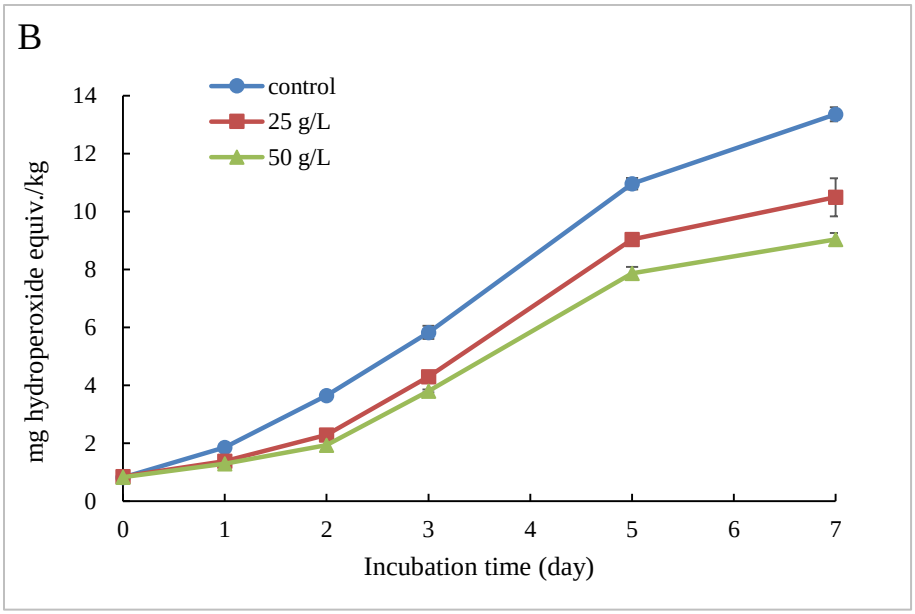
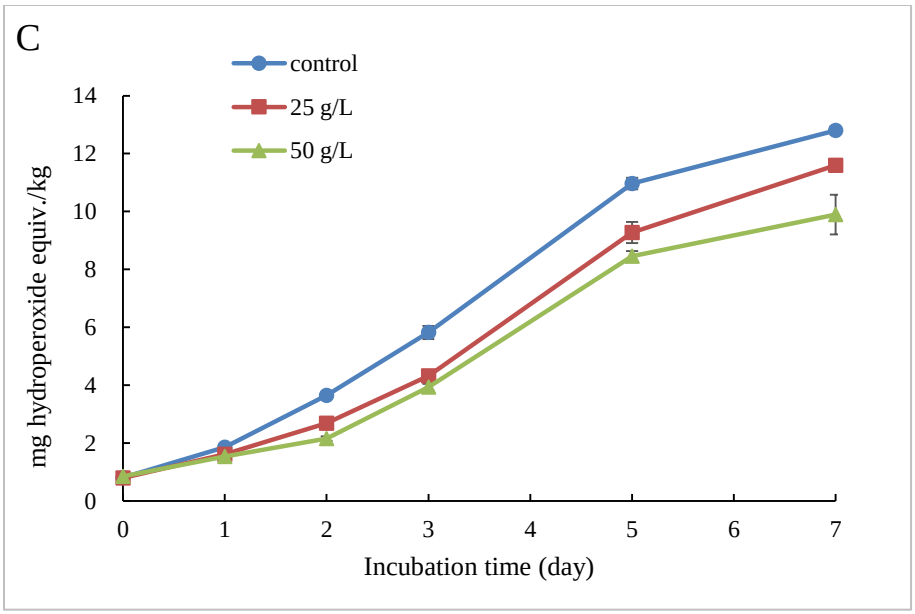


Figure 4.2 – TBARS value of corn oil with hydrolysates at 5 and 10 g/L, and BHT at 10 g/L. (A) Alcalase-hydrolyzed CGM; (B) Neutrase-hydrolyzed CGM; (C) Alcalase-hydrolyzed DDGS; and (D) Neutrase-hydrolyzed DDGS.


A
control
25 g/L
50 g/L
mg hydroperoxide equiv./kg
0
2
4
6
8
10
12
14
0
1
2
3
4
5
6
7
Incubation time (day)


B
control
25 g/L
50 g/L
mg hydroperoxide equiv./kg
0
2
4
6
8
10
12
14
0
1
2
3
4
5
6
7
Incubation time (day)



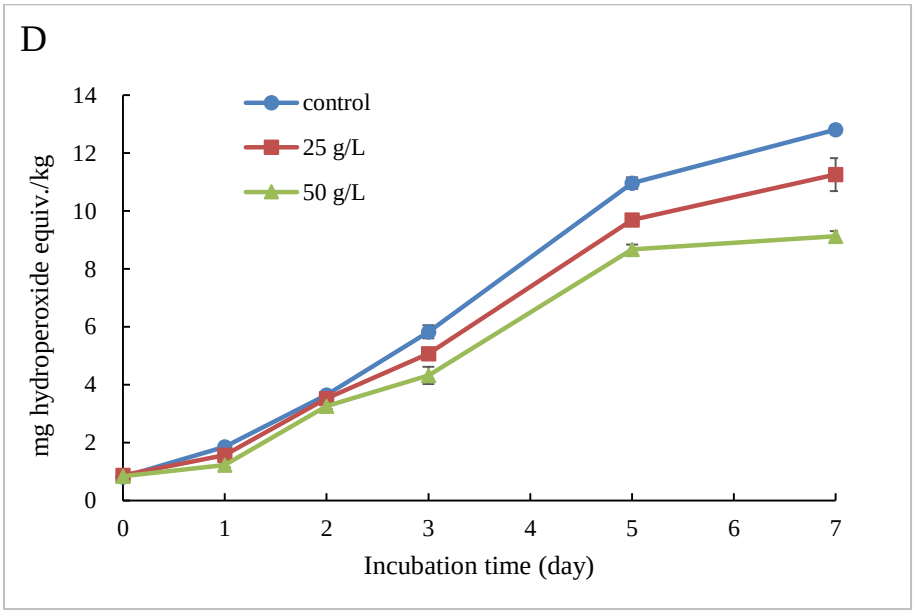
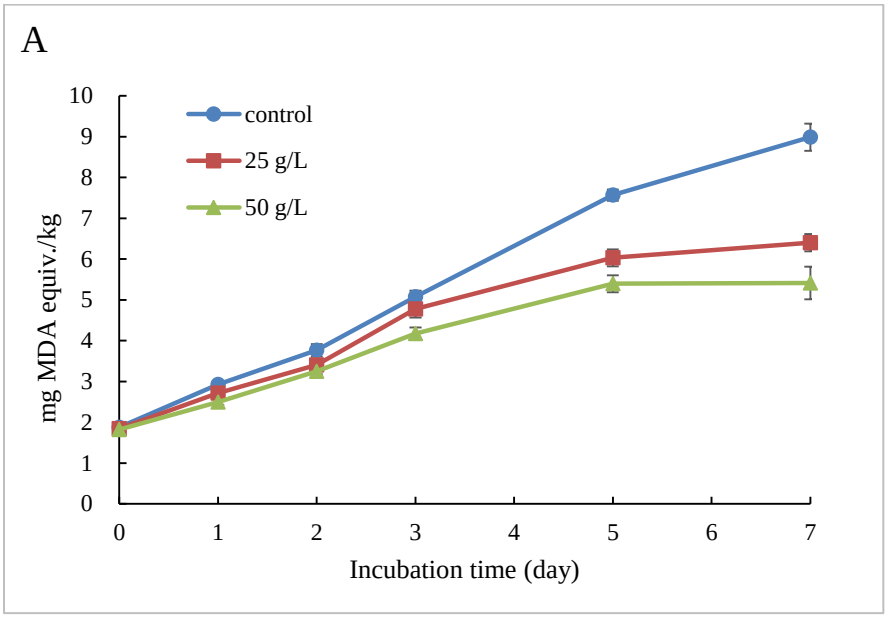
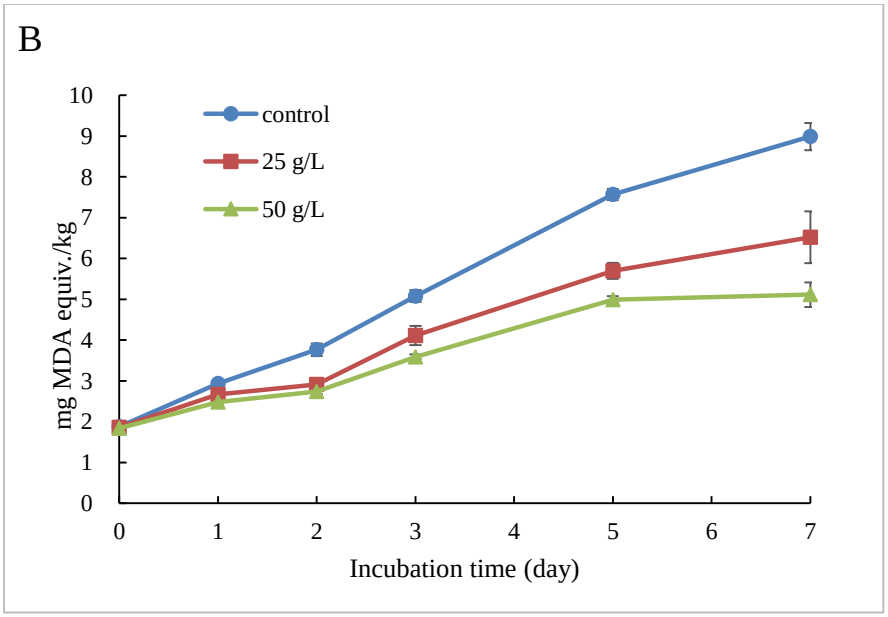
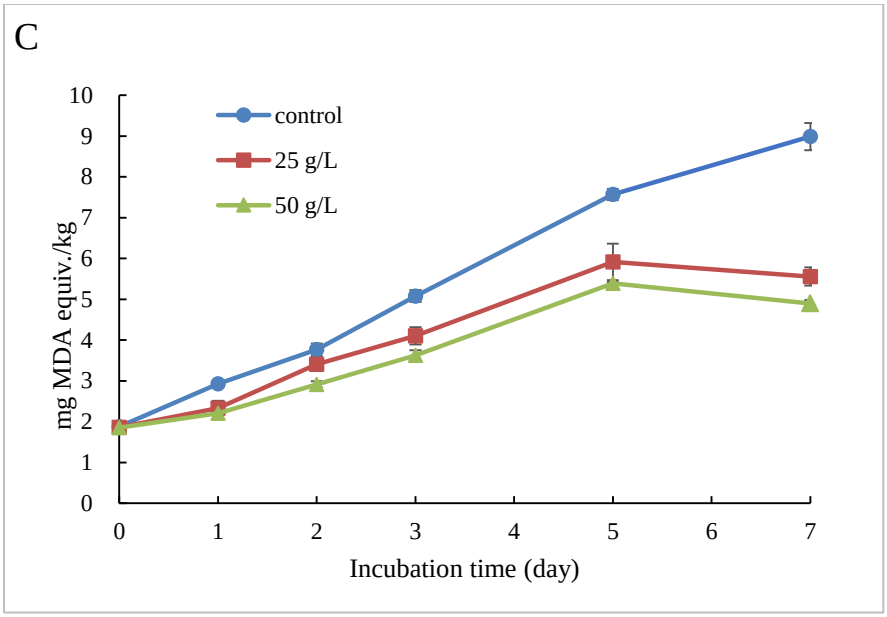


Figure 4.3 – PV of fish oil with hydrolysates at 25 and 50 g/L. (A) Alcalase-hydrolyzed CGM; (B) Neutrase-hydrolyzed CGM; (C) Alcalase-hydrolyzed DDGS; and (D) Neutrase-hydrolyzed DDGS.


A
control
25 g/L
50 g/L
mg MDA equiv./kg
Incubation time (day)
0
1
2
3
4
5
6
7
8
9
10


B
control
25 g/L
50 g/L
mg MDA equiv./kg
Incubation time (day)
0
1
2
3
4
5
6
7
8
9
10



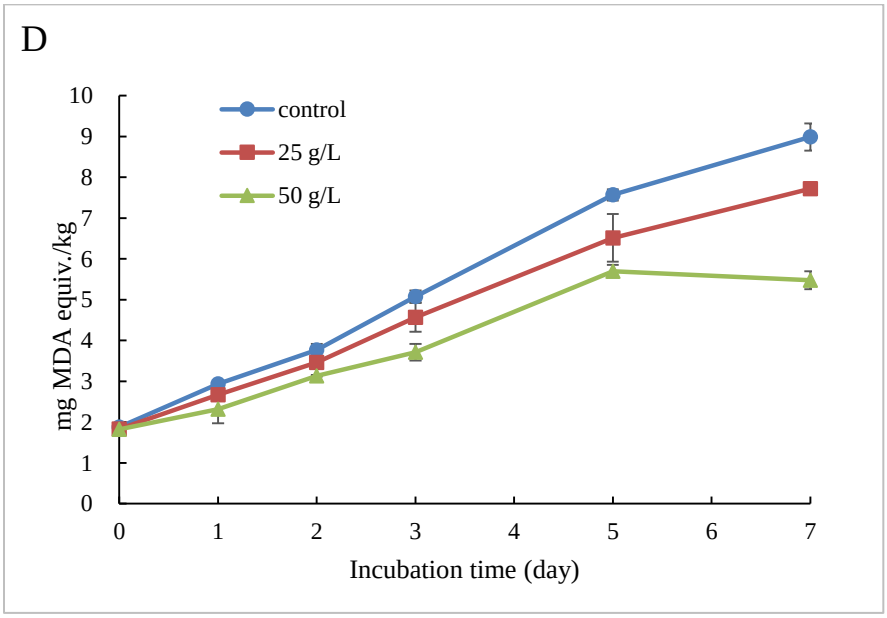
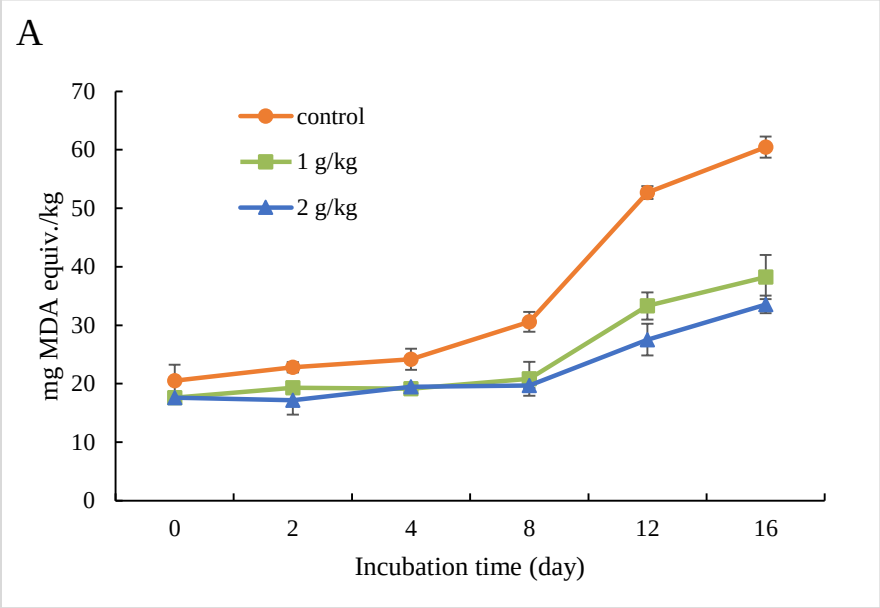
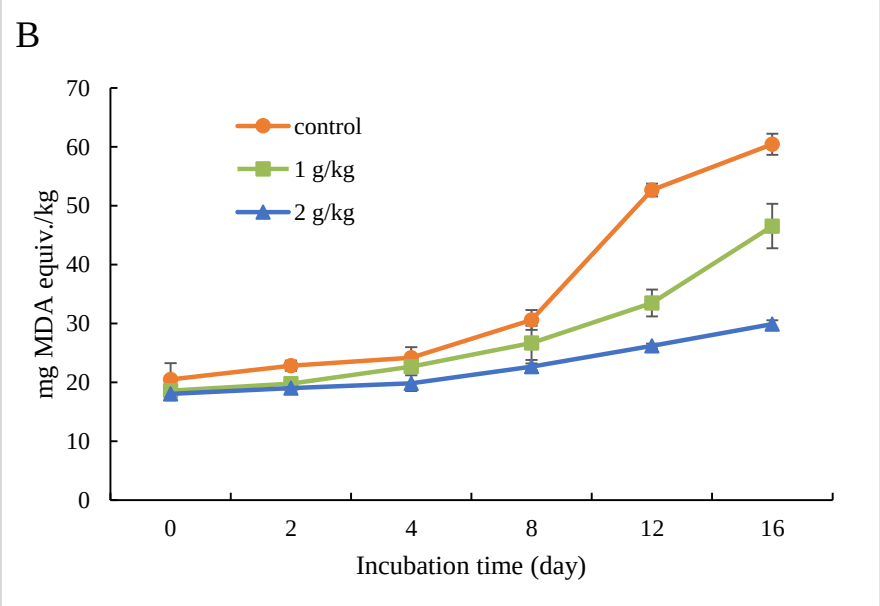
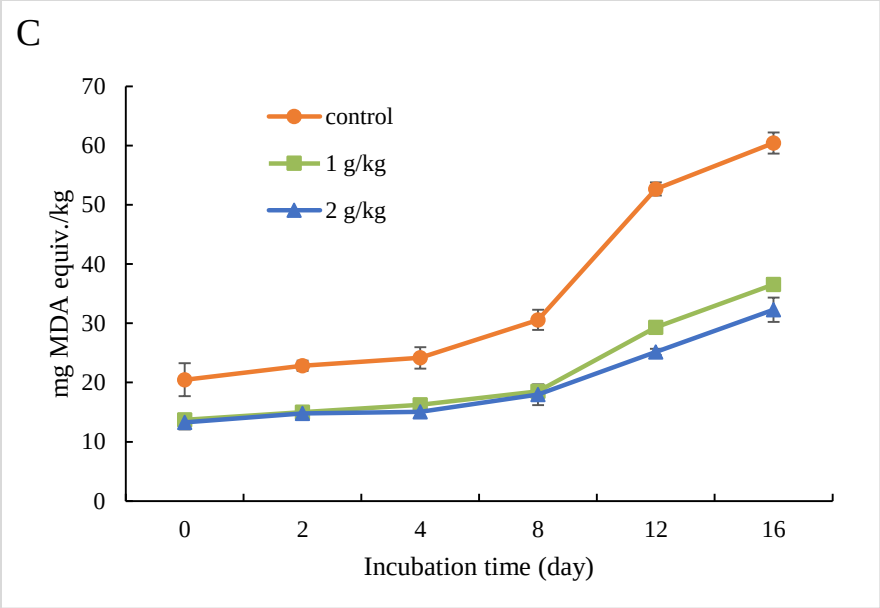


Figure 4.4 – TBARS value of fish oil with hydrolysates at 25 and 50 g/L. (A) Alcalase-hydrolyzed CGM; (B) Neutrase-hydrolyzed CGM; (C) Alcalase-hydrolyzed DDGS; and (D) Neutrase-hydrolyzed DDGS.


A
70
60
50
40
30
20
10
0
mg MDA equiv./kg
control
1 g/kg
2 g/kg
0
2
4
8
12
16
Incubation time (day)


B
70
60
50
40
30
20
10
0
mg MDA equiv./kg
control
1 g/kg
2 g/kg
0
2
4
8
12
16
Incubation time (day)



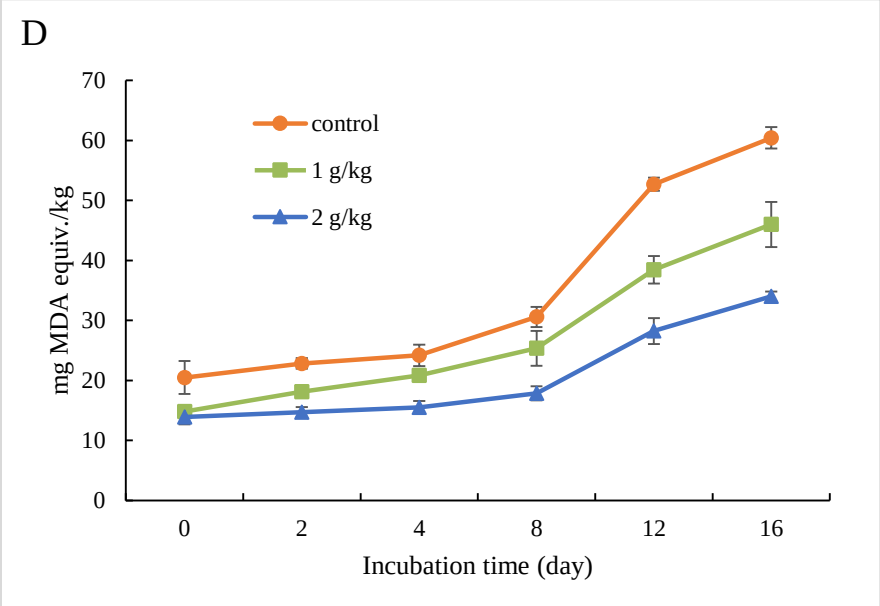
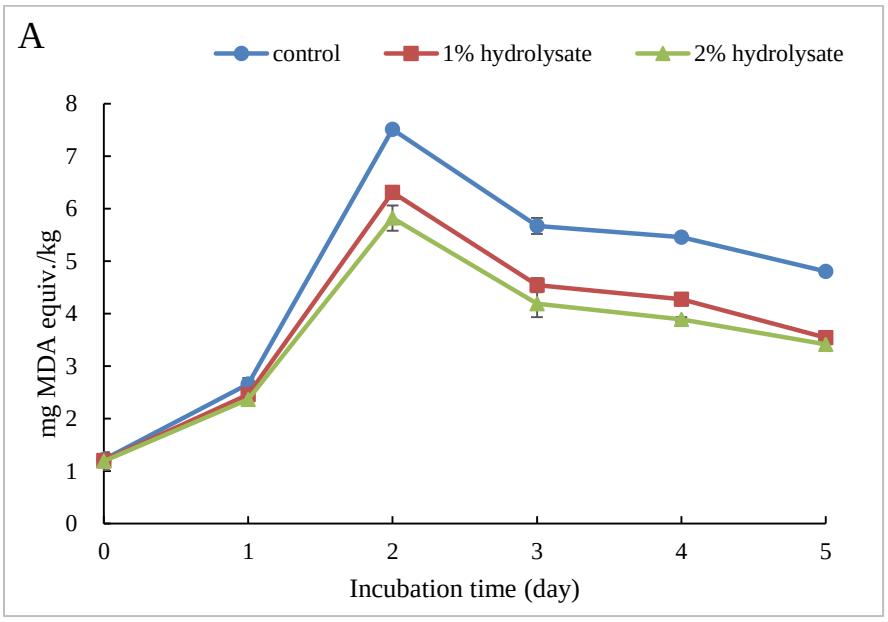
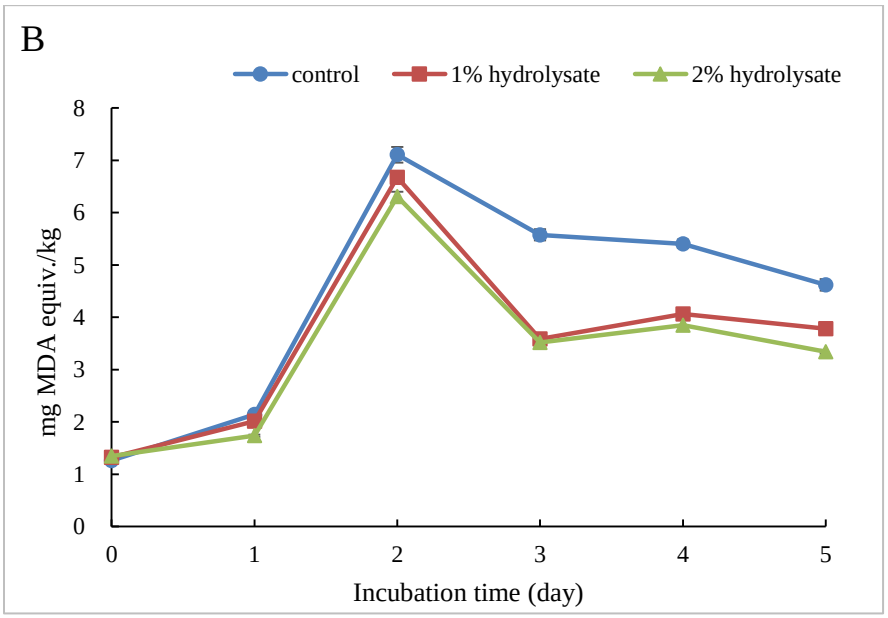
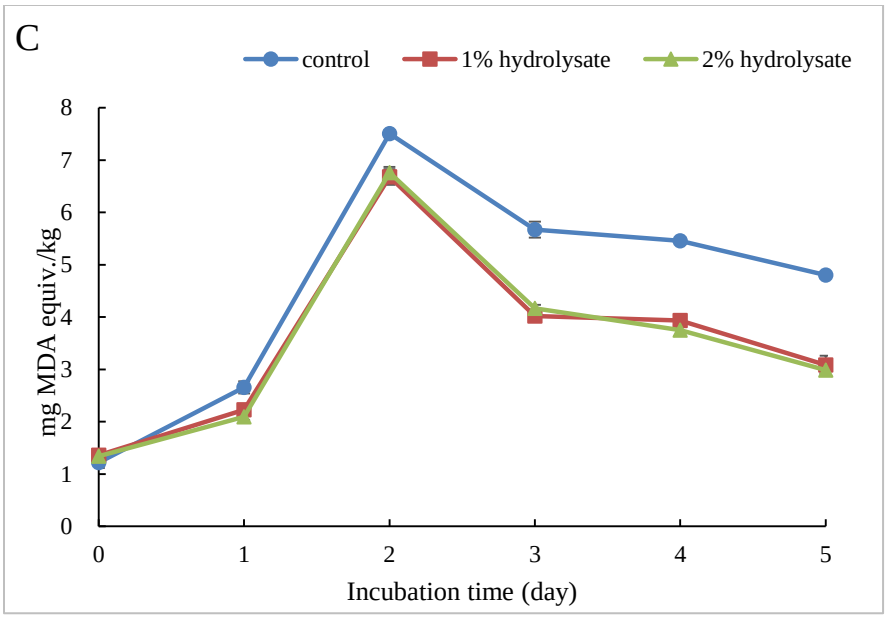


Figure 4.5 – TBARS value of ground pork with hydrolysates at 1 and 2 g/kg. (A) Alcalase-hydrolyzed CGM; (B) Neutrase-hydrolyzed CGM; (C) Alcalase-hydrolyzed DDGS; and (D) Neutrase-hydrolyzed DDGS.


A
control
1% hydrolysate
2% hydrolysate
mg MDA equiv./kg
0
1
2
3
4
5
6
7
8
0
1
2
3
4
5
Incubation time (day)


B
control
1% hydrolysate
2% hydrolysate
mg MDA equiv./kg
0
1
2
3
4
5
6
7
8
0
1
2
3
4
5
Incubation time (day)



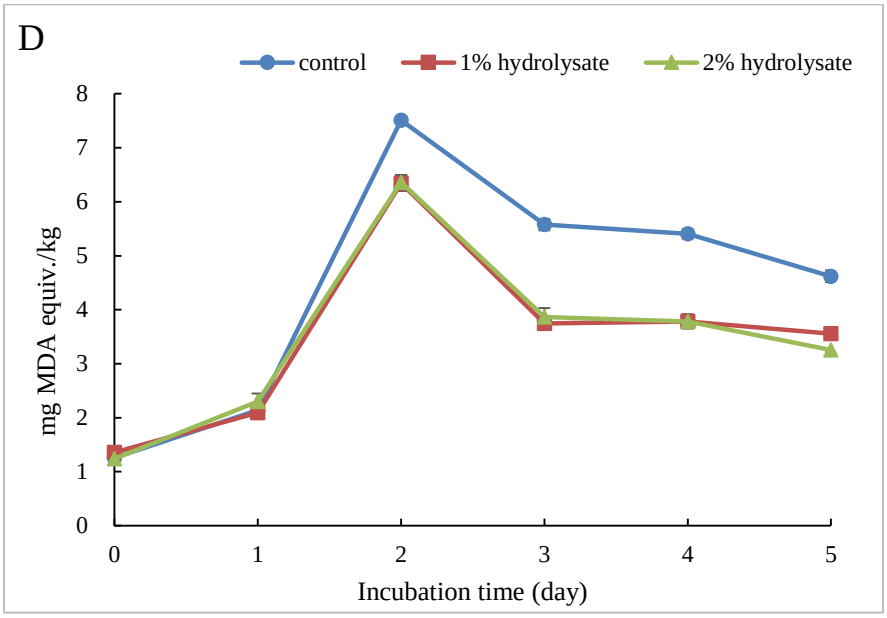
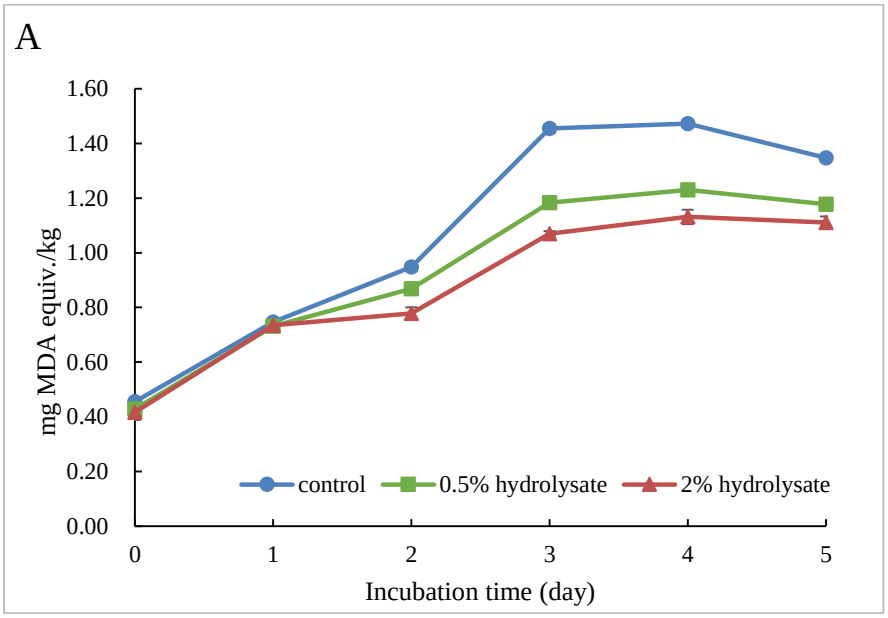
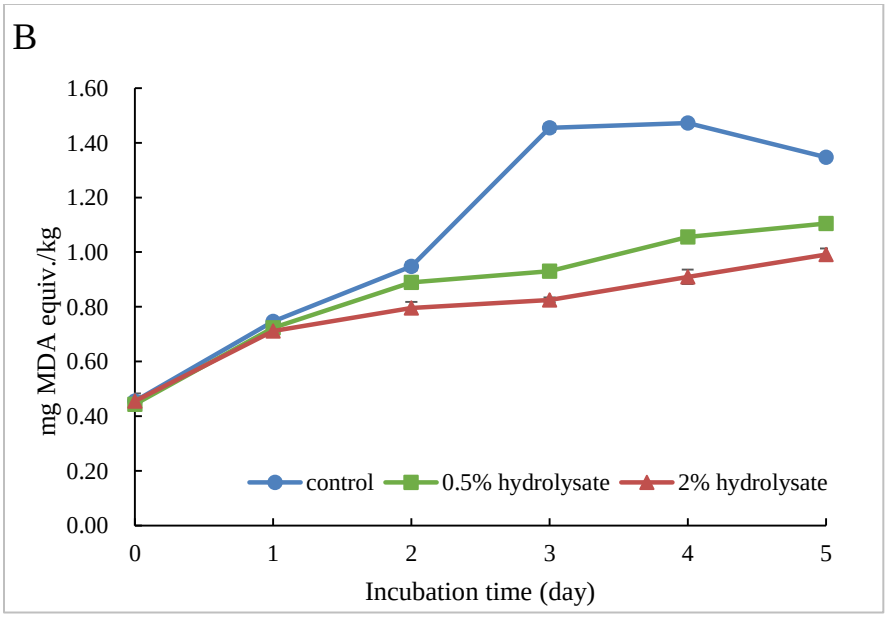


Figure 4.6 – TBARS value of cannie pet food with 1 and 2% hydrolysates (dry basis). (A) Alcalase-hydrolyzed CGM; (B) Neutrase-hydrolyzed CGM; (C) Alcalase-hydrolyzed DDGS; and (D) Neutrase-hydrolyzed DDGS.


A
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
mg MDA equiv./kg
control
0.5% hydrolysate
2% hydrolysate
0
1
2
3
4
5
Incubation time (day)


B
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
mg MDA equiv./kg
control
0.5% hydrolysate
2% hydrolysate
0
1
2
3
4
5
Incubation time (day)

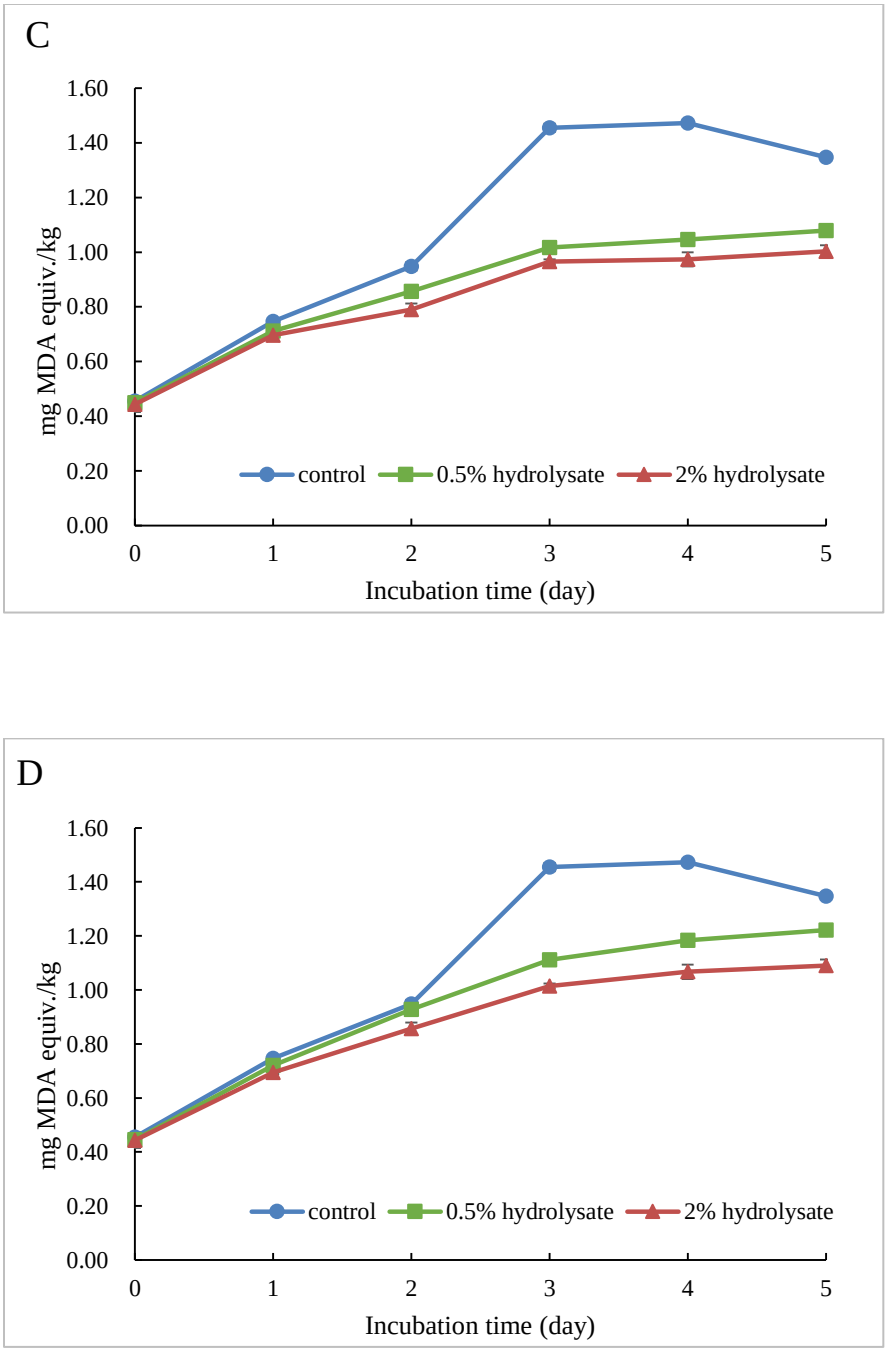


Figure 4.7 – TBARS value of pig feed with 0.5 and 2% hydrolysates (dry basis). (A) Alcalase-hydrolyzed CGM; (B) Neutrase-hydrolyzed CGM; (C) Alcalase-hydrolyzed DDGS; and (D) Neutrase-hydrolyzed DDGS.

Chapter 5 - Conclusions and Recommendations

5.1 Conclusions

Enzymatic hydrolysis of CGM and DDGS produced protein hydrolysates with desirable antioxidant properties and oxidation-inhibition performances in several oil-rich model systems. CGM was hydrolyzed by different types of enzymes including Neutrase, Alcalase and three plant enzymes (papain, ficin, and bromelain), and DDGS was hydrolyzed by Alcalase and Neutrase. In order to achieve a good balance of antioxidant yield, degree of hydrolysis as well as antioxidant activities of hydrolysates prepared using different enzymes, the enzyme specificity was a critical factor that should be carefully selected. Neutrase-hydrolyzed CGM was prepared under various reaction conditions, and the results showed that the enzyme-to-substrate ratio and reaction time were other two key factors dominating the yield and antioxidant activities of hydrolysates.

The hydrolysates were ultrafiltrated into different peptide fractions with different MW ranges. Among all the Neutrase-hydrolyzed CGM fractions, the fraction with MW of 3-5 kDa exhibited desirable antioxidant penitential according to DPPH scavenging activity, ABTS activity as well as metal chelating ability. The antioxidant activities of CGM hydrolysates were MW dependent. Among all the hydrolysates generated from CGM with three plant enzymes, the fraction with MW between 5-10 kDa produced by papain, fraction with MW below 1 kDa produced by ficin, and fraction with MW between 3-5 kDa produced by bromelain showed the strongest antioxidant activity and yield, respectively. The results demonstrated that low or medium size peptide fractions generally exhibited higher antioxidant activities.

Antioxidant performance of selected peptides was evaluated in several model systems. The antioxidative hydrolysates were effective in improving oxidative stability of O/W emulsion, bulk oils, and ground pork. They also demonstrated antioxidant performances in pet food and

feed containing fish oil. In addition, treatment with antioxidant CGM peptides (3-5 kDa) efficiently prevented the growth of HepG2 cancer cells with no toxicity. Overall, our study found that hydrolysates produced from corn co-produces have potential to be used as alternative antioxidants that can be added into food, feed and pet food products to improve quality and shelf stability by effectively retarding the production oxidation products, and they also have anti-cancer potentials.

5.2 Recommendations on Future Studies

According to the experiments and results of this study, future research could focus on:

(1) Further purification of hydrolysate fractions and investigating the correlation between peptide sequences and antioxidant properties of corn protein hydrolysates produced from CGM and DDGS;

(2) Manipulation and tailoring specific peptide sequences and compositions to further improve their antioxidant capabilities. Although CGM and DDGS hydrolysates demonstrated acceptable antioxidant activities, their performances in oil-rich ingredients, food, pet food, and animal feed need to be further enhanced. These hydrolysates have relatively poor solubility in oil/fat system, and the needed doses of antioxidant peptides were higher than current natural antioxidant such as rosemary extract.

(3) Investigating anticancer mechanisms of corn protein hydrolysates. Although the hydrolysates demonstrated prevention on the growth of human hepatocarcinoma (HepG2) cell, the mechanisms are still not clear, which requires more systematic study.