ANTIOXIDANT ACTIVITY OF FLAXSEED PROTEINS AND THEIR HYDROLYSATES

By

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Dedication

''To my beloved mother, Ruqia''

''To my dear husband, Mohamed''

''To my loving children, Saad and Abdulmalik ''

ABSTRACT

Certain components of flaxseed are known to exhibit antioxidant activity. The objective of this research was to study the antioxidant activity of flaxseed proteins and protein hydrolysates. Proteins and their hydrolysates were prepared from defatted, non-defatted, and demucilaged flaxseed with and without dialysis, and the antioxidant activities were examined by DPPH scavenging assay, reducing power assay, and metal ion chelating assay. The degree of hydrolysis (DH) of the proteins using bacterial protease was higher than using trypsin. Using bacterial protease, higher DH was observed for demucilaged and defatted flaxseed protein/dialysis (DDFPD, 30% DH), defatted flaxseed protein/dialysis (DFPD, 28% DH), and non-defatted flaxseed proteins/dialysis with 14% DH, compared to flaxseed protein/non-dialysis which were DDFPND 28% DH, DFPND 25% DH, and NDFPND with 12% DH. Proteins from dialysis treatment as well as their hydrolysates showed positive effect on antioxidant activity. DDFPD hydrolysates using bacterial protease showed higher DPPH radical scavenging activity (73.23%) and reducing power activity (0.15) at the concentration of 2.5 mg/ml as well as Fe^{2+} chelating ability (75%) at of concentration 1 mg/ml. SDS-PAGE and native-PAGE results of non-hydrolyzed samples showed no change in electrophoretic behavior as a result of the treatments; a major band corresponding to MW 48 KDa and three minor bands with MW of 16, 23, and 34 KDa. SDS-PAGE of DDFPNDH and NDFPNDH hydrolysates obtained using trypsin showed one resistant band.

RÉSUMÉ

Certaines composantes des graines de lin sont reconnues pour avoir des effets antioxydants. L'objectif du projet de recherche ci-présent est d'étudier les effets antioxydants des protéines des graines de lin et de leurs hydrolysâtes. Les protéines et leurs hydrolysâtes ont été préparé à partir de graines de lin dégraissées ou natures et démucilaginées, avec et sans dialyse. Leurs effets antioxydants ont été examinés avec le test de scannage DPPH, le test du pouvoir de réduction, et le test des ions métalliques chélates. Le degré d'hydrolyse (DH) des protéines était plus élevé avec l'utilisation d'une protéase bactériale qu'avec l'utilisation de trypsine. En utilisant la protéase bactériale, un DH plus élevé a été observé avec les graines démucilaginées et dégraissées avec dialyse/protéine (DDFPD, 30% DH), les graines dégraissées avec dialyse/protéine (DFPD, 28% DH), et les graines nature avec dialyse/protéines (14% DH), comparé aux graines protéine/sans dialyse qui ont eu comme résultat DDFPND 28%, DFPND 25% DH, et NDFPND avec 12% DH. Les protéines et leurs hydrolysâtes du traitement avec dialyse ont montré qu'il y avait bel et bien un effet antioxydant. Les hydrolysâtes, du test DDFPD et de l'utilisation du protéase bactériale, ont montré un niveau d'activité de radicaux libres plus élevé (DPPH 73.23%) ainsi qu'un pouvoir de réduction de (0.15) à une concentration de 2.5 mg/ml, de même qu'une habilité au Fe^{2+} de 75% à la concentration de 1mg/ml. Les résultats de SDS-PAGE et PAGE-original des échantillons non hydrolysés montrent qu'il n'y a aucun changement dans le comportement électrophorétique résultant du traitement: une bande majeure qui correspond au MW 48 KDa et trois bandes mineures avec un MW de 16, 23, et 34 KDa. Dans les échantillons d'hydrolysâtes SDS-PAGE du DDFPNDH et les hydrolysâtes du NDFPNDH obtenus en utilisant la trypsine ont montré une bande résistante.

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In the name of Allah, the Beneficent, the Merciful

‘’And these examples we present to the people, but none will understand them except those of knowledge.’’ (43) Al-Ankabut (Holy Quran).

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ABBREVIATIONS

ABTS	2,2'-azinobis (3-ethylbenzonthiazaline-6-sulfonic acid)
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
DFPD	Defatted Flaxseed Protein Dialysis
DFPND	Defatted Flaxseed Protein Non-Dialysis
DFPDH	Defatted Flaxseed Protein Dialysis Hydrolysates
DFPNDH	Defatted Flaxseed Protein Non-Dialysis Hydrolysates
DDFPD	Demucilaged and Defatted Flaxseed Protein Dialysis
DDFPND	Demucilaged and Defatted Flaxseed Protein Non-Dialysis
DDFPDH	Demucilaged and Defatted Flaxseed Protein Dialysis Hydrolysates
DDFPNDH	Demucilaged and Defatted Flaxseed Protein Non-Dialysis Hydrolysates
NDFPD	Non-Defatted Flaxseed Protein Dialysis
NDFND	Non-Defatted Flaxseed Protein Non-Dialysis
NDFPDH	Non-Defatted Flaxseed Protein Dialysis Hydrolysates
NDFPNDH	Non- Defatted Flaxseed Protein Non-Dialysis Hydrolysates
DH	Degree of Hydrolysis
DPPH	1,1-diphenyl-2-picrylhydrazyl
FPI	Flaxseed Protein Isolates
FRAP	Ferric ion Reducing Antioxidant Power
HAT	Hydrogen Atom Transfer

LDL	Low-Density Lipoprotein
MW	Molecular Weight
OPA	O-Phthaldialdehyde
ORAC	Oxygen Radical Absorbance Capacity
PA	Phytic Acid
PAGE	Polyacrylamide Gel Electrophoresis
PG	Propyl Gallate
SDS	Sodium Dodecyl Sulphate
SDG	Secoisolariciresinoldigluocisde
SET	Single Electron Transfer
TEAC	Trolox Equivalent Antioxidant Capacity
TBHQ	Tetra-Butyl Hydroquinone

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Oxidation has adverse effects on both food quality and biological systems. In all living aerobic organisms, harmful radicals can be formed during the metabolism involving oxygen and this can result in the occurrence of certain diseases such as atherosclerosis, and cancer (Zhang *et al*., 2011). Harmful radicals react readily with other groups or substances in the body, resulting in cell or tissue injury (Frankel, 1980). In foods, the problem of oxidative reactions not only produces undesirable odors and flavours but also losses in nutritional value of food products by formation of secondary reaction products after cooking and processing.

Antioxidants include substances that can prevent food lipid oxidation by delaying deterioration, rancidity or discoloration that occurs due to oxidation; these substances can act at different levels in an oxidative series, preventing free radicals and delaying the progress of many chronic diseases (Zhang *et al*., 2011). At low concentrations, antioxidants can react with free radicals to prevent the human body from the oxidative damage. Antioxidants significantly retard or inhibit oxidative reactions in foods by scavenging free radicals, chelating agents for transition metals, and quenching singlet oxygen, leading to decrease in free radical damage in biological systems (Choe and Min, 2009).

Oxidation of food can be protected by synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG), but their safety to health has to be considered. In recent years, there has been interest in identifying and characterising the activities of non-hazardous natural antioxidants for use in food products. Tocopherol, ascorbic acid, carotenoids, flavonoids, phospholipids, proteins, and amino acids are all considered to be natural antioxidants in foodstuff (Choe and Min, 2009). Proteins have received attention for their ability to inhibit lipid oxidation; therefore use of proteins and peptides as food additives, may increase the oxidative stability of foods (Elias *et al*., 2008). In addition, bioactive peptides can delay the oxidative reactions (Sarmadi and Ismail, 2010).

1.2 Objective of Research

The overall objective of this research is to investigate the antioxidant activity of proteins isolated from flaxseed meal and the antioxidant activity of enzymatic hydrolysates of these proteins. The specific objectives of this research were:

(i) To characterize of flaxseed protein/ protein hydrolysates by Sodium dodecyl polyacrylamide gel electrophoresis (SDS- PAGE) and Native-PAGE.

(ii) To investigate the antioxidant activity of flaxseed protein and their hydrolysates by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical-scavenging activity, reducing power and metal ion chelating activity.

(iii) To investigate the effects of dialysis treatment and flaxseed mucilage removal on the antioxidant activity of flaxseed protein / protein hydrolysates.

CHAPTER 2

LITERATURE REVIEW

2.1 Flaxseed

Flaxseed (*Linum usiatissimum*) is a member of the family linaceae (Rubilar *et al*., 2010). The seed known as flaxseed or linseed (Rubilar *et al*., 2010) is flat, oval with a tip and have a crisp and chewy texture and a pleasant, nutty taste; the color of flaxseed ranges from a deep brown to a light yellow depending on the amount of pigment in the outer seed coat (Flax Council of Canada, 1998).

The terms flaxseed and linseed are used interchangeably; flaxseed refers to use it as food while the linseed is used for industrial purpose (Singh *et al*., 2011). Flaxseed consumption in different forms as a food ingredient and for its medicinal properties in ancient Egypt and Greece, dates from 5000 BC (Oomah, 2001). Since then, common flaxseed has been cultivated as an oil crop or as a fiber crop, with fiber (linen) derived from the stem of fiber varieties and oil from the seed of flaxseed varieties (Toure and Xueming, 2010).

Recently, flaxseed has become one of the most important seed crops of the world, cultivated in over 2.6 million ha. India, Canada, China, United States, and Ethiopia are the most important flaxseed growing countries (Jhala and Hall, 2010). Canada is the world's largest producer of flaxseed and accounts for approximately 80% of the global flaxseed production (Singh *et al*., 2011).

2.2 Composition of Flaxseed

Flaxseed is considered as one of the main sources of phyotochemicals in the functional food arena. Flaxseed is rich in fat, dietary fibre, and proteins. It consists of 40-50% oil and meal, 23-34% protein, 4% ash, 5% viscous fiber (mucilage), and lignan (9-3 mg/g of defatted meal) (Tour and Xueming, 2010). Canadian-grown flaxseed contains 41% fat, 28% total dietary fibre, 20% protein, 7.7% moisture, 3.5% ash, and 1% simple sugars (Morris, 2003). This diversity in composition of flaxseed depends on the variety, the cultivar, and growing conditions (Morris, 2003; Tour and Xueming, 2010). In

addition, flaxseed is one of the richest sources of α-linolenic acid oil and lignans, and is considered as an essential source of high quality protein and soluble fibers (Oomah, 2001). The composition of flaxseed is shown in Table 2.1.

Table 2.1 The composition of flaxseed (El-Ramahi, 2003; Singh *et al*., 2011)

Nutrients	Whole flaxseed (%)
Moisture	5-8.3
Lipid	30-45
Protein	20-31.6
Carbohydrate	25-35
Total Dietary Fiber	24.5-46.8
Non-soluble Fiber	30
Soluble Fiber	10
Energy, Kcal	450-530
Minerals	2.4-4
Calcium, mg	170-520
Phosphate, mg	370-650
Iron, mg	2.7-10
Potassium, mg	750
Magnesium, mg	350
Copper, mg	0.7
Zinc, mg	2.0
Manganese, mg	7.0
Carotene, µg	30
Thiamin, mg	0.23-0.6
Riboflavin, mg	0.07-0.3
Niacin, mg	1-4.4
Vitamin B6, mg	0.8
Vitamin B12, mg	0.5

2.2.1 Flaxseed Oil

Oil content in flaxseed is nearly 41% on a dry basis with low saturated fatty acids (9%) and high in polyunsaturated fatty acids approximately (72-73%) (Cunnane *et al*.,1993; Hosseinian, 2006). α-linolenic acid (ALA 18:3, ω-3) represents up to 53% of the total fatty acids in flaxseed; this makes it the leading source of plant based ω-3 fatty acids (Oomah and Mazza, 1999; Hosseinian, 2006). Singh *et al*., (2011) reported that α-linolenic acid (C18:3) was in a range of 46-58%, oleic acid 19%, palmitic acid 6%, stearic acid 4%, and linoleic acid (18:2, ω-6) 16% of the total fatty acids. Singh *et al*., (2011)

also reported that flaxseed oil contains mainly triacylglycerols (98%) with lower contents of phospholipids (0.9%) and free fatty acids (0.1%).

2.2.2 Dietary Fiber

Flaxseed mucilage is a heterogenic polysaccharide which contributes largely of soluble fiber fractions (Kishk *et al*., 2011). Flaxseed contains both soluble and non-soluble fiber, which represent about 28% of the weight of full-fat flax seeds (Hosseinian, 2006; Ayad, 2010). The ratio of soluble to non-soluble fiber in flaxseed varies from 40:60 to 20:80 (Singh *et al*., 2011). Fiber in flaxseed can also be classified as dietary fiber or functional fiber (Hosseinian, 2006; Ayad, 2010). Celluloses and lignans are the major non-soluble fiber fractions in flaxseed while mucilage gums are the major soluble fiber fractions in flaxseed (Hosseinian, 2006).

Mucilage gums are polysaccharides which become viscous when mixed with water (Singh *et al*., 2011). Polysaccharides gums are important commercially in the food industries and seed gums (guar and locust bean gums) are using as a viscosity enhances and stabilisers (Kishk *et al*., 2011).

2.2.3 Flaxseed Proteins

Flaxseed proteins have physico-chemical characteristics and functional properties as shown in Figure 2.1. The protein content of flaxseed ranges from 10-31% with an average of 22%, which makes flaxseed a good source of proteins (Oomah and Mazza, 1993; Rubilar *et al*., 2010). Flaxseed proteins have been used in many food products to enhance some functional properties; these include flaxseed protein concentrate, flaxseed meal, and flaxseed protein isolate which contain 56-66%, 20-25%, and 87% protein, respectively (Rabetafika *et al*., 2011). For example, flaxseed protein concentrate has been added to ice cream to enhance the emulsion and viscosity properties (Wang *et al*., 2010). Flaxseed proteins have been fractionated into globulin “linin” which represents 40-80%, albumin “conlinin” 20-40%, glutelin13.5%, prolamin 6.5 %, oleosin 7.2%, cd-binding protein 7% and linusitin ‹1.0% (Ayad, 2010). Table 2.2 shows amino acid composition of flaxseed.

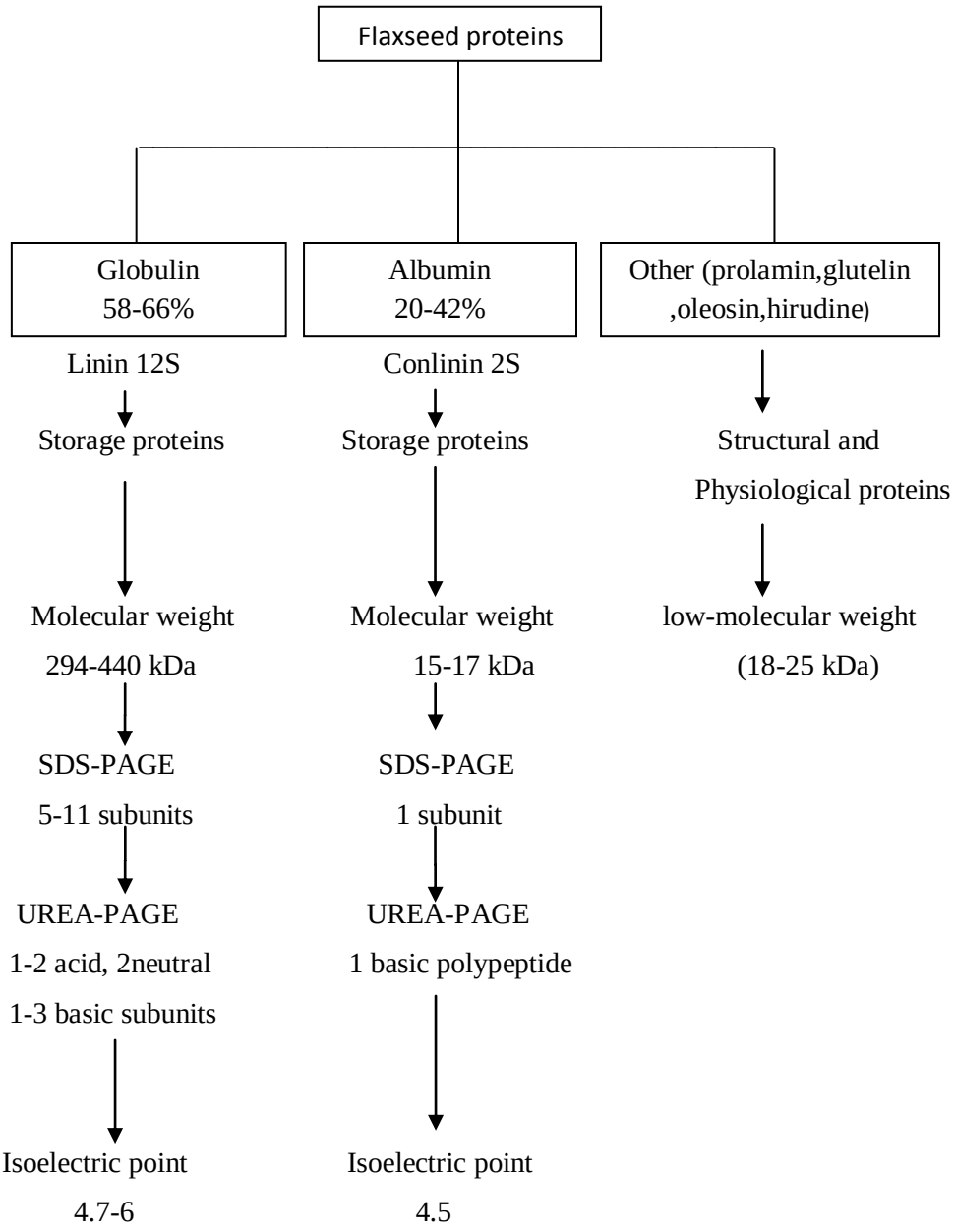

Figure 2.1 General physic-chemical characteristic of flaxseed proteins (Rabetafika *et al.*, 2011).

Table 2.2 Amino acid composition of flaxseed (Singh *et al*., 2011)

Amino Acid	Brown Flax	Yellow Flax (norlin) g/100g protein
	g amino acid/100 g protein	
Alanine	4.4	4.5
Arginine	9.2	9.4
Aspartic Acid	9.3	9.7
Cystine	1.1	1.1
Glycine	5.8	5.8
Histidine	2.2	2.3
Isoleucine	4.0	4.0
Leucine	5.8	5.9
Lysine	4.0	3.9
Methionine	1.5	1.4
Phenylalanine	4.6	4.7
Proline	3.5	3.5
Serine	4.5	4.6
Threinine	3.6	3.7
Tryptophan	1.8	NR[a]
Tyrosine	2.3	2.3
Valin	4.6	4.7

[a] Not Reported

2.2.3.1 Isolation and Characterization of Flaxseed Proteins

Several methods have been used for the isolation of flaxseed proteins contents (Ayad, 2010). The first method to isolate flaxseed protein was in 1892 by Osborne using salt extraction and precipitation of the protein by removal of the salt by dialysis. Ayad, (2010) reported that flaxseed protein fraction showed two intense bands for the albumin fraction with 22 and 24 KDa, for globulin - one band with molecular weight 23 KDa, and for glutelin - two intense bands at 22 and 35 KDa, by using sequential solvent extraction. Chung *et al*. (2005) reported that proteins from defatted flaxseed meal gave one a major band with molecular weight 365 KDa and five other predominant bands with molecular weights 20, 23 and 31 KDa and 40 and 48 KDa, respectively when fractionated by anion exchange chromatography. Vassel and Nesbit, (1945) classified flaxseed protein into the high molecular weight 12S globulin (linin) and the low molecular weight 2S albumin (conlinin). Table 2.3 shows the characteristics of high and low molecular weight of flaxseed proteins.

Table 2.3 Characteristics of the high-and low-molecular weight flaxseed proteins (Chung, 2001)

Property	High-MW fraction	low-MW fraction
Molecular Weigh t(KDa)	252-298;320	15-18
Sedimentation velocity	11S;12S ;11.45S	2S; 1.6S
Solubility	salt	water
Sub-unit composition		
SDS-PAGE	5	1
Urea-PAGE	6	N.A
Secondary structure (%)		
Alpha-helix	3-4	26
Beta-pleat structure	17	32
Aperiodic	~80	42
Component MW (KDa)		
Total number of amino acids 100g protein	871	885

2.2.3.1.1 High Molecular Weight Protein Fraction

Vassel and Nesbitt, (1945) was attempting to isolated the 12S protein from linseed using differential isoelectric precipitation at pH 5.7. The purified 12S (globulin) was shown to be homogenous, with an isoelectric point of pH 4.75, presenting 17% nitrogen, 0.6% sulphur and 0.54% carbohydrate (Chung, 2001). Youle and Huang, (1981) reported that the solubility of the high-molecular-weight proteins fractions in water, 0.05 M NaCl and 0.5 M NaCl were 41%, 61% and 82%, respectively. Dev and Sienkiewicz, (1987) isolated the major protein fraction by various procedures and characterized it as an 11S globulin (Figure 2.2).The salt seed globulins isolated from different dicotyledonous plants have the same structural and chemical properties (Marcone *et al*., 1998).

2.2.3.1.2 Low Molecular Weight Protein Fraction

The albumin (conlinin) was isolated from a dioxane-treated glycol extract of flaxseed meal by Vassel and Nesbitt, (1945). Youle and Huang, (1981) used 0.035 M sodium phosphate buffer, pH 7.5, in 1 M NaCl to extract the 2S protein from flaxseed and then centrifuged in a sucrose gradient from 5 to 30% sucrose in the same buffer. The protein fraction (2S) of flaxseed represented 42% of the total protein, was 93% soluble in water, 97% soluble in 0.05 M NaCl and 99% soluble in 0.5 M NaCl. Madhusudhan and Singh (1985b) isolated the low molecular weight protein from defatted flaxseed meal by CM-sephadex C-50 Chromatography. The 1.6 S fraction isolated has illustrated in (Figure 2.3). Youle and Huang, (1981) and Madhusudhan and Singh (1985) studied the characterization of the low molecular weight fraction of flaxseed meal and found high amounts of glutamate, glutamine, asparagine, aspartate, arginine and cysteine.

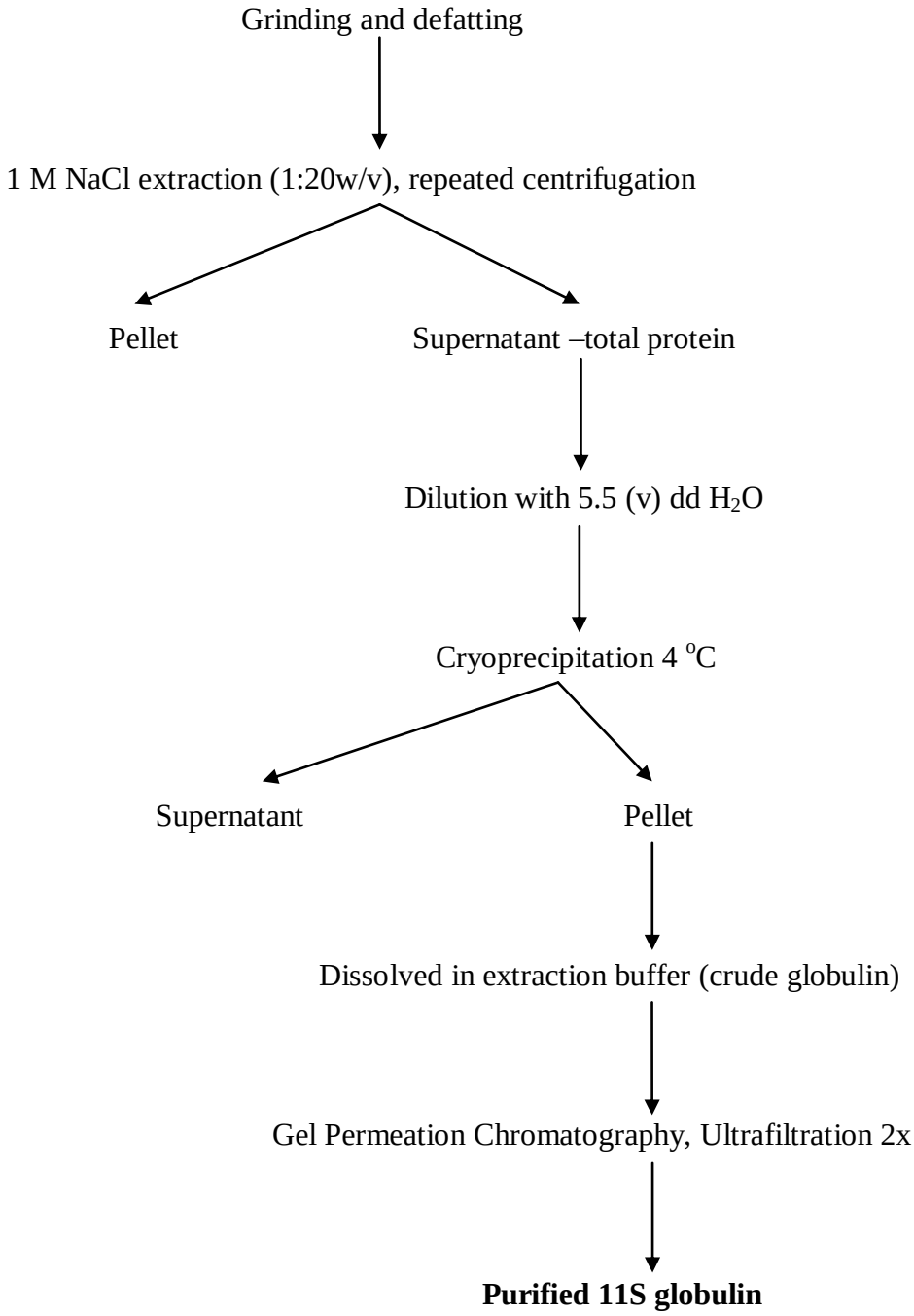

Figure 2.2 Simplified procedures for the isolation of 11S flaxseed globulin (Chung, 2001)

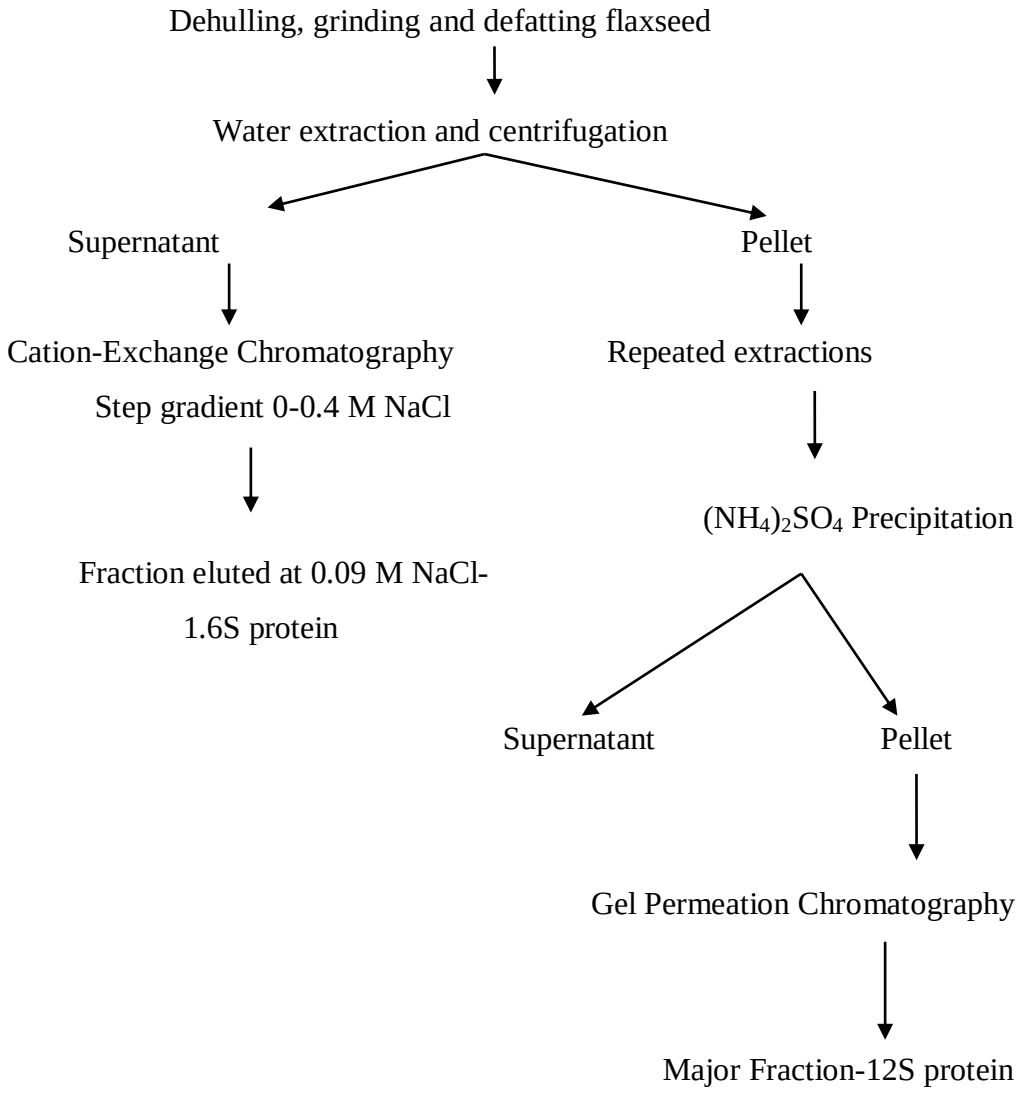

Figure 2.3 Simplified procedures for isolation of flaxseed 1.6S and 12S proteins (Chung, 2001)

2.2.3.2 Functional Properties of Flaxseed Proteins

2.2.3.2.1 Solubility

Solubility is one of the most important functional properties of proteins because it affects other functional properties of proteins (Chung, 2001). Solubility of flaxseed proteins showed a wide range of nitrogen extractability at different pH, depending on other factors such as protein concentration, salt concentration, heat treatment, solvent composition and ionic strength (Ayad, 2010). Oomah *et al*., (1994) reported that solubility of flaxseed protein in 1.28 M NaCl at pH 6.8 was 82% while the highest solubility was obtained at pH 8.0.

2.2.3.2.2 Viscosity

Most studies on viscosity of flaxseed protein focused on the viscosity contributed by the mucilage (Ayad, 2010). Madhusudhan and Singh, (1985c) measured viscosity of the isolated flaxseed proteins and found that intrinsic viscosity for the 12S linseed protein in phosphate buffer was 3.1 g/ml. Non-newtonian shear-thinning properties of the apparent viscosity depends on protein concentration, NaCl concentration, and solvent extraction whereas commercial processing (e.g. cleaning, solvent extraction, flaking, and pressing) showed no effect (Oomah and Mazza, 1998). Mueller *et al*., (2010) reported that temperature has influence on the viscosity; they found that viscosity of 20 g/L brown linseed and yellow fraction solution was 4.0-26 m, 3.8-10 pa/s respectively at a temperature of 20 ℃.

2.2.3.2.3 Foaming

The foaming capacity and stability of flaxseed isolate are 80% and 22% respectively whereas for soybean isolate, the foaming capacity and stability are 35% and 1.5%, respectfully (Chung, 2001). Wanasundara and Shahidi, (1997) reported that fractions of flaxseed protein showed higher foaming capacity compared to flaxseed protein isolate; this could be that smaller peptides are able to incorporate more air in the solution than the large one and increase the foaming capacity of the solution (Jamdar *et al*., 2010). Flaxseed protein isolate had maximum foaming capacity at pH 2 and pH 9 (El-Ramahi, 2003).

2.2.3.3 Structural properties of Flaxseed Proteins

2.2.3.3.1 Isoelectric Points of Flaxseed Proteins

Isoelectric precipitation of plant proteins is done at various pH. Chung, (2001) used pH 4.75, 4.5, and 3.55 to precipitate 12S protein, flaxseed meal protein and flaxseed extracts in sodium hexametaphosphate, respectively. The pI of the acidic and basic subunits of oat globulin is 5.9-7.2, 8.7- 9.2, respectively (Chung *et al*., 2005).

2.2.3.3.2 Hydrophobicity and Sulfhydryl (SH) and Disulfide

Wanasundara and Sshahidi, (1997) studied the effect of acylation on surface hydrophobicity of flaxseed protein isolate (FPI) by using 1-anilinonaphthalene-8-sulfonate (ANS) and reported a fluorescence intensity of the unmodified FPI is 200 units/ mg. The surface hydrophobicity of FPI was increased by alkylation (Madhusudhan and Singh, 1985a).

2.3 Antioxidant Compounds in Flaxseed

2.3.1 Phenolic Compounds and Lignans

Flaxseed has been reported to contain free phenolic acids, lignans, and glycosylated phenolic acids (Johnsson, 2004). Kozlowska *et al*., (1983) reported that the highest proportion of phenolic acids in flaxseed were ester bound and showed by extraction with 80% methanol that the amount of ester bound phenolic acids in flaxseed was 320 mg/Kg defatted flaxseed flour. Dabrowski and Sosulski, (1984) reported that the main phenolic acids in flaxseed are trans-ferulic (46%), trans-sinpaic (36%), *p*-coumaric (7.5%) and trans-caffeic (6.5%); they also reported that flaxseed has the highest amount of ferulic acid compared to other oilseeds. The ethanolic extracts of some phenolic compounds in flaxseed are associated with the role of phenolic compounds as antioxidants (Amarowicz *et al*., 1994). Table 2.4 shows the contents of phenolic compounds in different flaxseed preparations.

Table 2.4 The contents of phenolic compounds in different flaxseed preparations (Ayad, 2010)

phenolic compounds	NDFE[a] (mg/ 100g)	DFE [b] (mg/ 100g)
Ferulic acid	161	313
Coumaric acid	87	130
Caffeic acid	4	15
Chlorogenic acid	720	1435
Gallic acid	29	17
Protocatechuic acid	7	7
P-Hydroxybenzoic acid	1719	6454
Sinapic acid	18	27
Vanillin	22	42
Total	2767	8440
SDG[c]	2653	4793

[a](NDFE, non-defatted flaxseed extract);[b](DFE, defatted flaxseed extract; SDG[c], Secoisolariciresinoldigluocisde

2.3.2 Phytic Acid

Phytic acid (PA) is a common plant composition, constituting 1-5% of cereals, oilseed, nut and pollen (Sorour and Ohshima, 2010) and represent about 23-33 g/Kg dry weight of flaxseed (Ayad, 2010). PA has been reported to be antioxidant, anticarinogenic, and hypoglycaemic or hypoglycermic (Sorour and Ohshima, 2010). PA is an antioxidant agent because it is a strong chelator of mineral cation such as potassium, magnesium, and iron. Extensive research has been suggested that chelation properties of phytic acid may have some potential healthful effects such as lowering serum cholesterol and triglycerides and preventing heart disease and colon cancer (Zhou *et al*., 1995). The ability of PA to complex iron could eliminate the incidence of colon cancer on rats by reducing the amount of hydroxyl radical in the colon (Ayad, 2010).

2.4 Proteins and Peptides as Antioxidants

In recent years, many studies have been done on the ability of proteins to inhibit lipid oxidation in foods. Several food proteins have been shown to exhibit antioxidant activity in muscle foods (Elias *et al*., 2008), dairy products (Taylor and Richardson, 1980), gelatin (Wang and Xiong, 2005), soybean (Faraji *et al.*, 2004; Park *et al*., 2005), egg yolk (Sakanaka and Tachibana, 2006), and β-Lactoglobulin (Elias *et al*, 2005). Elias *et al*., (2005) reported that lipid oxidation in oil-in-water emulsions can be inhibited by whey proteins; β-Lactoglobulin showed antioxidant activity in Brij-stabilized oil-in-water emulsion. Park *et al.,* (2005, 2008) reported that soy protein inhibited the oxidation of ethyl esters of eicosapentaenoic acid in a maltodextrin-stabilized, freeze-dried emulsion in foods powder systems and that whey protein has antioxidant activity in cooked beef.

Many peptides from protein ingredients can also inhibit lipid oxidation in foods. Lipid oxidation in diverse muscle foods such as beef and tuna has been inhibited by hydrolysates of whey, casein, soy and egg yolk. Park *et al*., (2005) reported that soy peptides, gelatin peptides and carnosine inhibited the oxidation of freeze-dried ethyl esters of eicosapentaenoic acid encapsulated in maltodextrin. Antioxidative properties of peptides are associated with their composition, structure and hydrophobicity (Sarmadi and Ismail, 2010). Wang *et al.*, (2005) reported that Tyr, Trp, Met, Lys, Cys, and His have antioxidant activity. Peptides isolated from hydrolyzed alfalfa leaf using protease showed antioxidant properties (Xie *et al*., 2008). Peptides which have the sequence of Leu-Leu-Pro-His-His exhibited a strong inhibition of lipid peroxidation and His–His played an important role in the antioxidant activity of the peptides (Xiong, 2010).

Table 2.5 Antioxidant peptides derived from different sources (Sarmadi and Ismail, 2010)

Source of peptides	**Characteristic**	**Preparation**	**Antioxidant Activity**
Zein hydrolysates	Contains up to 60 % free amino acids and the rest short peptides (‹500 Da)	Pepsin, pancreatin and alcalase	Radical chelating and scavenging activities
Soy protein fractions	Peptides with molecular weight of ‹10 KDa	Ultrafiltration and hydrolysis by flavourzyme	Antioxidant activity in emulsion, radical scavengers, reducing power
Corn gluten meal	Peptides fraction of 500-1500 Da, 41.12% hydrophobic amino acids and ~12.7% aromatic amino acids	Alcalase	Lipid peroxidation, reducing power, scavenging activity
Alfalfa hydrolsate	Molecular weight ‹1000 Da	Alcalase	Reducing power, radical chelating and scavenging activities
Sunflower protein	Hydrolysate with 37% DH, enriched in certain amino acids, such as histidine and arginine	Pepsin and pancreatin	Copper-chelating activity
Peanut protein	Not specified	Alcalase	Inhibition of linoleic acid autoxidation, radical-scavenging activity, reducing power, and inhibitor of liver lipid oxidation

2.4.1 Factors Affecting Antioxidant Activity of Proteins

Antioxidant properties of protein are affected by several factors, including protein concentration, reactivity, and physical structure. Several protein antioxidant mechanisms such as metal chelation, free radical scavenging, hydroperoxide reduction and aldehyde adduction are dependent on amino acid composition (Elias *et al*., 2008). The tertiary structure of the protein plays a role in antioxidant activity; free radical scavenging amino acids of native β-lacoglobulin are buried within the protein and do not contribute to the proteins antioxidant properties (Elias *et al*., 2008). Taylor and Richardson, (1980) found that heating (70-130℃ for 30 min) skim milk in a methyl linoleate emulsion with haemoglobin increased reactive sulfhydryl groups which lead to an increase in the antioxidant properties of skim milk.

Enzymatic hydrolysis can increase the antioxidant properties of proteins. Increased antioxidant properties in hydrolyzed proteins has been reported for mackerel (Wu *et al*., 2003), soybean (Park *et al*., 2005), zien (Kong and Xiong, 2006), chickpea (Li *et al*., 2008; Zhang *et al*., 2011) rapeseed (Xue *et al*., 2009), pea seed (Pownall *et al*., 2010) and flaxseed (Ayad, 2010). Maillard reaction products, furans, reductones, Schiff base and aldehyde also have the ability to inhibit lipid oxidation by free radical scavenging and metal chelation; these products can be water soluble or produced on the surface of protein-stabilized emulsion droplet (Choe and Min., 2009; Elias *et al*., 2008).

2.5 Antioxidant Mechanisms of Proteins

2.5.1 Antioxidant Enzymes

The addition of an electron to molecular oxygen produces the superoxide anion which can promote oxidative reactions by formation perhydroxyl radicals that catalyzes lipid oxidation (Decker, 2002). However, the superoxide anion can be inhibited by superoxide dismutase (SOD) which is considered as an antioxidant defence in biological tissues; the active site of SOD isomers contains copper plus zinc or manganese that catalyze the conversion of superoxide anion to hydrogen peroxide according to the following chemical reaction [2.1] (Elias *et al*., 2008).

$$2\,O^{-}_{2} + 2H \xrightarrow{SOD} O_2 + H_2O_2 \quad \text{.....[2.1]}$$

Hydrogen peroxide is also an important oxidative substrate and can be decomposed to form hydroxyl radical that can extremely oxidize lipids or proteins; therefore, removal of hydrogen peroxide from biological tissues is essential to prevent oxidative damage. Catalase and ascorbate peroxidase inactivate hydrogen peroxide by mechanisms shown in the equation [2.2] and [2.3] respectively (Elias *et al*., 2008).

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad \text{..... [2.2]}$$

$$2\,\text{Ascorbate} + H_2O_2 \xrightarrow{\text{ascorbate peroxidase}} 2\,\text{monodehydroascorbate} + 2\,H_2O \text{... [2.3]}$$

2.5.2 Inactivation and Sequestration of Prooxidative Metals

Perooxidative transition metals such as iron and copper play an essential role in lipid oxidation due to their ability to reduce hydroperoxides as shown in equation [2.4] (Elias *et al*., 2008).

$$\text{Metal}^{n+} + \text{Lipid-OOH} \longrightarrow \text{Metal}^{(n+l)} + OH^{-} + \text{Lipid} - O\bullet$$

Protein chelators can inhibit oxidative reactions by many mechanisms. They can change the physical location of transition metals, and reduce the chemical reactivity of transition metals, or hamper sterically the interaction of transition metals and lipids (Diaz *et al*., 2003; Elias *et al*., 2008). Lipid oxidation reactions occur at specific locations depending on the physicochemical properties of the food system; at the surface of oil-in-water emulsions, the reaction of metals and lipid can occur yet protein inhibit the reaction by changing the physical location of aqueous metals especially at pH values below the isoelectric point of protein that prevent access of metals to the water–oil interface by electrostatic repulsion (Elias *et al*., 2008).

Some proteins have the ability to chelate and store or transport inactive metals; ferritin protein can reduce iron's oxidative activity by interfering with its redox cycling capacity. After binding ferritin to ferrous (Fe^{+2}), the latter is oxidized to the ferric (Fe^{+3}) that becomes sequestered and unavailable to participate in redox reactions (Elias *et al*.,

2008). In addition, proteins can bind strongly to copper which is considered a more effective catalyst than iron. Bovine serum albumins (BSA) inhibit copper-catalyzed oxidation of low density lipoprotein (LDL) (Elias *et al*., 2008).

2.6 Determination of Antioxidant Activity

There are several methods to measure antioxidant capacity of compounds; these methods can be divided into two categories (i) methods such as oxygen radical absorbance capacity (ORAC) and β-carotene bleaching are based on the hydrogen atom transfer (HAT) in which both the substrate and antioxidant act as a competitive reaction (ii) methods such as Trolox equivalent antioxidant capacity (TEAC), DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical–scavenging activity and ferric ion reducing antioxidant power (FRAP) are based on the capacity of an antioxidant to give an electron to an oxidant (Sarmadi and Ismail, 2010). Due to the diversity of antioxidant mechanisms, the antioxidant capacity can be measured by more than one assay. Antioxidant determination can be affected by solvent, affinity substrate to antioxidant and purity of substrate, and it is recommended to use different methods with different mechanisms to measure antioxidant capacity of foods (Moure *et al*., 2006b). The most widely used methods to measure the antioxidant capacity are DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical–scavenging activity (Scalzo, 2008), trolox equivalent antioxidant capacity (TEAC) (Mariken *et al*., 2004) and oxygen radical absorbance capacity (ORAC) assay (Gillespie *et al*., 2007).

2.6.1 DPPH Assay

The DPPH method which known also as stable organic free radical (Prior *et al*., 2005; Noipa *et al*., 2011) is simple, rapid, sensitive, and stable (Nopia *et al*., 2011). Factors affecting the DPPH method are the nature of the solvent, concentrations of sample, reaction time and pH (Noipa *et al*., 2011). DPPH method based on the measurement of the reducing of alcoholic $DPPH^{\bullet}$ solution at wavelength 517 nm by an antioxidant which donates the hydrogen, leading to the formation of DPPH-H (non-radical form) as shown in equation [2.5] (Koleva *et al*., 2002). Figure 2.4 shows the DPPH mechanism.

$$DPPH^{\bullet} + AH \longrightarrow DPPH\text{-}H + A^{\bullet} \quad [2.5]$$

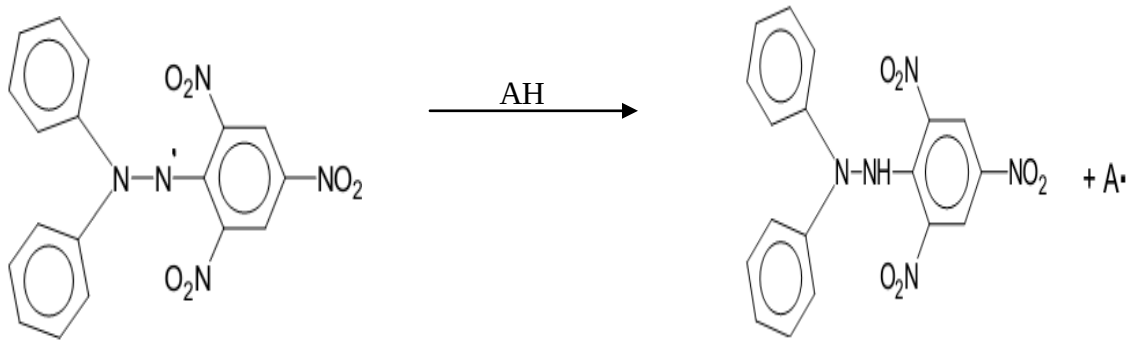

Figure 2.4 DPPH chemical structures and its reaction with a scavenger indicated by A-H (Roberto, 2008)

2.6.2 TEAC Assay

The principle of this method is the ability of an antioxidant to scavenge the 2-2`-azinbis-(3-ethylhbenzothiazoline-6-sulfonate ($ABTS^{\bullet+}$), producing a colorless product (Mariken *et al*., 2004). TEAC assay is simple and rapid and it can be done in 30 min and it can be done with different range of pH (Prior *et al*., 2005). Figure 2.5 shows the ($ABTS\bullet^{+}$) structure.

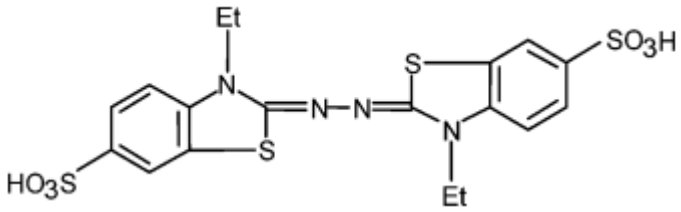

Figure 2.5 Structure 2-2`-azinbis-(3-ethylhbenzothiazoline-6-sulfonic acid) ($ABTS\bullet^{+)}$ (Mariken *et al*., 2004).

2.6.3 ORAC Assay

The ORAC or phycoerythyrin assay which used to determine the antioxidant capacity of foods and nutraceuticals is based on the use of phycoerytrin (fluorescent probe) as target of free radicals, leading to change in the fluorescence intensity (Gillespie *et al*., 2007; Moreno, 2002). This method is simple, sensitive to temperature, and it can be used for both hydrophilic and hydrophobic antioxidant (Mariken *et al*., 2004).

2.7 Types of Antioxidants

2.7.1 Synthetic Antioxidants

Synthetic antioxidants are fundamentally phenolic antioxidants such as butylatedhydroxy toluene (BHT) butylatedhydroxy anisole (BHA), tertiary butylhdroquinone (TBHQ) and propyl gallate (PG) (Figure 2.6) (Pokorny *et al*., 2001). Synthetic antioxidants are usually added to the fat or oil food at the level of 0.02% (Hosseinian, 2006). Some synthetic antioxidants such as BHA and BHT are used in combination with resulting synergistic effects. BHA is also synergistic with PG. (Pokorny *et al*., 2001).

2.7.2 Natural Antioxidants

Natural antioxidants have been widely used because of their ability to delay the development of off-flavors in foods, decreasing demand for synthetic antioxidants and health issues (Gordon, 2003). Natural antioxidants are mainly plant phenolic compounds that may exist in all parts of the plant (Gordon, 2003). Plant phenolics can act as radical scavengers, metal chelators, singlet oxygen quenchers, or reducing agents (Decker, 2003). Several natural food components such as oils and oilseeds, proteins and protein hydrolysates, fruits and vegetables have antioxidant properties (Decker, 2003).

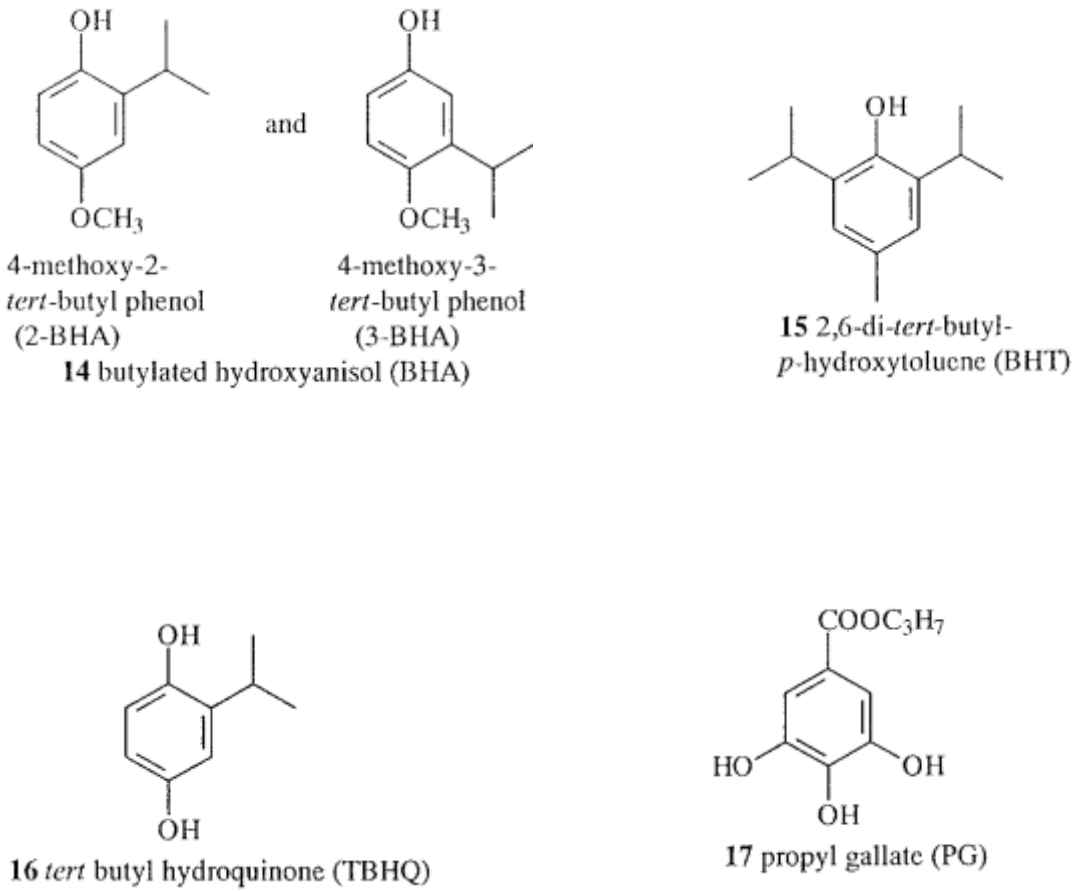

Figure. 2.6 Types of synthetic antioxidants (Pokorny *et al*., 2001)

CHAPTER 3

MATERAILS AND METHODS

3.1 Material

Flaxseed was obtained from the Bunge Canada (Winnipeg, Manitoba). Samples were ground by using a coffee grinder, and then were passed though #20 mesh screen (1 mm, ASTME II) and kept in plastic container at 4 ℃ before use. O-phthaldialdehyde (OPA), DPPH (1, 1-diphenyl-2-picrylhydrazyl), ferrozine (3-(2-pyridyl)-5, 6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt), potassium ferricyanide, ferric chloride, and pyridine were purchased from Sigma Aldrich. All other chemicals used were of analytical grade.

3.2 Extraction and Isolation of Protein from Flaxseed

3.2.1 Preparation of Defatted Flaxseed Protein

Flaxseed protein extraction was carried out as described by Marambe *et al.*, (2008) with some modifications. The crude fat was removed from flaxseed by using the Soxhlet unit (AOAC, 2005). A suspension was prepared by adding 1 L distilled water to the defatted flaxseed meal (meal to solvent ratio of 1:10 w/v) and the mixture was adjusted to pH 8.5 using 1 M and 0.1 M NaOH solution. The mixture was stirred for 1 h using a magnetic stirrer at ambient temperature; the suspension was centrifuged at 8,000 xg for 30 min. The proteins were precipitated at pH 3.8 using 0.1 M HCl. The supernatant was centrifuged at 8,000 xg for 30 min. The protein precipitate was suspended in distilled water and the pH was adjusted to 7.0 using 0.1 M NaOH. The protein solution was then dialyzed against distilled water using a dialysis membrane (Spectra/por, MWCO3500) at room temperature for 48 h and then lyophilized. Figure 3.1 shows the steps of extraction of flaxseed protein.

3.2.2 Preparation of Demucilaged and Defatted Flaxseed Protein

Demucilaged and defatted flaxseed protein was prepared according to Marambe *et al.*, (2008) with some modifications. Flaxseed was stirred with 0.5 M $NaHCO_3$ (1:8 w/v, 50 ℃) for 1 h and then the seeds were rubbed though #20 mesh screen and washed thoroughly with distilled water several time. After draining and oven-drying at 45 ℃ for 24 h, the demucilaged flaxseed were ground using a coffee grinder, and passed though #20 mesh screen (1 mm, ASTME II). The demucilaged flaxseed was defatted using a Soxhlet unit. The flaxseed meal was air dried at room temperature, sieved and used for protein extraction as described in Section 3.2.1

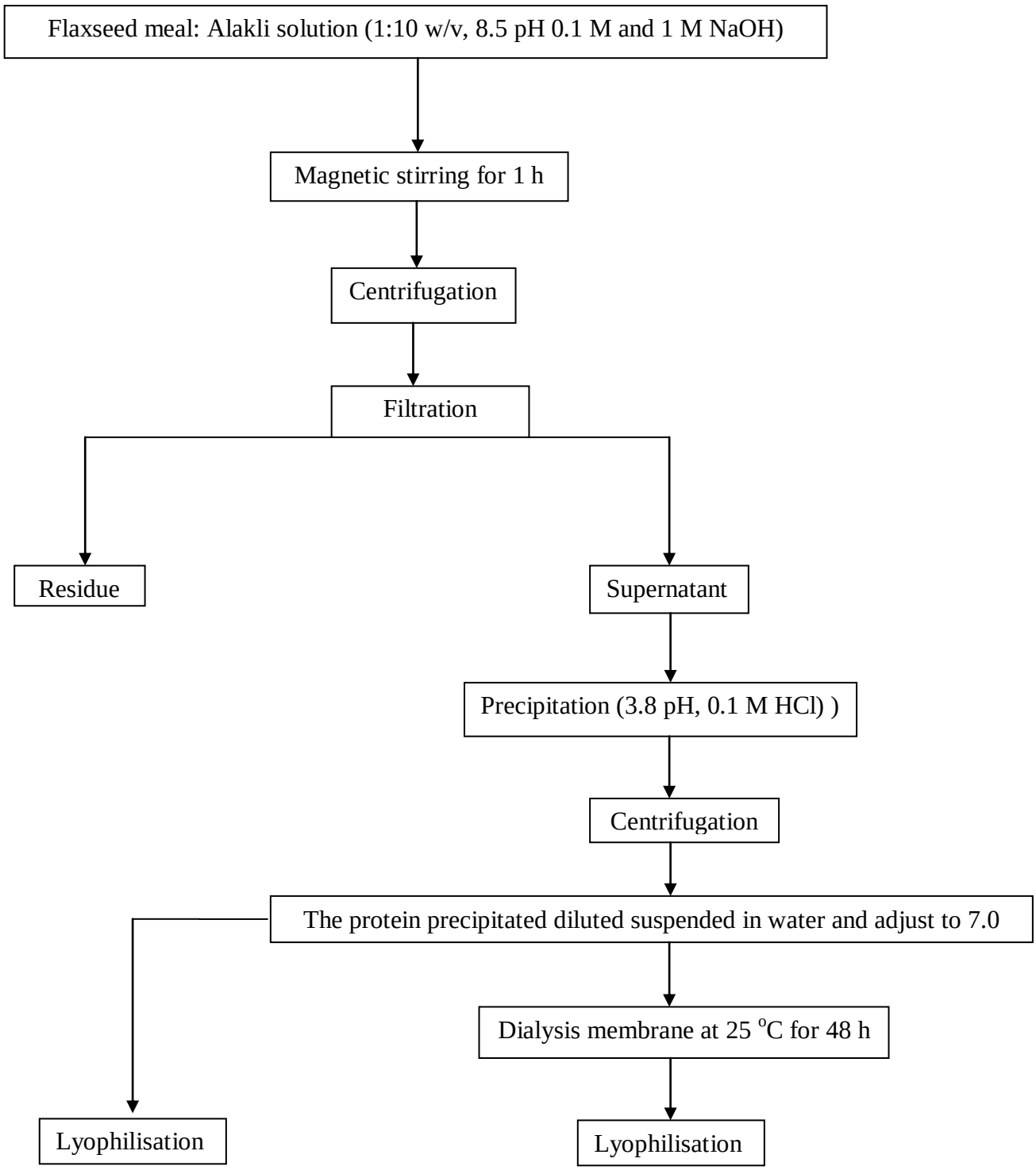

Figure 3.1 Steps of extraction of flaxseed protein

3.2.3 Enzymatic Hydrolysis of Flaxseed Protein

The enzymatic hydrolysis of flaxseed protein isolate was carried out according to Adebiyi *et al*. (2008) with some modifications. A dispersion of flaxseed protein (1% w/v) was brought to pH 8.0 with 2 N NaOH with mixing and was incubated with trypsin and bacterial protease (1:20 enzyme to protein ratio) in 50 mM sodium phosphate buffer at 37 ℃ for 2 h. At time intervals between 0 to 120 min, tubes of the digest were removed from the water bath at 0, 15, 30, 45, 60 and 120 min. The enzyme reaction was stopped by heating at 95 ℃ in boiling water for 10 min. The protein hydrolysates were then centrifuged at 8,000 xg for 30 min, and the supernatant was lyophilized.

3.2.4 Determination of Degree of Hydrolysis

The degree of hydrolysis (DH) was determined by the o-phthaldialdehyde (OPA) method as described by Ayad (2010). The OPA reagent was prepared by combining the 25 ml 100 mM sodium tetraborate, 2.5 ml 20% SDS (w/w) and 40 mg OPA (dissolved in 1 ml methanol) and 100 µl 2-Mercaptoethanol and diluting to a final volume of 50 ml with distilled water. Samples of 50-100 µl of protein hydrolysis were added directly to 2 ml OPA reagent, mixed and incubated for 2 min at ambient temperature and the absorbance was measured at 340 nm. The degree of hydrolysis was calculated using the following equation:

$$DH\ (\%) = (MW\ \Delta_{340\ nm})/\ (d.e.p) \times 100$$

Where MW = Average molecular weight of amino acids (120)

Δ_{340nm} = Absorbance at 340 nm

d = Dilution factor

e = Average molar absorption of amino acids (6000 $M^{-1}\ cm^{-1}$)

p = protein concentration

3.3 Determination of Antioxidants Activity

3.3.1 DPPH Scavenging Activity Assay

The antioxidant activity of flaxseed protein and protein hydrolysates was measured by using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay according to Li *et al*. (2008) and You *et al*. (2009). 2 ml of each sample solution at different concentration (1-2.5 mg/ml) was added to 2 ml 0.1 mM DPPH dissolved in 95% ethanol. The mixture was mixed and left at room temperature for 30 min in the dark place; the absorbance of the mixture was measured at 517 nm. In the blank the ethanol solution (95%) used instead of the sample. The scavenging activity assay was calculated using the following equation:

DPPH scavenging activity (%) =

Blank absorbance − Sample absorbance/Blank absorbance × 100

3.3.2 Reducing Power Activity Assay

The reducing power of flaxseed protein and protein hydrolysates was carried out as described by Cumby *et al.*, (2008) with some modifications. Flaxseed protein and protein hydrolysates samples were dissolved in a 0.2 M phosphate buffer (pH 6.6) at different concentrations of 1, 1.5, 2 and 2.5 mg/ml. 2.5 ml of samples was added to 2.5 ml potassium ferricyanide solution (10 mg/ml) and incubated at 50 ℃ for 20 min. After incubation, 2.5 ml of deionized water and 0.5 ml of ferric chloride solution (1.0 mg/ml) was added to the mixture. For the blank, distilled water was used instead of samples. The absorbance of the mixture was read at 700 nm.

3.3.3 Metal Ion Chelating Activity Assay

The ability of flaxseed protein and protein hydrolysates to chelate Fe^{+2} was determined according to the method which described by Zhang *et al*. (2011) with some modifications. An aqueous solution was prepared by adding 1 ml of iron (II) chloride 20 µM to 1 ml 0.5 mM ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt). 0.5 ml of flaxseed protein /protein hydrolysates was added to the aqueous solution and the absorbance was measured at 562 nm. In the blank, the flaxseed sample was

replaced with distilled water. The Fe^{+2} chelating activity of the proteins and their hydrolysates were calculated using the following equation:

Metal ion chelating activity (%) =

Blank absorbance - Sample absorbance/Blank absorbance × 100

3.5 Characterization of Flaxseed Protein and Protein Hydrolysates

3.5.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.5.1.1 Sample Preparation

SDS-PAGE was carried out according to Laemmli (1970), using 12% acrylamide in separation gels and 4% acrylamide in stocking gels. Samples of freeze dried isolate protein at concentration (5-20 mg/ml) were diluted in sample buffer (10% glycerol. 0.05% bromophenol Blue, 5% 2-Marcoptoethanol, Tris-HCl, pH 6.8 and 2% SDS). Samples were heated at 95 ^{0}C for 5 min before loading (10-15 µl) into each well.

3.5.1.2 Electrophoresis Running and Staining and Destaining Condition

The gels were run for 1-2 h at a constant voltage of 100 V/gel using a mini protein III cell unit (Bio-Rad, Hercules, CA). The gels were stained over night about 16 h in solution of Coomassie Brilliant Blue R-250. The gels were destained by fixing solution (20% methanol and 10% acetic acid solution) and repeated as necessary.

3.5.2 Native –PAGE

Native-PGAE was carried out using the technique as described by Davis (1964) with 14% separating el and 4% stocking gel using a Mini-protein III electrophoresis cell unit (Bio-Rad, Hercules). Freeze dried of flaxseed protein isolates at concentration (5-20 mg/ml) was dissolved in sample buffer (Tris-HCl 1.5 M, pH 6.8, glycerol, bromophenol Blue). The electrophoresis was performed at constant current 120 V/gel for 2-2.5 h.

3.5.2.1 Electrophoresis Staining and Destaining Condition

The procedure is the same as that mentioned in section 3.5.1.2 for protein staining and destaining conditions.

3.6 Statistical Analysis

All the experimental data were performed in duplicate and the data obtained were subjected to one way analysis of variance (ANOVA). Data were analyzed ($p < 0.05$ was regarded as significant).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Enzymatic Hydrolysis

4.1.1 Enzymatic Hydrolysis of Isolated Flaxseed Protein using Trypsin

Figure 4.1 and Table 4.1 show the results of the degree of hydrolysis (DH) for defatted, flaxseed protein/dialysis hydrolysates (DFPDH) and defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH) compared to the control. The DH values of flaxseed proteins/dialysis were significantly different ($p < 0.05$) to that of flaxseed proteins/non-dialysis. The DH of defatted flaxseed protein/dialysis hydrolysates (DFPDH) and defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH) ranged from 8.97-17.06% DH and 7.36-12.29% DH, respectively. At 120 min DFPDH showed the highest rate of hydrolysis of 17.06% DH. Defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) showed similar levels of 2.93-3.36% DH and 2.45-2.64% DH.

Figure 4.2 and Table 4.2 show the results of the degree of hydrolysis (DH) for demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH) and demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH) compared to the control. At 120 min of hydrolysis, the DH of DDFPDH showed the highest value with16.34% DH compared to DDFPNDH with DH of 10.68%. The control samples for demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) showed DH levels of 2.39-3.33% and 1.88-2.38%, respectively.

Figure 4.3 and Table 4.3 show the results of the degree of hydrolysis (DH) for non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), and non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH) compared to the control. NDFPDH and NDFPNDH showed DH levels of 8.55% and 7.23% at 120 min, respectively. Control sample of non-defatted flaxseed protein/dialysis (NDFPD) showed an increase in DH with time of hydrolysis with 3.8-5.55% DH compared to non-defatted flaxseed protein/non-dialysis (NDFPND) which little change in DH of 1.73-2.67%. These findings

are in agreement with those reported by other researchers. Zhu *et al*. (2008) reported that the degree of hydrolysis of zein protein ranged from 20 to 40% by using alcalase; Ayad (2010) Found that DH of flaxseed protein fraction was 9.4-24.5%; Singh (2011) reported that the maximum DH of soy protein and chickpea protein by trypsin was 11.5% and 12.3% DH, respectively while non-hydrolyzed soy protein and chickpea protein was 0.9% and 1.2% at 120 min.

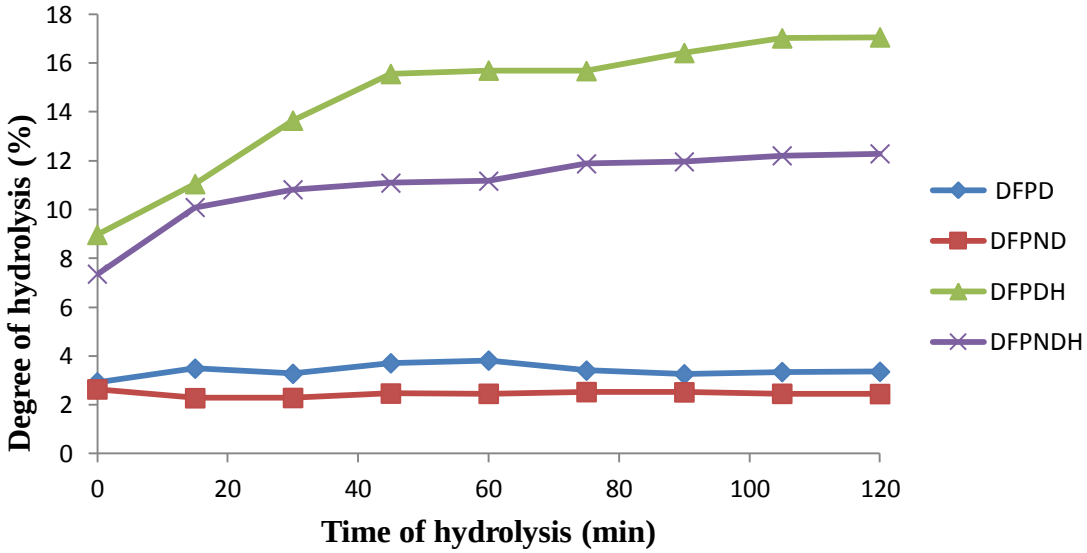

Figure 4.1 Enzymatic hydrolysis of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) by trypsin.

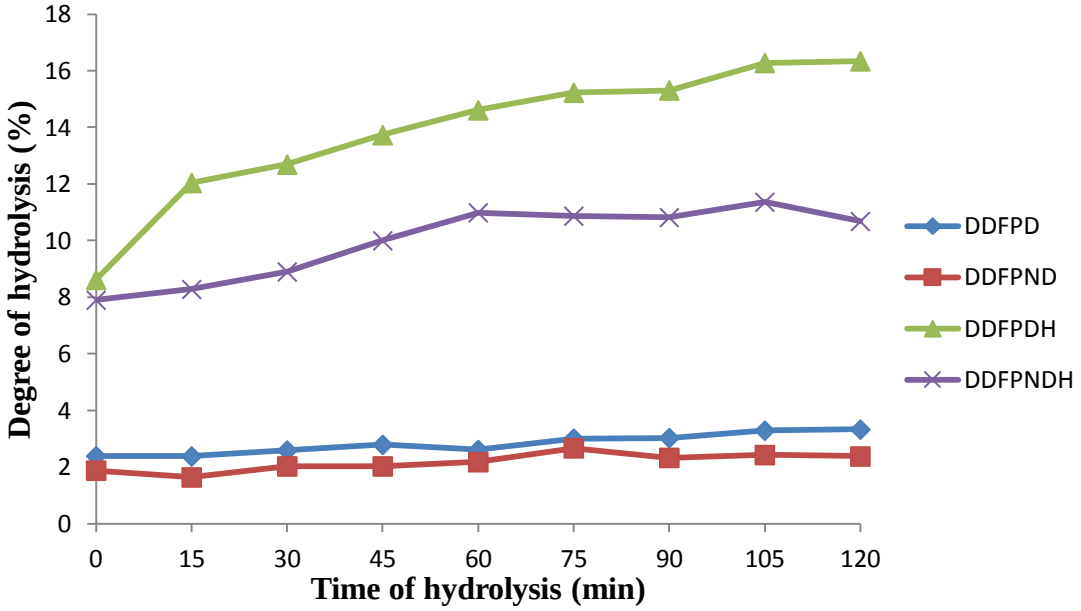

Figure 4.2 Enzymatic hydrolysis of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD), and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) by trypsin.

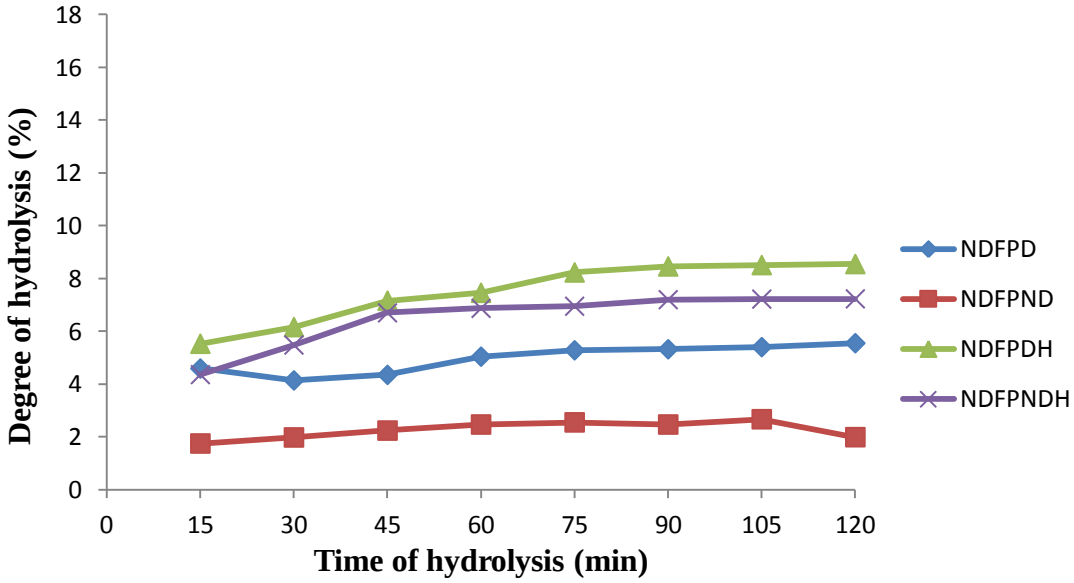

Figure 4.3 Enzymatic hydrolysis of non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non- defatted flaxseed protein non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND) by trypsin.

Table 4.1 % DH of isolated defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) by trypsin.

Time (min)	%DH of DFPD(control)	%DH of DFPDH	%DH of DFPND (control)	%DH of DFPNDH
0	2.93	8.97	2.64	7.36
15	3.49	11.05	2.28	10.09
30	3.28	13.65	2.29	10.82
45	3.71	15.56	2.47	11.09
60	3.82	15.70	2.46	11.17
75	3.41	15.68	2.53	11.88
90	3.26	16.42	2.52	11.96
105	3.35	17.02	2.46	12.21
120	3.36	17.06	2.45	12.29

Results are average of duplicate determination
DH= Degree of Hydrolysis

Table 4.2 % DH of isolated demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) by trypsin

Time (min)	% DH of DDFPD (control)	% DH of DDFPDH	% DH of DDFPND (control)	% DH of DDFNDH
0	2.39	8.62	1.88	7.9
15	2.38	12.04	1.64	8.28
30	2.59	12.69	2.03	8.90
45	2.79	13.73	2.03	10.00
60	2.61	14.61	2.18	10.99
75	3.01	15.23	2.67	10.87
90	3.03	15.31	2.33	10.82
105	3.29	16.28	2.43	11.36
120	3.33	16.34	2.38	10.68

Results are average of duplicate determination
DH= Degree of Hydrolysis

Table 4.3 % DH of isolated non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non- defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND) by trypsin.

Time (min)	% DH of NDFPD (control)	% DH of NDFPDH	% DH of NDFPND (control)	% DH of NDFPNDH
0	3.8	4.57	1.73	4.09
15	4.59	5.53	1.75	4.36
30	4.14	6.16	1.98	5.48
45	4.35	7.16	2.24	6.70
60	5.04	7.46	2.47	6.87
75	5.28	8.23	2.55	6.95
90	5.32	8.46	2.47	7.20
105	5.40	8.51	2.67	7.22
120	5.55	8.55	2.99	7.23

Results are average of duplicate determination
DH= Degree of Hydrolysis

4.1.2 Enzymatic Hydrolysis of Isolated Flaxseed Protein using Bacterial Protease

Figure 4.4 and Table 4.4 show the results of the degree of hydrolysis (DH) for defatted flaxseed protein/dialysis hydrolysates (DFPDH) and defatted flaxseed protein non-dialysis hydrolysates (DFPNDH) compared to the control. The DH values of flaxseed proteins/dialysis were higher ($p < 0.05$) than flaxseed proteins/non-dialysis. The DFPDH gave maximum DH at 120 min with 28.43% DH compared to DFPNDH which gave 25.33%. DH Control samples of defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) showed similar DH levels of 2.93-3.36% DH and 2.45-2.64% DH

Figure 4.5 and Table 4.5 show the results of the degree of hydrolysis (DH) for demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH) and demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH) compared to the control. After 2 h of hydrolysis, the highest degree of hydrolysis (DH) was obtained for DDFPDH with 30.19% DH followed by DDFPNDH with of 28.46% DH. Non-hydrolyzed of demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) gave 2.39-3.33% DH and 1.88-2.38% DH for DDFPD and DDFPND, respectively.

Figure 4.6 and Table 4.6 show the results of the degree of hydrolysis (DH) for non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH) and non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH) compared to the control. NDFPDH and NDFPNDH showed DH of 14.23% and 12.69%, respectively. In the non-hydrolyzed treatment non-defatted flaxseed protein/dialysis (NDFPD) showed a significant increase ($p < 0.05$) in DH with increase in the time of hydrolysis (3.8-5.55% DH) compared to non-defatted flaxseed protein/non-dialysis (NDFPND) which showed little change DH (1.73-2.67%). These findings are in agreement with other researchers. Marambe *et al.* (2008) reported that the maximum degree of flaxseed protein hydrolysis was 70% using Flavourzyme (enzyme to substrate ration 80 LAPU/ g protein); Pena-Ramos and Xiong (2002) found that DH% of soy protein in a liposomal system increased with an increase in the time of hydrolysis and reached 20.6% DH at 6 h using Flavourzyme; the DH of whey

protein isolate increased with increase time of hydrolysis (enzyme to substrate ratio 2:100) and ranged from19.3 to 38.5% DH(Peng *et al*., 2010).

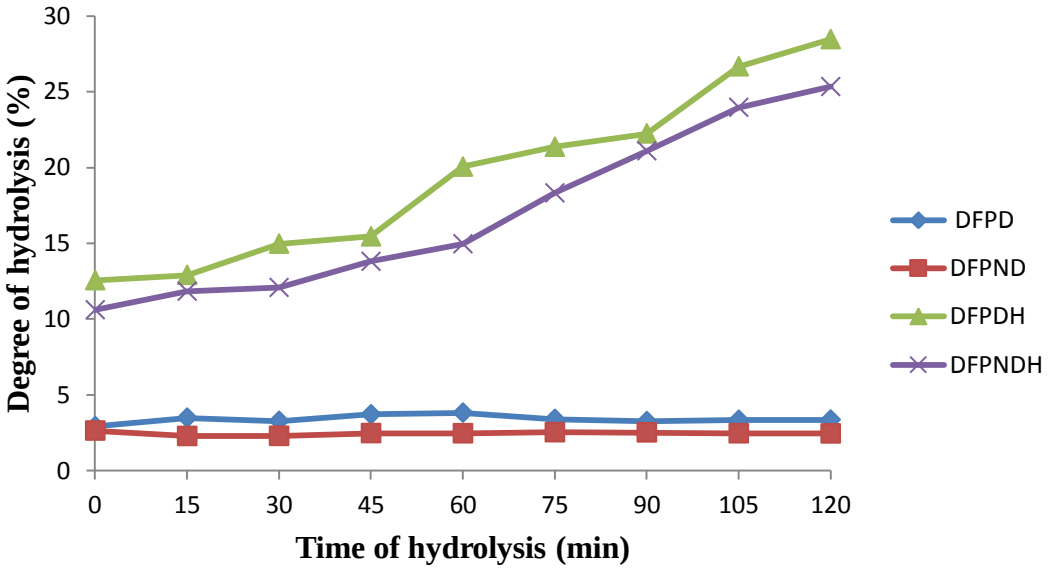

Figure 4.4 Enzymatic hydrolysis of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) by bacterial protease.

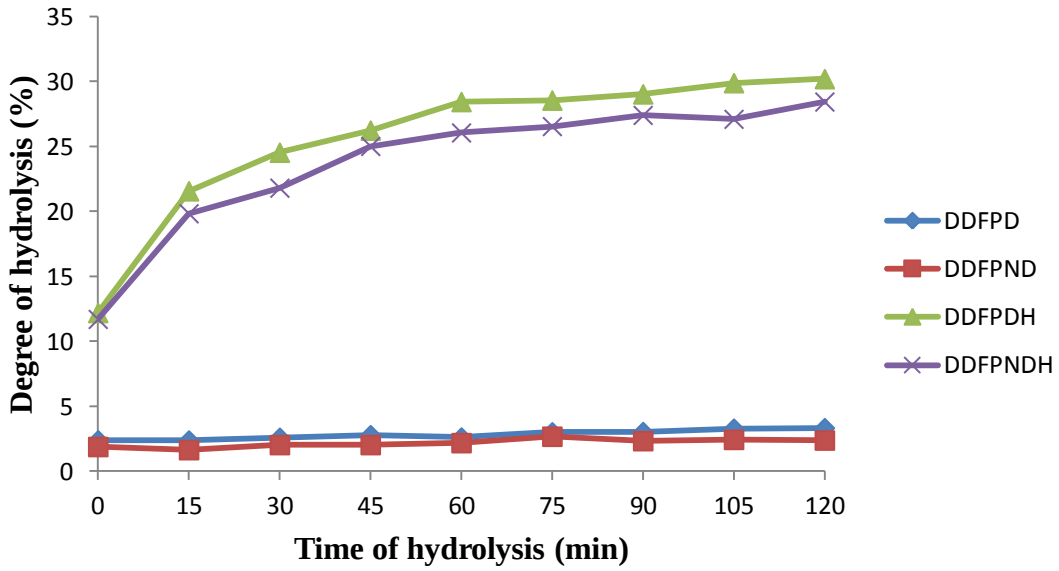

Figure 4.5 Enzymatic hydrolysis of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) by bacterial protease.

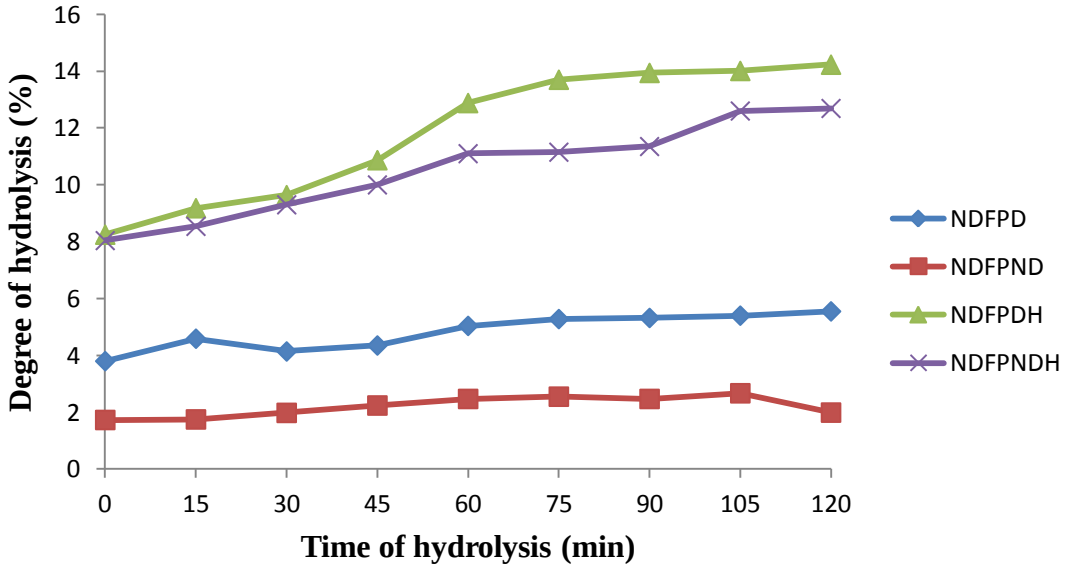

Figure 4.6 Enzymatic hydrolysis of non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non- defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND) by bacterial protease.

Table 4.4 % DH of isolated defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) by bacterial protease.

Time (min)	%DH of DFPD(control)	%DH of DFPDH	%DH of DFPND (control)	%DH of DFPNDH
0	2.93	12.56	2.64	10.61
15	3.49	12.91	2.28	11.83
30	3.28	14.97	2.29	12.08
45	3.71	15.45	2.47	13.80
60	3.82	20.05	2.46	14.96
75	3.41	21.36	2.53	18.32
90	3.26	22.24	2.52	21.09
105	3.35	26.67	2.46	23.96
120	3.36	28.43	2.45	25.33

Results are average of duplicate determination
DH= Degree of Hydrolysis

Table 4.5 % DH of isolated demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) by bacterial protease.

Time (min)	% DH of DDFPD (control)	% DH of DDFPDH	% DH of DDFPND (control)	% DH of DDFNDH
0	2.39	12.19	1.88	11.70
15	2.38	21.56	1.64	19.83
30	2.59	24.53	2.03	21.80
45	2.79	26.23	2.03	25.01
60	2.61	28.43	2.18	26.05
75	3.01	28.54	2.67	26.54
90	3.03	29.02	2.33	27.10
105	3.29	29.88	2.43	27.40
120	3.33	30.19	2.38	28.46

Results are average of duplicate determination
DH= Degree of Hydrolysis

Table 4.6 % DH of isolated non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non- defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND) by bacterial protease.

Time (min)	% DH of NDFPD (control)	% DH of NDFPDH	% DH of NDFPND (control)	% DH of NDFPNDH
0	3.8	8.25	1.73	8.04
15	4.59	9.18	1.75	8.54
30	4.14	9.64	1.98	9.30
45	4.35	10.87	2.24	10.00
60	5.04	12.88	2.47	11.10
75	5.28	13.70	2.55	11.15
90	5.32	13.94	2.47	11.35
105	5.40	14.01	2.67	12.60
120	5.55	14.23	2.99	12.669

Results are average of duplicate determination
DH= Degree of Hydrolysis

4.2 Antioxidant Activity

4.2.1 Antioxidant Activity of Flaxseed Proteins and Protein Hydrolysates using Trypsin

4.2.1.1 DPPH Scavenging Activity

The DPPH radical scavenging activity was used to evaluate the ability of flaxseed protein treatments to act as free radical scavengers in order to evaluate the antioxidant activity. In general, the flaxseed proteins/dialysis treatments showed greater ($p < 0.05$) than flaxseed proteins/non-dialysis treatments; similar to hydrolysis treatment which showed significantly affect ($p < 0.05$) compared with non-hydrolyzed samples. Figure 4.7 shows the results for the DPPH scavenging activity of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND). The DFPDH showed the highest scavenging activity of 61.1% followed by DFPNDH 58.09%; DFPD 51.1% and DFPND 36.43% at protein concentration (2.5 mg/ml).

Figure 4.8 shows the results of DPPH radical-scavenging activity of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND). The DDFPDH showed the highest DPPH radical-scavenging ability of 70.53% at protein concentration (2 mg/ml) compared to the non-hydrolyzed DDFPD with 40.26% of DPPH scavenging activity at (2mg/ml). Non-dialysis treatment showed 60.39% of DPPH scavenging activity for DDFPNDH and 32.58% of DPPH scavenging activity for DDFPND at 2 mg/ml, respectively.

Figure 4.9 shows the results of scavenging activity of non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein non-dialysis/hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND). The results showed an increase in the scavenging activity with increase in protein concentration from (1-2.5 mg/ml) and are

similar to the results shown in Figures 4.7 and 4.8. NDFPDH exhibited a scavenging activity range of 35.64-59.06% compared to NDFPNDH with scavenging activity ranging from 24.50-53.83%. The hydrolyzed flaxseed proteins have highest DPPH radical-scavenging activity in the range of 24.50-70.53% compared to non-hydrolyzed flaxseed protein isolates with 21.23-59.30% radical-scavenging activity; this could be due to protein hydrolysates and peptides an increase in hydrophobicity which wil increase the solubility in the lipid and therefore enhances the antioxidant activity (Singh, 2010 and Zhu *et al*., 2005). These results are similar to those obtained by Ayad (2010) who reported that the antioxidant activity using DPPH scavenging activity of hydrolyzed flaxseed protein fractions were higher than non-hydrolyzed as well for commercial soybean protein isolate; the radical-scavenging activity ranged from 45-90% comparing to non-hydrolyzed results with 31-81%. Singh (2011) also reported that isolated soybean hydrolysates and isolated chickpea protein hydrolysates showed radical scavenging activity higher than control samples (non-hydrolysates), with 16.5-32%, 3.2-26.8% and 6-10.6%, 1.8-8.3%, respectively at protein concentration 2.5-10 mg/ml.

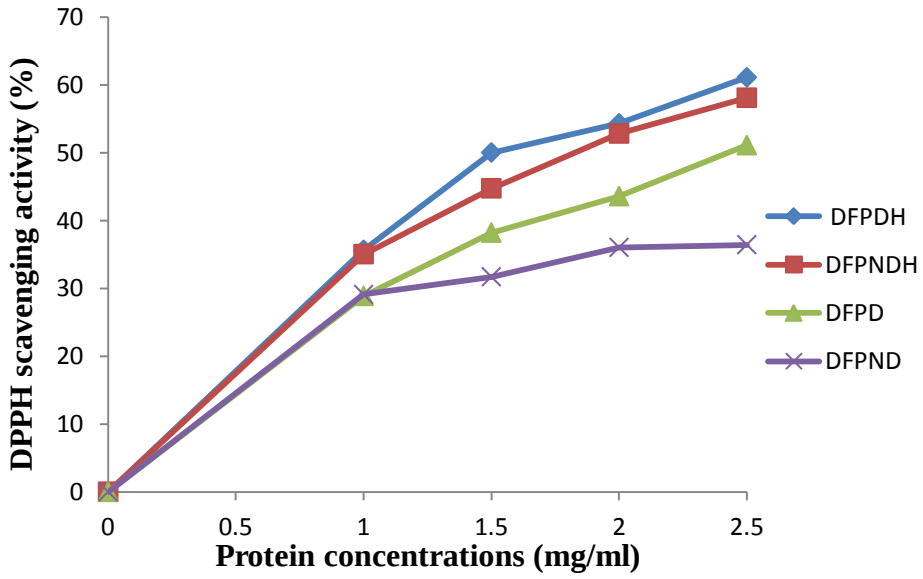

Figure 4.7 DPPH scavenging activity curve for defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) by trypsin.

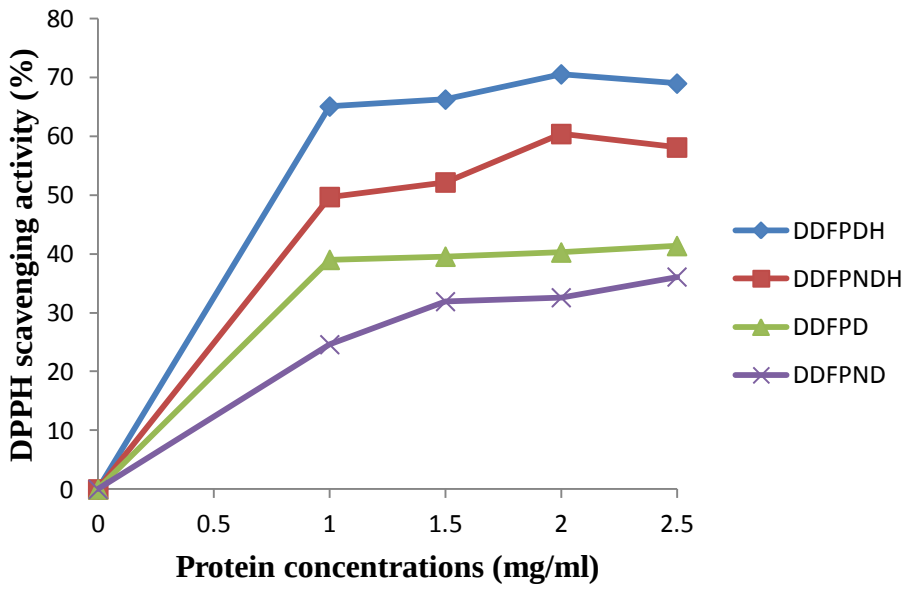

Figure 4.8 DPPH scavenging activity curve for demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) by trypsin.

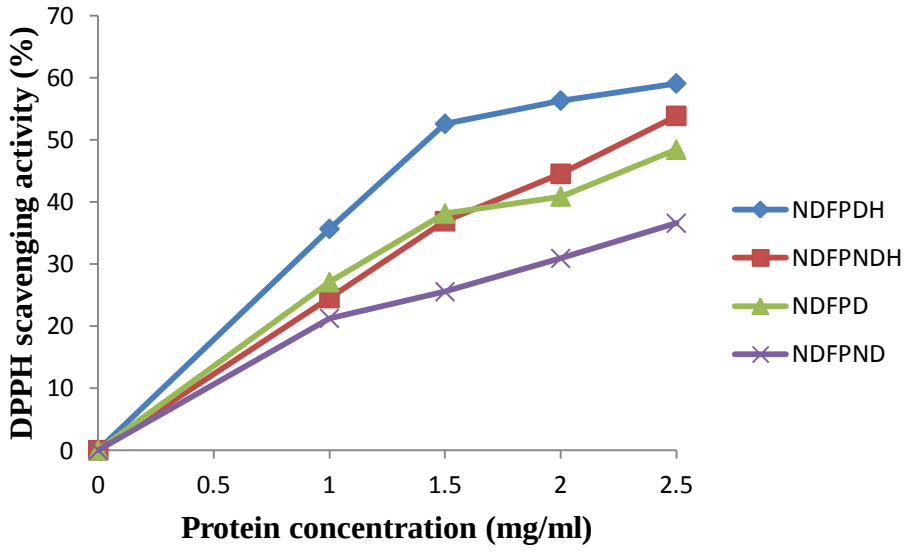

Figure 4.9 DPPH scavenging activity curve for non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND) by trypsin.

4.2.1.2 Reducing Power Activity

The reducing power assay is the most effective method to evaluate the ability of protein and protein hydrolysates to donate electrons (Xie *et al*., 2008). Figure 4.10 shows the results of reducing power of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND). DFPDH showed reducing power of 0.12 which is higher ($p < 0.05$) than DFPNDH with reducing power of 0.092; while control samples DFPD and DFPND showed reducing power of 0.08 and 0.06, respectively at 2.5 mg/ml.

Figure 4.11 shows the reducing power of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND). DDFPDH showed the strong reducing power 0.15 followed by DDFPNDH 0.12, while non-hydrolyzed DDFPND and DDFPND showed reducing power 0.09 and 0.08, respectively.

Figure 4.12 shows the results of reducing power of non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD), and non-defatted flaxseed protein/non-dialysis (NDFPND). In contrast of the defatted flaxseed protein isolates and demucilaged and defatted flaxseed protein isolates, the non-defatted flaxseed protein isolates showed the lowest reducing power with 0.07, 0.06 and 0.058, 0.05 for NDFPDH, NDFPNDH, NDFPD and NDFPND, respectively. These results are in agreement with those reported by other researchers. Zhu *et al*. (2008) found that the reducing power of zein protein hydrolysates was 0.0652 to 0.082 at protein concentration 1-16 mg/ml by using alcalase while Zhao *et al*. (2012) reported that the maximum reducing power of rice dreg (a by-product from rice syrup production contain $\geq$ 50% protein) protein hydrolysates using trypsin was 0.3% at 10 mg/ml.

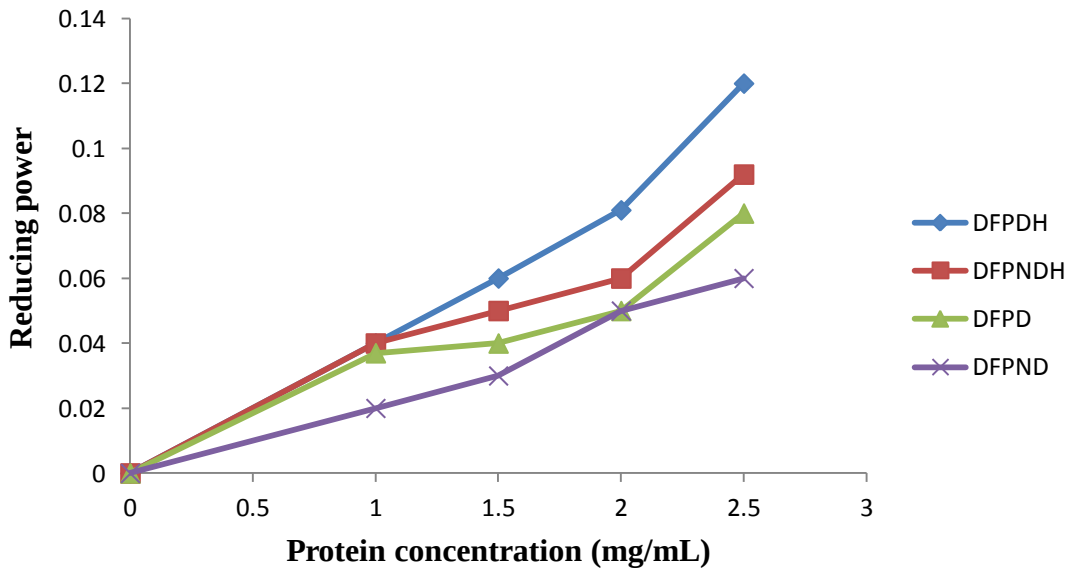

Figure 4.10 Reducing power of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) using trypsin.

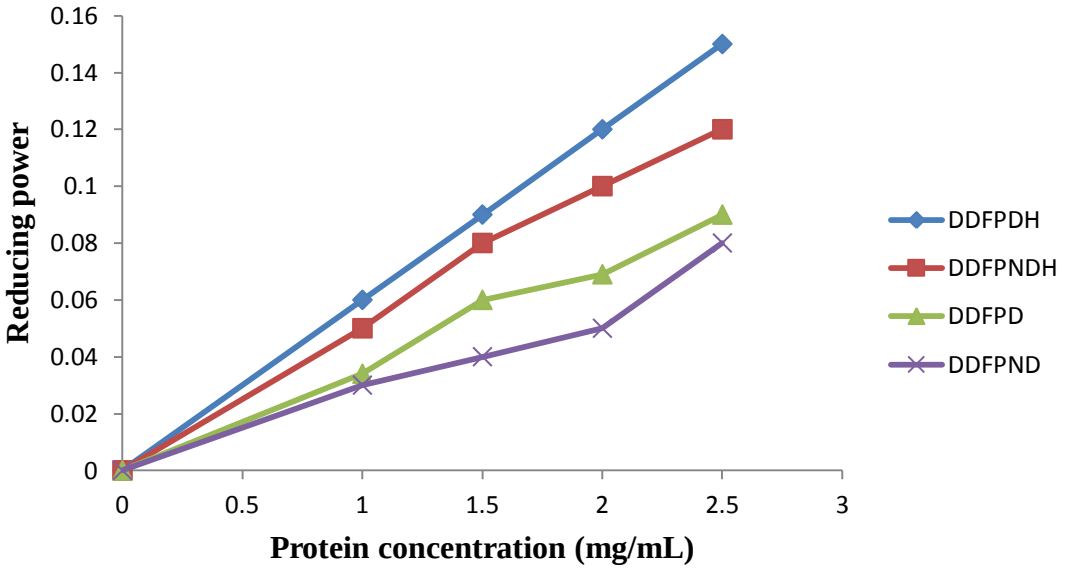

Figure 4.11 Reducing power of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and dimucilgae and defatted flaxseed protein/non-dialysis (DDFPND) using trypsin.

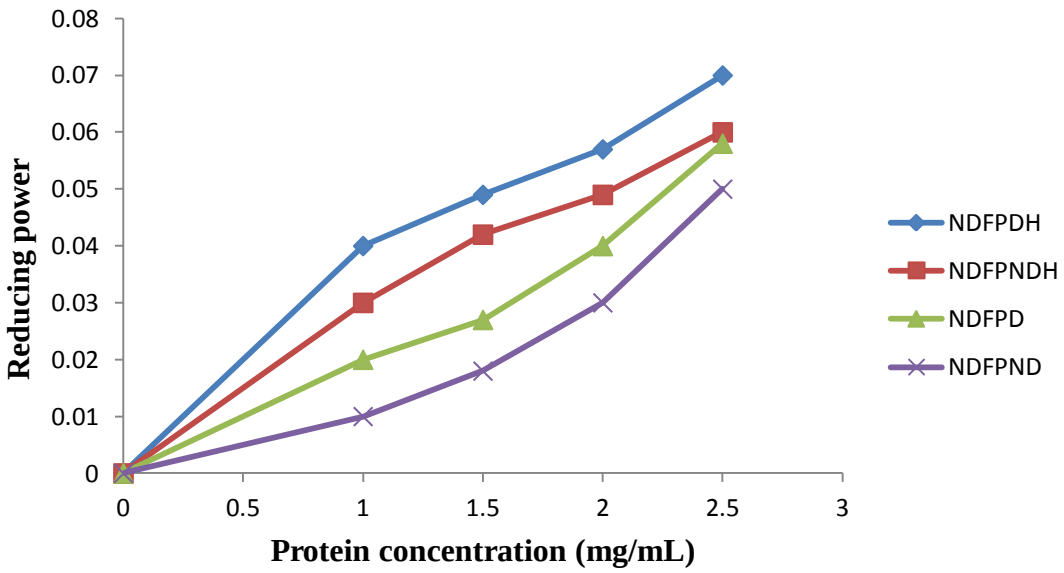

Figure 4.12 Reducing power of non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non- defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND) using trypsin.

4.2.1.3 Metal Ion Chelating Activity

Figure 4.13 shows the results of Fe^{+2} chelating activities of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), comparing to their corresponding non-hydrolyzed samples. The dialysis treatment of flaxseed proteins showed the ability to chelate the iron higher ($p < 0.05$) than non-dialysis treatment. The highest Fe^{+2} chelating activities were estimated to be 60.1% for DDFPDH and 50.68% for DDFPNDH compared to the other treatments. DFPDH and DFPNDH showed strong chelating activities which were 47.2% and 45.7% compared to DFPD and DFPND which exhibited significantly ($p < 0.05$) weaker Fe^{+2} chelating activities 24.6% and 20.2%, respectively. Non-hydrolyzed samples of all flaxseed proteins isolates showed similar ability to chelate for Fe^{+2} ion; Fe^{2+} chelating activities ranged from 20.13% to 26.01%. These results are in agreement with the results that reported by other researchers. Jamdar *et al*. (2010) showed that the chelating activity of peanut protein hydrolysates at 20% DH, 30% DH and 40%DH were 66%, 78% and 85%, respectively.

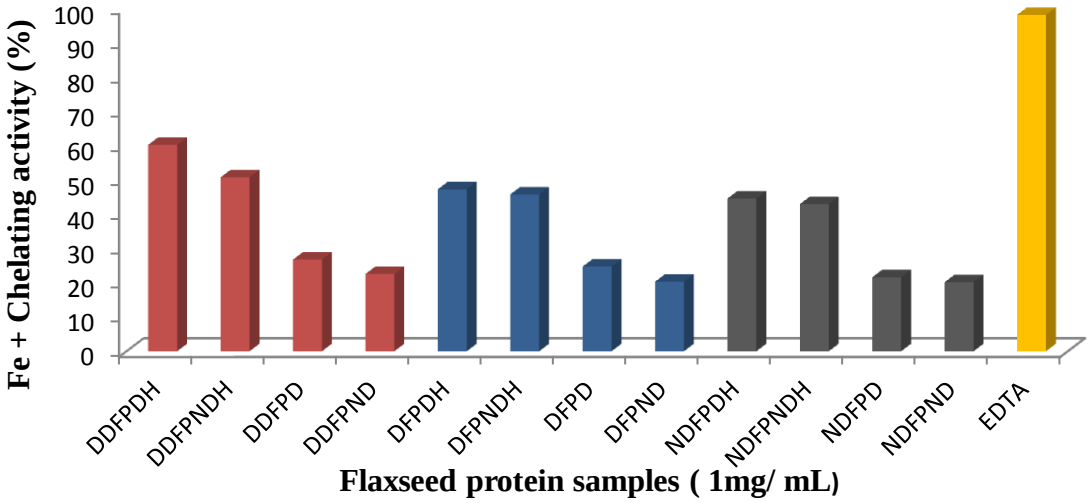

Figure 4.13 Fe^{+2} chelating activities of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD), defatted flaxseed protein/non-dialysis (DFPND), demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD), and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND), non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD), non-defatted flaxseed protein/non-dialysis (NDFPND) using trypsin and (EDTA) as control.

4.2.2 Antioxidant Activity of Flaxseed Proteins and Protein Hydrolysates using Bacterial Protease

4.2.2.1 DPPH Scavenging Activity

Figure 4.14 shows the results for the DPPH scavenging activity of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD), and defatted flaxseed protein/non-dialysis (DFPND). In general, the flaxseed proteins/dialysis showed higher ($p < 0.05$) DPPH scavenging activity than flaxseed proteins/non-dialysis; this is because of that removing the salt led to increase protein concentration and increase in the repulsive forces between the ionic groups of the surfactant molecules which affect on the antioxidant activity (Noipa *et al*., 2011). Defatted flaxseed protein hydrolyzed and non-hydrolyzed results showed an increase in the antioxidant activity with increasing protein concentration. The maximum DPPH scavenging activity was observed in DFPDH compared to DFPNDH with 64.19% and 60.34%, respectively. Non-hydrolyzed samples for DFPD and DFPND exhibited a range of 28.88-51.10% DPPH radical scavenging activity.

Figure 4.15 shows the DPPH scavenging activity of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND). The DDFPDH showed the highest values of DPPH radical scavenging activity with a range of 64.16-73.23% in comparison to the results obtained for DDFPNDH with 49.68-58.10% DPPH radical scavenging activity at protein concentration of 1-2.5 mg/ml.

Figure 4.16 shows the results the DPPH scavenging activity of non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND). In comparison with defatted flaxseed protein isolates and demucilaged and defatted flaxseed protein isolates, the non-defatted flaxseed protein isolates also showed an increase in the scavenging activity with

increasing in the protein concentrations (1-2.5 mg/ml). NDFPDH and NDFPNDH showed DPPH radical scavenging activities of 59.06% and 53.83% at 2.5 mg/ml, respectively. These results are in agreement with those of other researchers. Xie *et al*. (2008) reported that the highest DPPH radical scavenging of alfalfa protein hydrolysates was 80% at concentration 1.8 mg/ml using alcalase Cumby *et al*. (2008) reported that the DPPH radical scavenging of canola protein increased with an increase of protein concentration and reached to 73.2% at 10 mg/ml using flavourzyme.

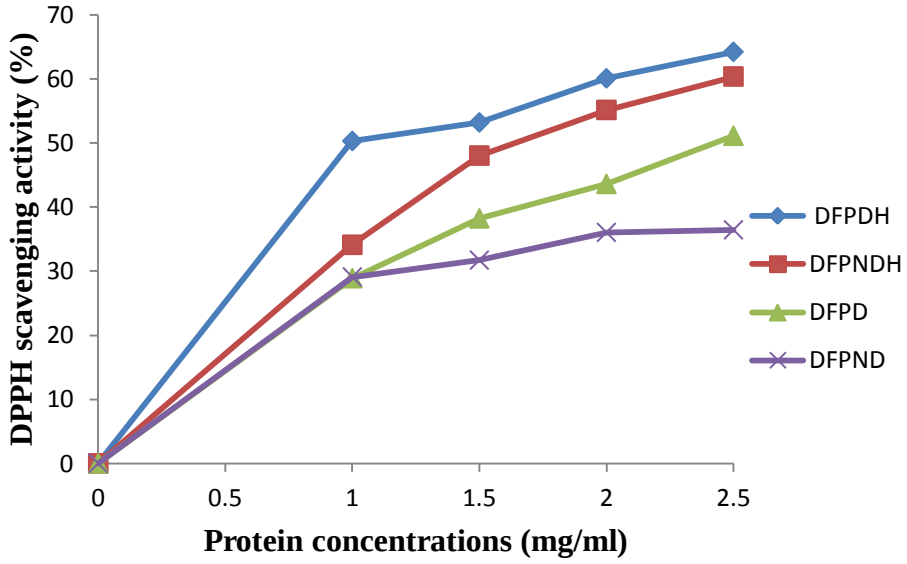

Figure 4.14 DPPH scavenging activity curve for defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) using bacterial protease.

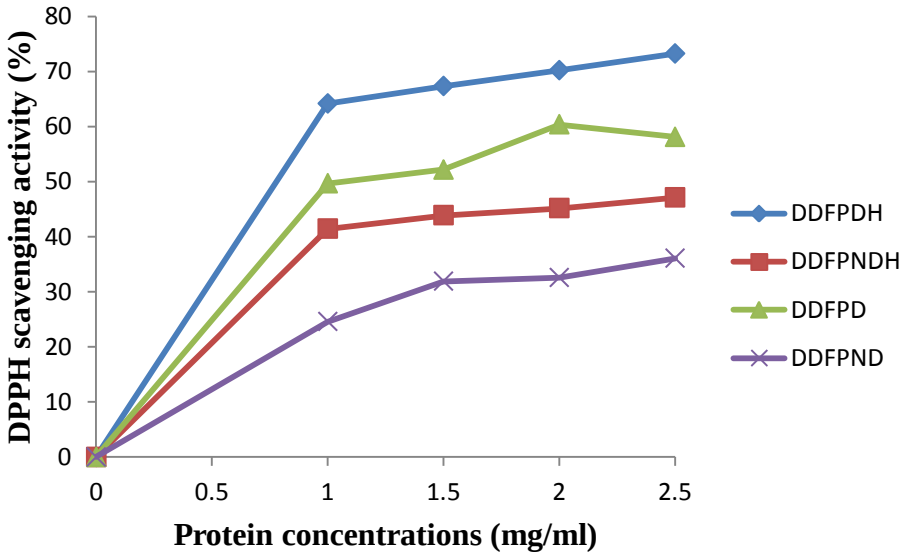

Figure 4.15 DPPH scavenging activity curve for demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) using bacterial protease.

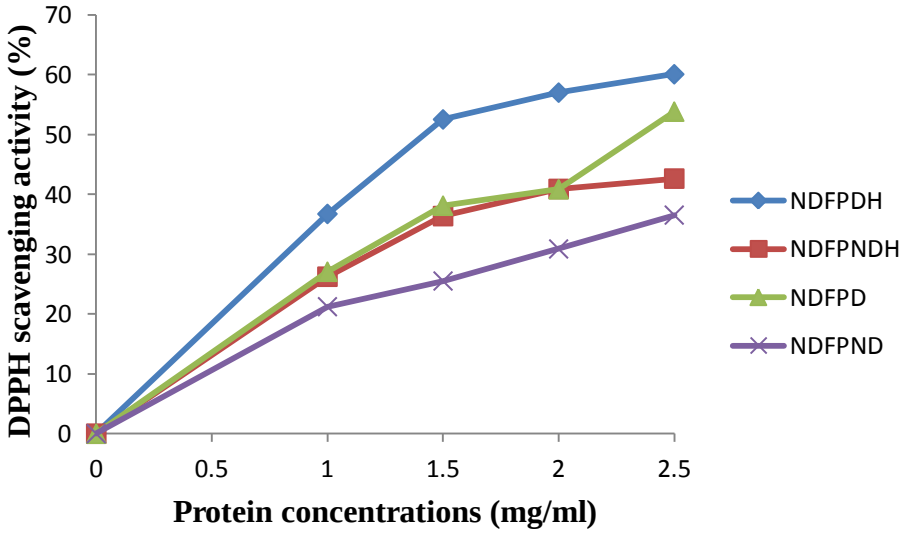

Figure 4.16 DPPH scavenging activity curve for non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND) using bacterial protease.

4.2.2.2 Reducing Power Activity

Figure 4.17 shows the results of reducing power of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND). The results showed an increase in the reducing power with increase protein concentrations (1-2.5 mg/ml). The reducing power of DFPDH was 0.14 compared to DFPNDH which was 0.1, while non-hydrolyzed DFPD and DFPND gave reducing power values of 0.08 and 0.06, respectively.

Figure 4.18 shows the results of reducing power of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND). The results were similar with those from defatted flaxseed protein isolates with an increase in DPPH scavenging activity for demucilaged and defatted flaxseed protein isolates with increase in protein concentration (1-2.5 mg/ml). The maximum reducing power activity was 0.17 obtained with DDFPDH and 0.13 with DDFPNDH.

Figure 4.19 shows the results of reducing power of non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND). The non-defatted flaxseed protein isolates showed lower ($p < 0.05$) reducing power activity as compared with defatted flaxseed protein isolates and demucilaged and defatted flaxseed protein isolates. NDFPDH and NDFPNDH showed reducing power activity of 0.08 and 0.07 respectively, while NDFPD and NDFPND gave reducing power of 0.058 and 0.05, respectively. These results are consistent with those obtained by other researchers. Xie *et al*. (2008) reported that the reducing power of alfalfa protein hydrolysates increased with an increase of the protein concentration and the highest reducing power was 0.69 at 2.0 mg/ml; similar

results have been shown with peanut protein hydrolysates (Jamadar *et al*., 2010). Zhao *et al*., (2012) reported that the reducing power of rice dreg (a by-product from rice syrup production contain ≥ 50% protein) protein hydrolysates ranged from 0.01to 0.4 using flavourzyme.

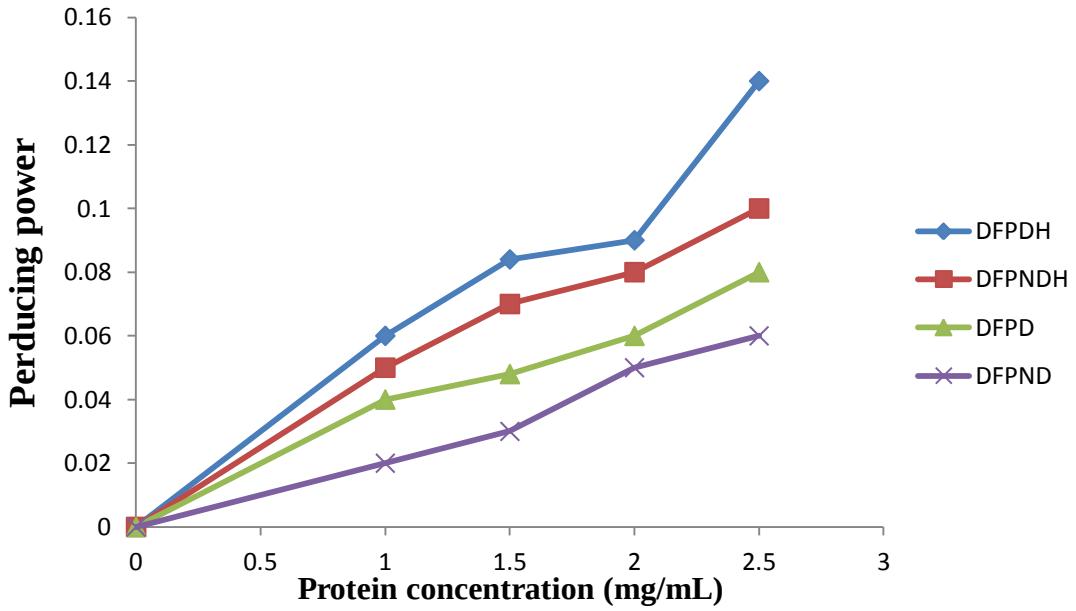

Figure 4.17 Reducing power of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein/non-dialysis (DFPND) using bacterial protease.

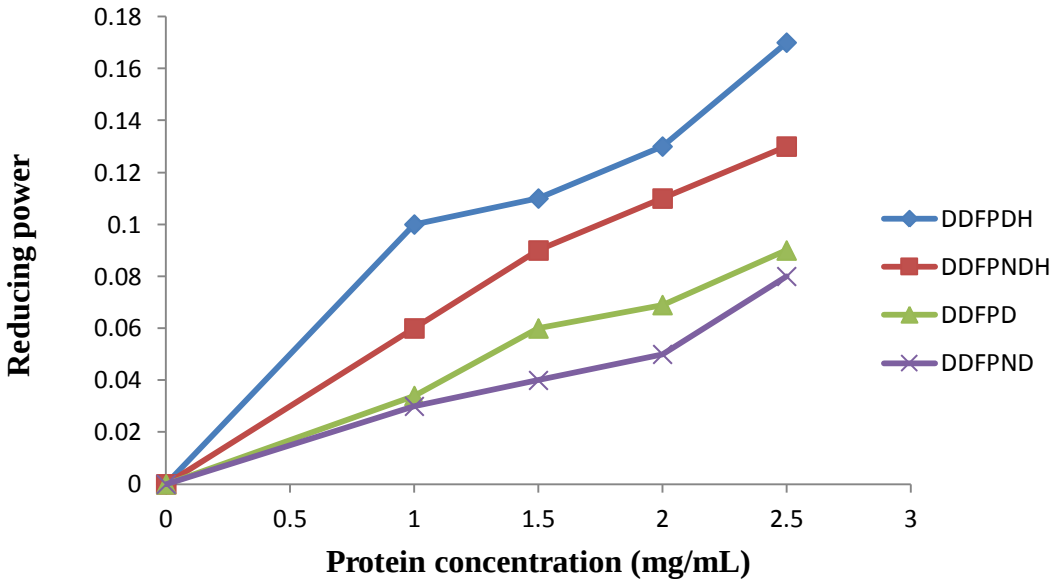

Figure 4.18 Reducing power of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) using bacterial protease.

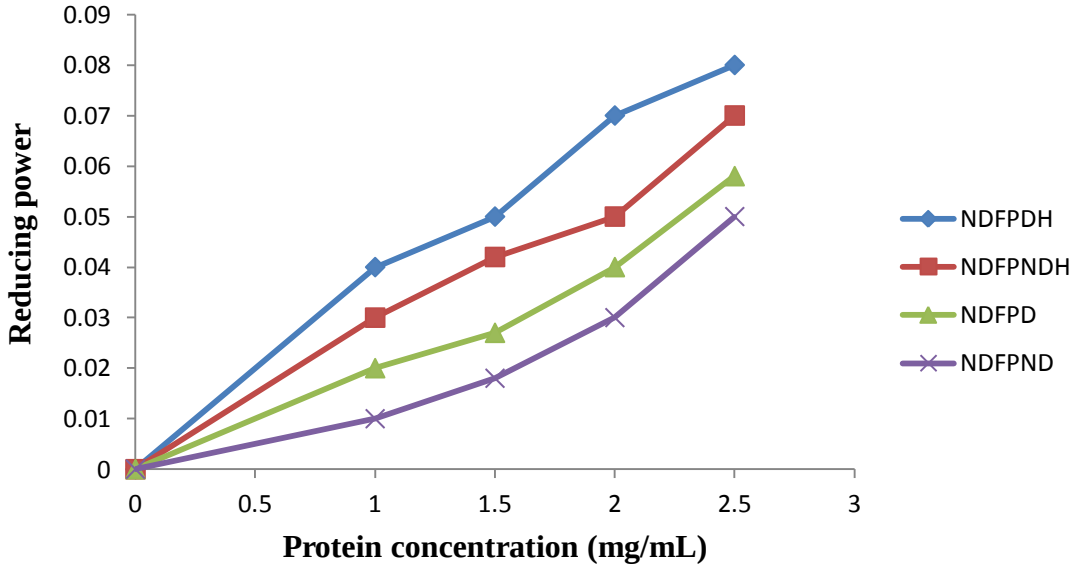

Figure 4.19 Reducing power of for non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND) using bacterial protease.

4.2.2.3 Metal Ion Chelating Activity

Figure 4.20 shows the ability to chelate the metal ions Fe^{2+} for defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), and non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH). Flaxseed proteins from the dialysis treatment showed the ability to chelate the iron higher ($p < 0.05$) than proteins from non-dialysis treatment. Compared to their non-hydrolyzed samples, Fe^{+2} chelating activities were highest in DDFPDH (75.87%), while Fe^{+2} chelating activities of DDFPNDH was 71.28%; DFPDH and DFPNDH showed Fe^{+2} chelating activities with 65.76% and 48.58%, respectively. Non-defatted flaxseed protein isolates had lower ability to chelate the iron activity as compared with other flaxseed proteins treatments; Fe^{+2} chelating activities for NDFPDH and NDFPDNH were 63.10% and 58.33%, respectively. Non-hydrolyzed samples of all flaxseed proteins isolates showed similar ability to chelate for ions Fe^{+2}. The ability of the non-hydrolyzed flaxseed proteins to chelate Fe^{+2} ranged from 20.13% to 26.01%. These results are in agreement with the findings reported in other studies. Carrasco-Castilla *et al*. (2012) reported that phaselolin hydrolysates had the iron chelating activity up to 81% at 210 min of hydrolysis.

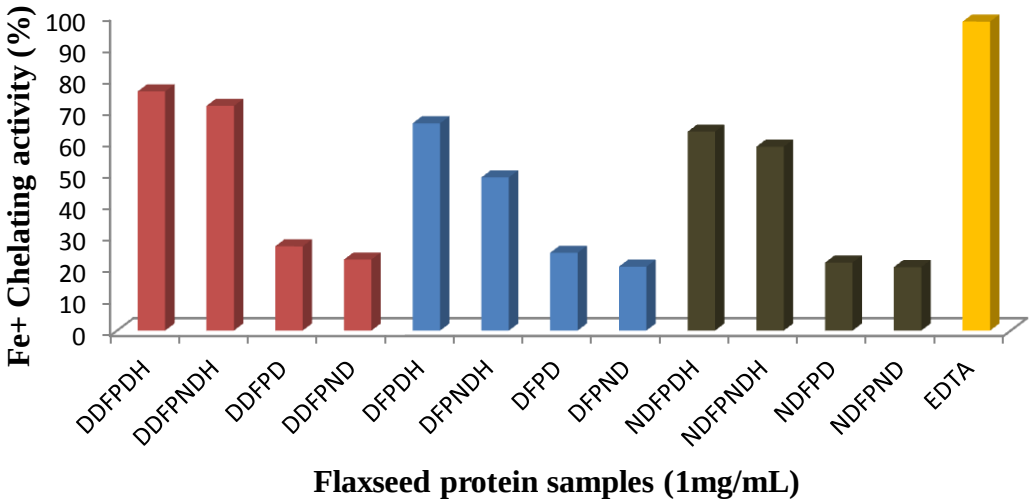

Figure 4.20 Fe^{2+} chelating activities of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD), defatted flaxseed protein/non-dialysis (DFPND), dimucilgae and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), dimucilgae and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), dimucilgae and defatted flaxseed protein/dialysis (DDFPD), and dimucilgae and defatted flaxseed protein/non-dialysis (DDFPND), non-defatted flaxseed protein/dialysis hydrolysates (NDFPDH), non- defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD), non-defatted flaxseed protein/non-dialysis (NDFPND) by bacterial protease and (EDTA) as control.

Table 4.7 The antioxidant activity of flaxseed proteins/ proteins hydrolysates

Flaxseed proteins	Antioxidants activity of flaxseed proteins		
	DPPH scavenging activity (%)	Reducing power activity	Metal ion chelating activity (%)
DFPD	51.1	0.08	24.67
DFPND	36.43	0.06	20.26
DFPDH [a]	61.1	0.12	47.19
DFPNDH [a]	58.09	0.092	45.69
DFPDH [b]	64.19	0.14	65.76
DFPNDH [b]	60.34	0.1	48.58
DDFPD	41.36	0.09	26.71
DDFPND	36.09	0.08	22.51
DDFPDH [a]	68.96	0.15	60.15
DDFPNDH [a]	58.10	0.12	50.68
DDFPDH [b]	73.23	0.17	75.87
DDFPNDH [b]	47.12	0.13	71.28
NDFPD	48.39	0.05	21.56
NDFPND	36.54	0.05	20.13
NDFPDH [a]	59.06	0.07	44.5
NDFPNDH [a]	53.83	0.06	42.87
NDFPDH [b]	60.08	0.08	63.1
NDFPNDH [b]	42.64	0.07	58.33

[a] Hydrolyzed by trypsin
[b] Hydrolyzed by bacterial protease

4.3 Characterization of Flaxseed Proteins and Protein Hydrolysates

4.3.1 SDS-PAGE Native–PAGE of Isolated Flaxseed Proteins and Protein Hydrolysates using Trypsin

Figure 4.22 shows SDS-PAGE and Native-PAGE of defatted flaxseed protein dialysis hydrolysates (DFPDH), defatted flaxseed protein non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein dialysis (DFPD) and defatted flaxseed protein non-dialysis (DFPND). DFPD gave four bands; three minor bands with molecular weight of 34, 23, and 16 KDa and the major band corresponding to 48 KDa. DFPND showed similar results to that obtained from DFPD. DFPNDH and DFPDH gave four bands with MW 48, 33, 22, and 16 KDa.

Figure 4.23 shows SDS-PAGE and Native-PAGE of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND). DDFPD gave four bands; the major band showed MW 48 KDa and the other minor bands with MW 34, 23, and16 KDa. Similar results were observed for DDFPND. DDFPDH showed three bands with molecular weight 35, 22, and 14 KDa while DDFPNDH gave three bands with MW 35, 22 and 14 KDa which were resistant to hydrolysis.

Figure 4.24 shows the result of SDS-PAGE and Native-PAGE of non-defatted flaxseed proteins/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND). NDFPD and NDFPND both gave four bands, a major band MW 48 KDa and three minor bands with MW 34, 23, and 16 KDa. NDFPDH showed three bands with MW 33, 24 and 14 KDa while NDFPNDH gave four bands, one band resistant to hydrolysis with MW 14 KDa and three bands with MW 33, 24, and 22 KDa. Native-PAGE results of NFPD, NDFPND, NDFPDH, and NDFPNDH. NDFPD and NDFPND gave one band. These findings are similar to that obtained with defatted flaxseed protein isolates; Ayad (2010) reported that flaxseed protein fractions, globulin gave one intense band with MW 23 KDa, while albumin and glutelin were identified as two intense bands with MW 22, 24 KDa and 22,

23 KDa respectively; two minor bands with MW 9, 33 KDa has been observed from albumin protein fraction and three minor bands observed from glutelin protein fractions with MW 35, 45, and 55 KDa. While globulin showed three minor bands with MW 10, 24 and 33 KDa; Native- PAGE of globulin, glutelin, and albumin showed one band.

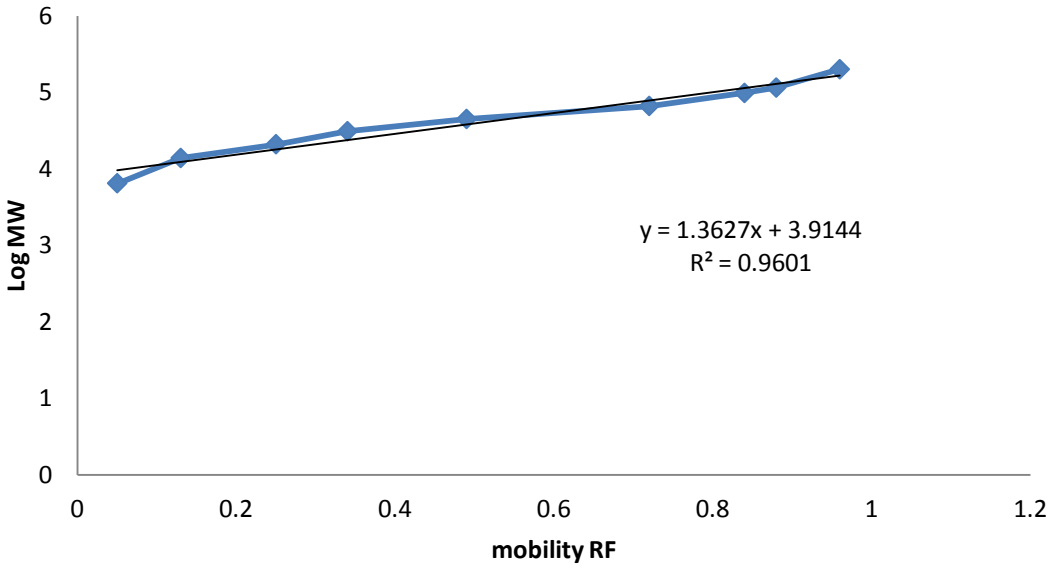

Figure 4.21 Standard curve generated by plotting the log of the molecular weight of protein standards vs. the relative mobility.

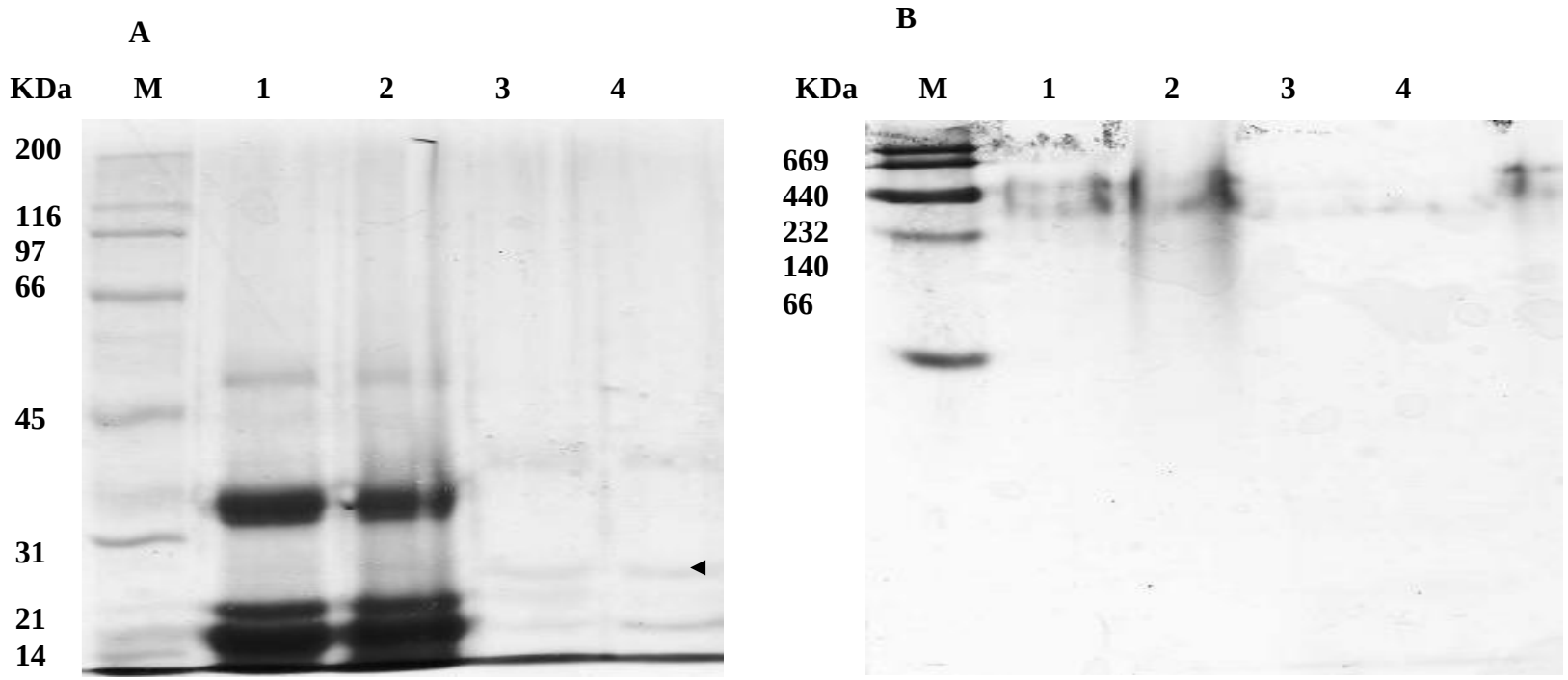

Figure 4.22 SDS-PAGE (**A**) and Native-PAGE (**B**) of defatted flaxseed protein/dialysis (DFPD) (control) (1), defatted flaxseed protein/non-dialysis (DFPND) (control) (2), defatted flaxseed protein/dialysis hydrolysates (DFPDH) (3), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH) (4) using trypsin and standard protein markers (M).

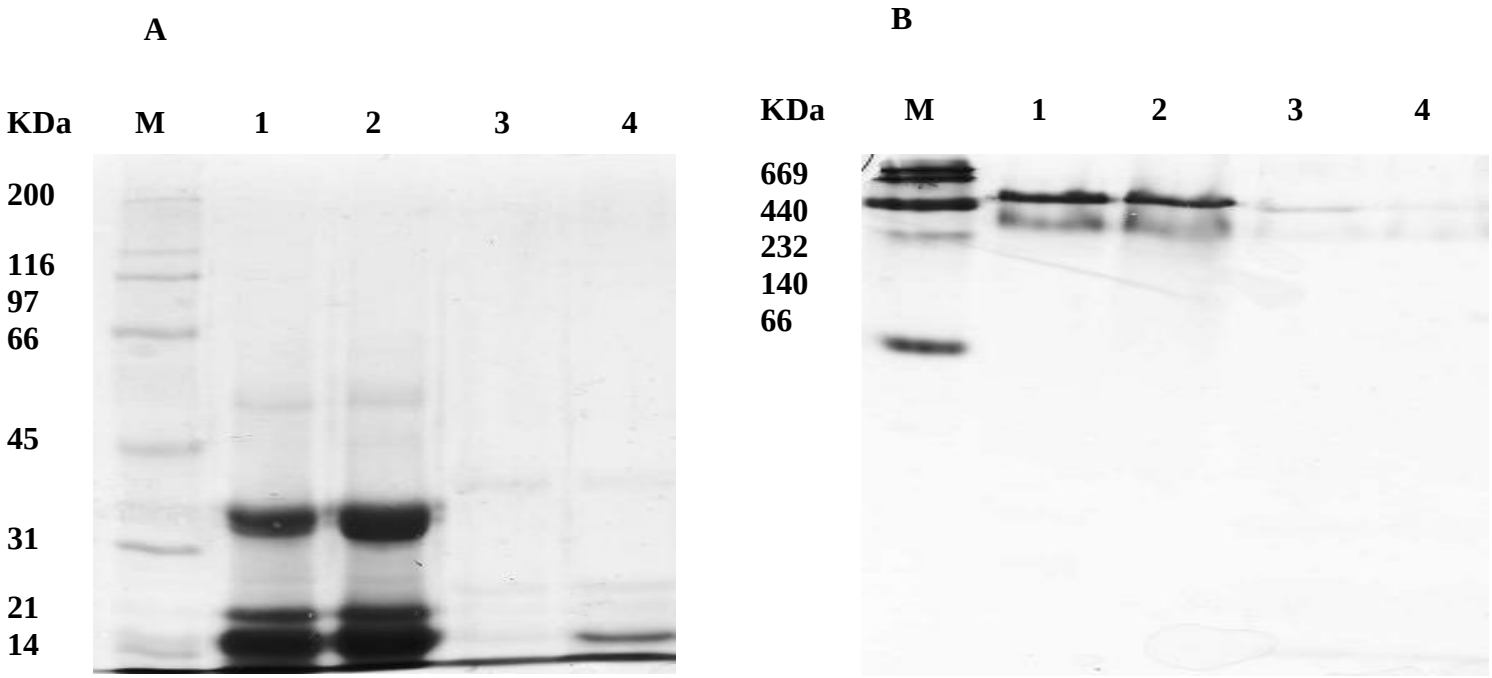

Figure 4.23 SDS-PAGE (**A**) and native-PAGE (**B**) of demucilaged and defatted flaxseed protein/dialysis (DDFPD) (control) (1), demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) (control) (2), demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH) (3), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH)(4) using trypsin and standard protein markers (M).

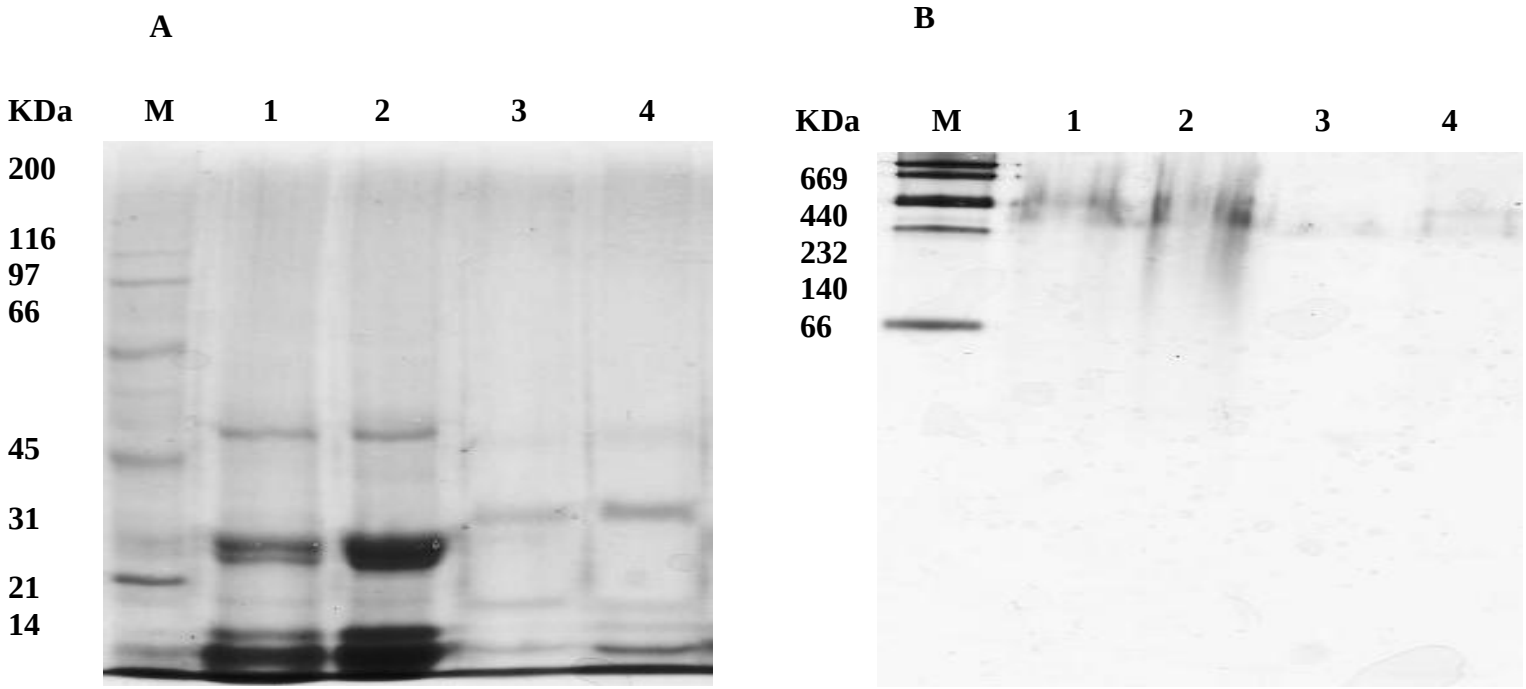

Figure 4.24 SDS-PAGE (**A**) and Native-PAGE (**B**) of non-defatted flaxseed protein isolates/dialysis (NDFPD) (control) (1), non-defatted flaxseed protein isolates/non-dialysis (NDFPND) (control) (2), non-defatted flaxseed protein isolates/dialysis hydrolysates (NDFPDH) (3), non-defatted flaxseed protein isolates/non-dialysis hydrolysates (NDFPNDH) (4) using trypsin and standard protein markers (M).

4.3.2 SDS-PAGE and Native –PAGE of Isolated Flaxseed Proteins and Protein Hydrolysates using Bacterial Protease

Figure 4.25 shows SDS-PAGE and Native-PAGE of defatted flaxseed protein/dialysis hydrolysates (DFPDH), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH), defatted flaxseed protein/dialysis (DFPD) and defatted flaxseed protein non/dialysis (DFPND). DFPD gave four bands; three minor bands with MW 34, 23, and 16 KDa, one of which was a major band corresponding to 48 KDa. DFPND showed similar results obtained with DFPD. DFPNDH and DFPDH gave four bands with MW 48, 33, 22, and 16 KDa. Native-PAGE of DFPD, DFPND and the hydrolysates of these proteins DFPDH and DFPNDH gave on band.

Figure 4.23 shows SDS-PAGE and Native-PAGE of demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH), demucilaged and defatted flaxseed protein/dialysis (DDFPD) and demucilaged and defatted flaxseed protein/non-dialysis (DDFPND). DDFPD gave four bands, the major band with MW 48 KDa and the other minor bands with MW 34, 23, and16 KDa. Similarly result has shown in DDFPND. DDFPDH and DDFPNDH showed no bands. Native-PAGE of DDFPD and DDFPND and the hydrolysates of these proteins DDFPDH and DDFPNDH showed one band.

Figure 4.24 shows the SDS-PAGE and Native-PAGE of non-defatted flaxseed proteins/dialysis hydrolysates (NDFPDH), non-defatted flaxseed protein/non-dialysis hydrolysates (NDFPNDH), non-defatted flaxseed protein/dialysis (NDFPD) and non-defatted flaxseed protein/non-dialysis (NDFPND). NDFPD and NDFPND both gave four bands; the major band was MW 48 KDa and there minor bands with MW 34, 23, and 16 KDa. NDFPDH showed two bands with MW 31 and 16 KDa while NDFPNDH gave one band with MW 16 KDa. Native-PAGE results of NFPD, NDFPND, NDFPDH NDFPNDH, NDFPD and NDFPND showed one band.

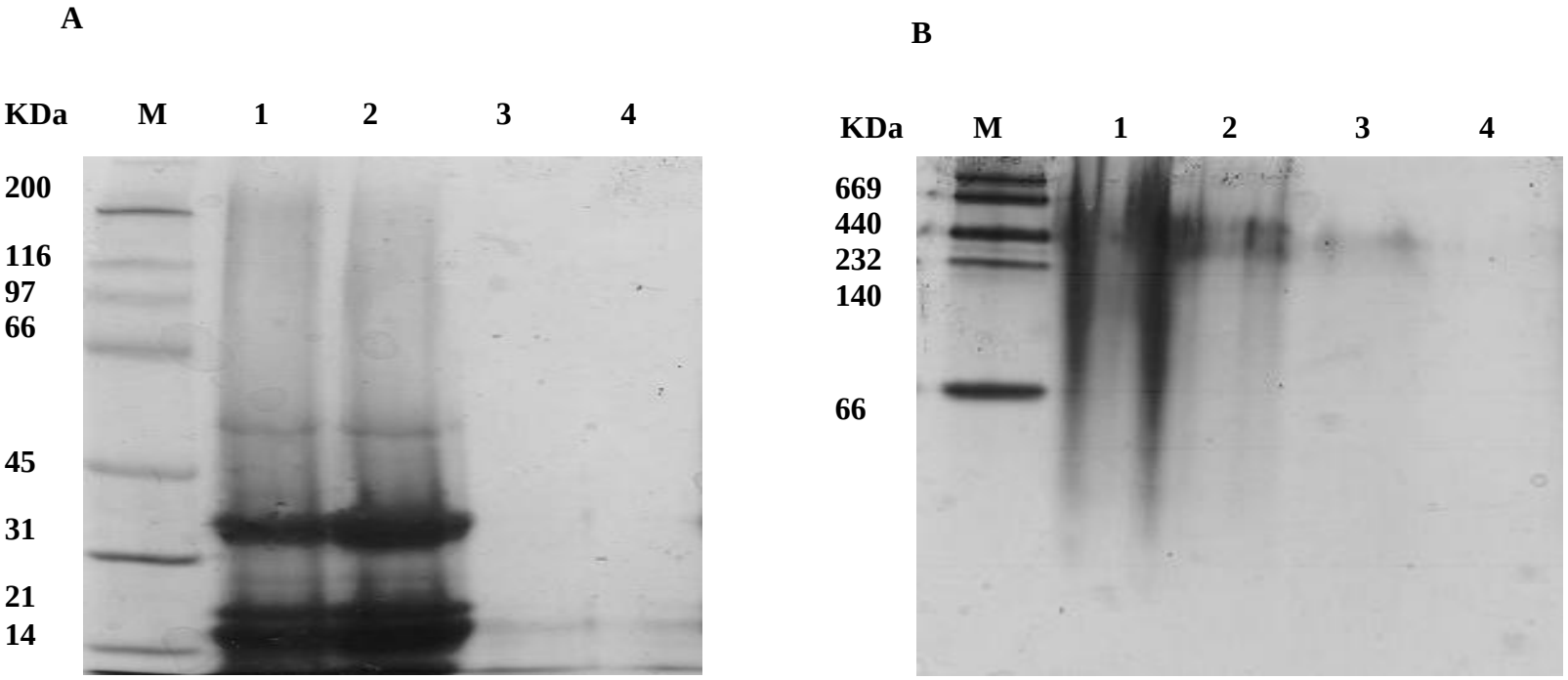

Figure 4.25 SDS-PAGE (**A**) and native-PAGE (**B**) of defatted flaxseed protein/dialysis (DFPD) (control) (1), defatted flaxseed protein/non-dialysis (DFPND) (control) (2), defatted flaxseed protein/dialysis hydrolysates (DFPDH) (3), defatted flaxseed protein/non-dialysis hydrolysates (DFPNDH) (4) using bacterial protease and standard protein markers (M).

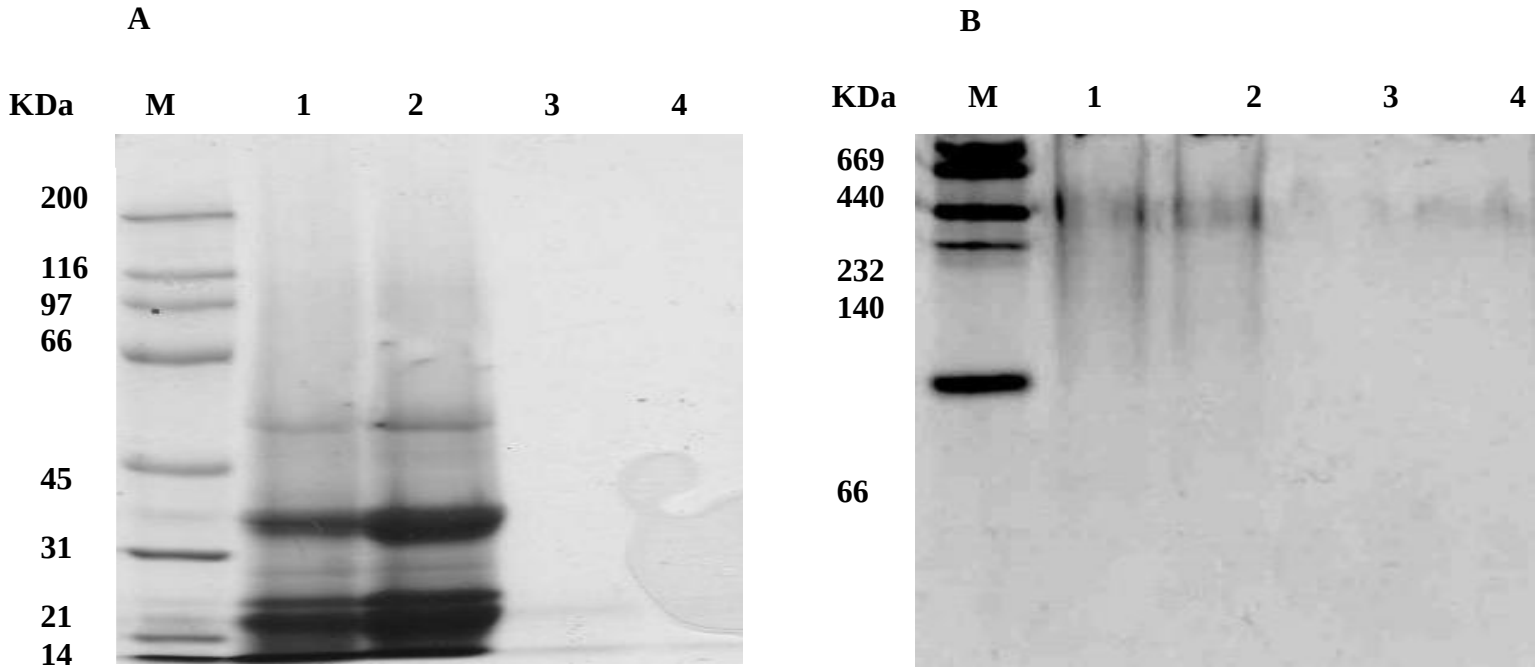

Figure 4.26 SDS-PAGE (**A**) and native-PAGE (**B**) of demucilaged and defatted flaxseed protein/dialysis (DDFPD) (control) (1), demucilaged and defatted flaxseed protein/non-dialysis (DDFPND) (control) (2), demucilaged and defatted flaxseed protein/dialysis hydrolysates (DDFPDH) (3), demucilaged and defatted flaxseed protein/non-dialysis hydrolysates (DDFPNDH)(4) using bacterial protease and standard protein markers (M).

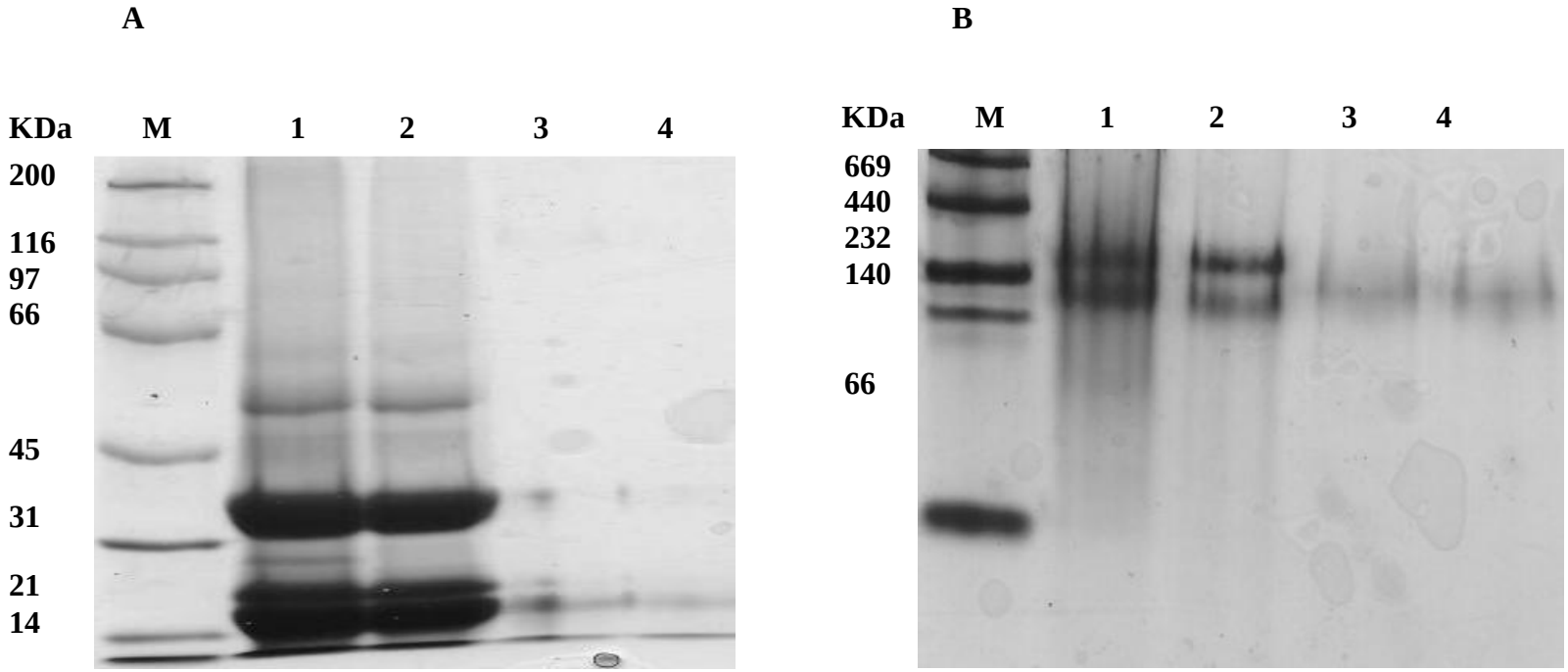

Figure 4.27 SDS-PAGE (**A**) and native-PAGE (**B**) of non-defatted flaxseed protein isolates/dialysis (NDFPD) (control) (1), non-defatted flaxseed protein isolates/non-dialysis (NDFPND) (control) (2), non-defatted flaxseed protein isolates/dialysis hydrolysates (NDFPDH) (3), non-defatted flaxseed protein isolates/non-dialysis hydrolysates (NDFPNDH) (4) using bacterial protease and standard protein markers (M).

GENERAL CONCLUSION

The degree of hydrolysis of demucilaged and defatted flaxseed protein, defatted and non-defatted flaxseed protein increased with increase in the time of hydrolysis for both dialysis and non-dialysis treatments using bacterial protease and trypsin. Using bacterial protease, the highest degree of hydrolysis for demucilaged and defatted flaxseed protein dialysis was 30 % DH as compared with defatted and non-defatted flaxseed protein/dialysis which were 28 % and 14 %, respectively. Unhydrolyzed samples for demucilaged and defatted flaxseed protein and defatted flaxseed protein showed unchanged level of degree of hydrolysis with range from 2.39 to 3.82 % (DH) while non-defatted flaxseed protein/dialysis showed DH increase from 3.8 to 5.55% with the time of hydrolysis.

Dialysis treatment showed positive effect on the antioxidant properties of flaxseed proteins. The antioxidant activity of flaxseed proteins increased with increase of protein concentrations. Hydrolyzed demucilaged and defatted flaxseed protein/dialysis showed antioxidant activity as follows: 73.23% DPPH scavenging activity, 0.15 reducing power activity and 75.8% iron chelating activity; these are higher compared with hydrolyzed defatted and non-defatted flaxseed proteins. Non-hydrolyzed demucilaged and defatted flaxseed protein/dialysis showed antioxidant activity as follows:- 58 % DPPH scavenging activity, 0.09 reducing power activity at 700 nm and 26 % iron chelating activity at 1 mg/ml.

SDS-PAGE results confirmed the hydrolysis of demucilaged and defatted flaxseed protein and defatted and non-defatted flaxseed protein; four bands with different molecular weights were observed for dialysis and non-dialysis unhydrolyzed demucilaged and defatted flaxseed protein and defatted and non-defatted flaxseed protein. SDS-PGAE showed one resistant band for hydrolyzed demucilaged and defatted flaxseed protein/non-dialysis and hydrolyzed non-defatted flaxseed protein/non-dialysis.

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