Isolation of Cadmium-Binding Components from Proteins of Flaxseed (*Linum usitatissimum* L.)

by

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

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Food Science)

THE UNIVERSITY OF BRITISH COLUMBIA

July 2006

ABSTRACT

Flaxseed contains the toxic heavy metal cadmium (Cd) at concentrations often exceeding the recommended maximum dietary intake limit. The objectives of this study were to investigate the distribution of Cd-binding components in flaxseed, and to establish a protocol for separating Cd-binding components from storage proteins. The results indicated that over 80% of Cd in flaxseed (NorMan cultivar) was extracted with proteins into Tris buffer. Fractionation of protein extracts by ion exchange chromatography yielded a major Cd-binding fraction eluting at 0.1 M NaCl with over 50% of the eluted Cd and less than 8% of the eluted proteins, while the major storage proteins were eluted into the 0.25 M NaCl fraction containing over 60% of the eluted protein and less than 25% of the eluted Cd. The Cd level in flaxseed was strongly influenced by growing location. Similar trends in percent distribution of Cd and protein for 15 flaxseed samples (5 cultivars grown in 3 locations) suggest that this fractionation protocol could form the basis for developing an industrial process to produce flaxseed proteins with low Cd content for use as food ingredients.

Further separation of the 0.1 M NaCl eluted major Cd-binding fraction by size exclusion chromatography resulted in three peaks. The major components in the first two peaks were a 19 kDa protein and a 14 kDa protein, while 0.6-0.9 kDa constituents in the third peak were comprised of unusual amino acids or organic acids and a 649.7 Da component tentatively identified as Cd-(γ-Glu-Cys)$_2$Gly. The second and third peaks bound over 40% of the Cd contained in the flaxseeds.

This study also demonstrated *in vitro* protective effects of flaxseed components against toxicity by Cd and H_2O_2. The protein extract and the major Cd-binding fraction at a concentration of 110 µg/mL reduced Cd (300 µM) toxicity in THP-1 cell culture by 14% and

44%, respectively, and reduced H_2O_2 (0.06%) toxicity by 48% and 89%, respectively. The protein extract was also found to promote THP-1 cell growth in a dose dependent manner. Further investigations should be conducted to explore the underlying mechanisms and potential applications of flaxseed constituents.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

0.10 M NaCl fraction	Fraction eluted by 0.1 M NaCl from DEAE Sephacel ion exchange chromatography
0.25 M NaCl fraction	Fraction eluted by 0.25 M NaCl from DEAE Sephacel ion exchange chromatography
0.45 M NaCl fraction	Fraction eluted by 0.45 M NaCl from DEAE Sephacel ion exchange chromatography
0.50 M NaCl fraction	Fraction eluted by 0.50 M NaCl from DEAE Sephacel ion exchange chromatography
1.0 M NaCl fraction	Fraction eluted by 1.0 M NaCl from DEAE Sephacel ion exchange chromatography
AAFC	Agriculture and Agri-Food Canada
AAPH	2,2'-azobis(2-amidino-propane)
Cd	Cadmium
DEAE	Diethylaminoethyl
DPBS	Dulbecco's phosphate buffered saline
FM	Flaxseed cultivar Flanders grown in Morden
FP	Flaxseed cultivar Flanders grown in Portage la Prairie
G50	Sephadex G50 column
HMW	High molecular weight complex
HMW peak	The first peak eluted from Sephadex G50 size exclusion chromatography
ICP-MS	Inductively coupled plasma – mass spectrometry
IEF	Isoelectric focusing
kDa	Kilo Daltons
LMW	Low molecular weight complex

MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MCF	Major Cd-binding fraction
ME	2-Mercaptoethanol
MPF	Major storage protein fraction
MT	metallothionein
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
MWCO	Molecular weight cutoff
MMW	Medium molecular weight complex
N/A	Not applicable
PC	Phytochelatin
PE	Protein extract from flaxseed defatted powder
Peak A	The second peak eluted from Sephadex G50 size exclusion chromatography
Peak B	The third peak eluted from Sephadex G50 size exclusion chromatography
PMS	Phenazine methosulfate
ppb	Parts per billion
QAE	Quaternary aminoethyl
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH & SS	Sulfhydryl (SH) and disulfide (SS) groups
TIC	Total ion current
UV/Vis	Ultraviolet/visible absorbance

Dedicated

to my Father and Mother

ACKNOWLEDGEMENTS

I am sincerely grateful to the members of my family and my in-laws for their support and encouragement throughout my Ph.D. studies. I especially want to thank my wife Hua Li, for her support and understandings. I also want to thank my beloved daughter Liya, for the happiness she gave to me through the most challenging times, which is one of the most important spiritual supports for my study and life in university.

It is most difficult to express my deep gratitude and the feeling of dearly debt to my father and mother, from whom I always get encouragement for my academic careers, but I never had a chance to fulfill the primary duty of filial piety. Because of this, I dedicate this thesis to my father and mother to express my great thankfulness for their understandings.

CO-AUTHORSHIP STATEMENT

Part of the work summarized in Chapter 2 was published in the Journal of Agricultural and Food Chemistry 51(3): 814-821, 2003, entitled "Distribution of Cadmium-Binding Components in Flax (*Linum usitatissimum* L.)".

The thesis author (Bo Lei) is the principal author and his supervisor (Professor E. C. Y. Li-Chan), Dr. B. D. Oomah and Dr. G. Mazza are the co-authors for this contribution.

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CHAPTER 1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1   INTRODUCTION

Flax (*Linum usitatissimum* L.) is an economically important oilseed crop in Canada, which contributes over 35% of the world flaxseed production (Figure 1-1). Canada is the largest producer and exporter of flaxseed in the world, and the world flaxseed production is largely influenced by the production in Canada (Agriculture and Agri-Food Canada, 2005). Apart from its well-known value as an oilseed crop, flaxseed has turned out to be a functional food ingredient with increasing importance in the world market (Morris, 2005), because of its medicinal effects that may help reduce the risk of cardiovascular diseases, cancers and gastrointestinal disorders (Cunnane et al., 1995; Hasler, 1998; Stavro et al., 2003; Thompson, 2003; Mozaffarian, 2005). Flaxseed has been listed as one of the 40 cancer-preventive foods (Caragay, 1992).

However, flaxseed often accumulates significant amounts of cadmium (Cd) that exceed the recommended dietary critical limit of 0.3 μg Cd/g seed (0.3 ppm) (Marquard et al., 1990; Moraghan, 1993; Cieslinski et al., 1996; Becher et al., 1997). Cd is a toxic element that can accumulate in the human kidney through the food chain and cause renal tubular dysfunction and pulmonary emphysema (Bernard, 2004). International marketing of flaxseed to Northern European nations requires meeting strict Cd limits (Li et al., 1997). The German guideline value for Cd in flaxseed is 0.3 ppm (Marquard et al., 1990). The Codex Alimentarius of FAO/WHO has adopted the maximum limit of 0.1 ppm in cereal grains traded on the international market, and the same limit for flaxseed is now under debate (FAO/WHO, 2003). The Cd content of 109 accessions from the world collection of flaxseed

1

ranges from 0.075 to 2.775 ppm, with over 40% of the population higher than 1.4 ppm (Oomah & Kenaschuk, 1995). A range of 0.14 to 1.37 ppm of Cd contents has been reported for flaxseed grown in North America (Li et al., 1997). Therefore, intensive research is needed to identify the mechanism of Cd accumulation in flaxseed in order to reduce the risk of Cd toxicity and to ensure continued safety with increased flaxseed utilization in food and medicinal industries.

1.2   FLAXSEED: AN IMPORTANT OILSEED & FUNCTIONAL FOOD

Oilseed flax, commonly called linseed outside North America, is commonly grown in Canada and in the USA on neutral and calcareous soil (Moraghan, 1993). Flaxseed is recognized as having about 40% oil, 30% dietary fiber, 20% protein, 4% ash, and 6% moisture (Daun et al., 2003). Estimates of proximate composition vary considerably according to the samples obtained and the methodology employed to measure each of the components. According to the Canadian Grain Commission, Canadian grown flaxseed contains 39.8-45.6% oil, 30.5-36.8% fiber, 17.4-24.1% protein, and 4.2-4.9% moisture (Daun et al., 2003). Flaxseed is also characterized as being made up of about 45% oil and 55% meal on a dry basis (Daun et al., 2003). The defatted flaxseed meal contains 7% ash, 44% protein, 9% crude fiber, and 40% nitrogen-free constituents (calculated by difference from 100% minus other components). The proximate composition of whole flaxseed and defatted meal varies considerably, depending on cultivar, growing condition, seed processing, and analytical methods (Bhatty, 1995).

The major nutrients of flaxseed are oil and protein; the latter is concentrated in the defatted meal, which is more commonly known as linseed meal, the by-product of the

flaxseed-crushing industry. The high level of α-linolenic acid (18:3n-3) (57% of total fatty acid) in flaxseed oil is commercially exploited in the manufacture of several industrial products, such as paints and plastics (Oomah & Mazza, 1993). α-Linolenic acid derives most of its importance in nutrition and health because it is the "parent" n-3 unsaturated fatty acid (PUFA). Flaxseed oil contains a very high level of unsaturated fatty acids. The level of α-linolenic acid in the oil of individual farm samples of Canadian flaxseed harvested in 2002 was found to range from 52 – 63% (Daun et al., 2003). This makes flaxseed the richest commonly accessible food source of α-linolenic acid (Cunnane, 2003).

According to Oomah and Mazza (1993), the flaxseed protein contents in the collection of world accessions are in the range of 20.9 to 48.1% (nitrogen % x 5.41). The major storage proteins of flaxseed are 11-12S globulin- and 2S albumin-type (Wanasundara & Shahidi, 2003). The total flaxseed protein (albumin and globulin together) has an amino acid pattern comparable to soybean protein (Wanasundara & Shahidi, 2003). The percentage ratio of the essential to total amino acids of flaxseed protein is well above the 36% value that is reported for ideal proteins as recommended by FAO/WHO in 1973 (Wanasundara & Shahidi, 2003). The Fischer ratio (branched chain amino acids/aromatic amino acids) of flaxseed is high and comparable to that of soybean protein (Oomah, 2001). Proteins with a high Fischer ratio are suitable for developing functional foods for special needs, such as malnutrition associated with burns, cancer, liver failure, and trauma, and for nutritional support of children with chronic or acute diarrhea or milk protein allergies (Wanasundara & Shahidi, 2003).

The consumption of the various forms of flaxseed as a functional food ingredient could be traced back to 5000 BC. The new information from the research in the last decade

has generated increasing interest in flaxseed, which contains biologically active phytochemicals believed to possess many medicinal effects. It is expected that flaxseed may become one of the most useful functional foods, because it contains high levels not only of α-linolenic acid, but also phenolic compounds known as lignans (>500 µg/g, as dry basis) (Oomah, 2001). These and other flaxseed components are particularly attractive for incorporation in the diet in the development of foods with specific health advantages. Flaxseed polysaccharide gum or mucilage may have nutritional value as a source of dietary fiber (Daun et al., 2003). The consumption of flaxseed has been evaluated and believed to be related to reducing or preventing the risk of diabetes, coronary heart disease, colon and rectal cancer (Setchell et al., 1981; Oomah & Mazza, 2000; Oomah, 2001; Thompson, 2003). A 25 kDa protein isolated from flaxseed has been demonstrated to have antifungal properties (Borgmeyer et al., 1992).

Another major reason for the use of flaxseed as a food ingredient is the uniqueness of its food functionality. The high content of mucilage material of flaxseed, which has thickening and emulsifying properties, has potential for use as a stabilizer and thickening agent in food products (Mazza & Oomah, 1995). Flaxseed protein has been underutilized because it mainly exists in the defatted meal, which is normally used as feed or discarded. However, the protein fraction contains a favourable ratio of amino acids, with lysine, threonine and tyrosine as the limiting amino acids. It is a good source of the sulphur amino acids methionine and cystine (Oomah & Mazza, 1993). In addition to the nutritional characteristics, flaxseed protein provides prominent functional roles in foods. These functional characteristics include solubility, rheological behaviour, emulsifying capacity, and foaming and whipping ability (Oomah & Mazza, 1993). There is increasing use of whole

flaxseed as an ingredient in a variety of foods, including breakfast foods and breads (Hocking & McLaughlin, 2000).

1.3   FLAXSEED: HIGH Cd CONCENTRATION AND HUMAN HEALTH

With a density of 8.6 g/cm^3, Cd is classified as a heavy metal, and belongs to a group of metals with density higher than 5.0 g/cm^3, including chromium (Cr), mercury (Hg), lead (Pb), aluminium (Al), silver (Ag) and tin (Sn) (Toppi & Gabbrielli, 1999). It is a group IIB transition element (atomic number Z = 48, MW = 112.411). Cd is generally considered a soft ion with a ligand atom preference (in decreasing order) of $I^->Br^->Cl^->F^-$ and a preference for donor atoms in ligands of S>N>O. Its strong preference for SH containing ligands (particularly polythiols) is undoubtedly the principal basis for its toxicity in biological systems (Wagner, 1993).

Cd can be a potential threat to a wide range of biota because (i) it is not essential physiologically, and it is not metabolized, (ii) it is toxic to humans at concentrations lower than those toxic to plants, (iii) it is more mobile and available than other metals, and (iv) its effects on humans are cumulative. The major route of exposure for the nonsmoking general population is via food; the contribution from other pathways to total uptake is slight (Singh & McLaughlin, 1999). Because the half-life of Cd in the human body is about 20 years, the consumption of foods containing high levels of Cd may result in chronic toxicity (Jackson & Alloway, 1992; FAO/WHO, 1995).

Concerned about the transfer of Cd through food chain, WHO has set a maximum provisional tolerable intake limit for an adult at 60-70 µg Cd per day (WHO, 1972). The Codex Alimentarius Commission of FAO/WHO has proposed limits on the concentration of

Cd in cereal grains and oilseeds traded on the international markets; for flaxseed, the suggested limit is 0.1 ppm (FAO/WHO, 2003). Evidence from Germany (Marquard et al., 1990) and North America (Moraghan, 1993) indicates that flaxseed can accumulate undesirably high levels of Cd in its seed. Cd levels in flaxseed can easily exceed 0.3 ppm (Marquard et al., 1990). From historical information and the FDA's research, according to Vanderveen (1995), flaxseed either as whole seed or defatted meal is safe to be used as a food ingredient in bread. The human studies suggested that consumption of flaxseed at < 50 g/day does not present a significant health risk (Jenkins, 1995). However, the relatively high level of Cd in flaxseed may be a major concern if flaxseed is to be promoted as a functional food, which may increase the amount of flaxseed in the human diet, hence, the Cd.

Flaxseed often accumulates higher levels of Cd compared to cereal crops (Grant & Bailey, 1997). The Cd content of flaxseed is of the greatest concern as a Cd reservoir and as the pathway of Cd to man and animal. Although Cd has no essential function in plants, it is readily taken up from the soil and may accumulate to high levels in edible plant parts, consequently entering the human food chain (Jackson & Alloway, 1992). Cd is generally regarded as the element most likely to accumulate in the human food chain (Tahvonen, 1996).

Renal tubular damage is probably the most common adverse health effect related to Cd exposure in humans (Jarup et al., 2000). In several areas of Japan, Cd pollution of soil is subsequently bioaccumulated into rice grown for human consumption. These Cd-exposed cohorts have a high incidence of renal injury (Iwata et al., 1993). Likewise, Belgian, Chinese, and German populations have been identified as groups exposed to a sufficient amount of environmental Cd to manifest renal tubular damage (Jarup et al., 1998; Klaassen et al., 1999).

The accumulation of Cd in the renal cortex leads to renal tubular dysfunction, disturbances in calcium metabolism, hypercalciuria and the formation of renal stones (Tahvonen, 1996). Cd may alter human metabolism by competing with Fe, Cu, Zn, Mn and Se for ligands in biological systems (Kozlowska et al., 1993). Cd binds to the thiolate (S⁻) groups of enzymes, and to phospholipids and nucleic acids, and can interfere with oxidative phosphorylation. Cd may displace Zn in metallo-enzymes resulting in changes to their activities (Tahvonen, 1996).

Cd accumulates in renal tubular cells, with a half-life of 17 to 30 years, until a critical concentration of about 180 ppm in renal cortex is reached (Nordberg, 1992). Experimental and epidemiological studies are providing increasing evidence that Cd is carcinogenic, and that this effect, which is considered to be stochastic in character, can be considered to be the critical effect (Tahvonen, 1996). Recently, Cd has been categorized as "Known to be Human Carcinogens" by U.S. Department of Health and Human Services (2002). There are no specific methods that can effectively eliminate Cd. This is why the emphasis is on prevention of accumulation over long periods (Baldwin and Marshall, 1999).

1.4 Cd CONCENTRATIONS IN SOIL AND PLANTS

In areas not subject to gross anthropogenic pollution, Cd concentrations generally fall in the range of 0.1-2.0 ppm (Kabata-Pendias & Pendias, 1991). The average contents of Cd in surface soils worldwide lie between 0.06 and 1.1 ppm with a mean of 0.53 ppm (Kabata-Pendias & Pendias, 1991). The concentrations of Cd in surface agricultural soils of Canada ranges from 0.10 to 8.10 ppm with a mean of 0.56 ppm (Frank et al., 1976; McLaughlin et al., 1999). In comparison, the Canadian data for Cd in drinking water ranges from 3×10^{-6} to

9.7×10^{-5} ppm with a mean of 4.4×10^{-5} ppm (Dabeka et al., 1987). The level of Cd in the soil appears to be increasing over time (Jones and Johnston, 1989; Grant et al., 1998). According to Wagner (1993), non-polluted soil solutions contain Cd concentration ranging from 0.04 μM (≈ 0.004 ppm) to about 0.32 μM (≈ 0.036 ppm), while soil solutions with Cd concentration varying from 0.32 to about 1 μM (≈ 0.112 ppm) can be regarded as polluted to a moderate level. In soil solutions containing Cd concentration as high as 35 μM (≈ 3.934 ppm), Cd-'hyperaccumulating' species are almost the only ones that can grow (Sanita di Toppi & Gabbrielli, 1999).

Plants readily take up Cd from soil solution, but unlike the other heavy metals, Cd is not phytotoxic at the low concentrations (0.3 ppm) that pose concern from a human health viewpoint (McLaughlin et al., 1999). The phytotoxic concentration is 10 to 20 ppm (Kabata-Pendias & Pendia, 1991). Cd concentration in plants varies considerably among species and cultivars; moreover, different plant parts accumulate different amounts of Cd (Florjin & Beusichem, 1993; Page et al., 1981; Cieslinski et al., 1996). Most plants retain over 50% of the Cd taken up in their roots (Jarvis et al., 1976; Obata & Umebayashi, 1993). Strong root retention of Cd may occur in white lupin so that little Cd is transported to the shoot in the xylem. Further, Costa and Morel (1993) reported that some of the Cd taken up by white lupin root cells is actively excreted back out of the roots, thus reducing net plant uptake (Hocking & McLaughlin, 2000).

The concentration levels found in most vegetables, including bulbs, roots and tubers, are normally well below 0.05 ppm, whereas slightly higher average levels may be found in leafy vegetables such as spinach. Rice most frequently contains less than 0.1 ppm Cd (FAO/WHO, 1998). Cd concentration in flaxseeds grown at uncontaminated sites in North

America varied from 0.14 to 1.37 ppm (Li et al., 1997). Marquard et al. (1990) found that both geographic location and flaxseed cultivar influenced Cd accumulation in flaxseeds.

1.5 MAJOR MECHANISMS OF Cd STORAGE AND DETOXIFICATION IN PLANTS

Plants achieve the detoxification of heavy metal accumulation by a combination of biophysical and biochemical mechanisms. The biophysical mechanisms include (i) cell wall immobilization, such as Cd bound to pectic sites and histidyl groups of the cell wall of bush bean roots and leaves (Leita et al., 1996); (ii) preventing Cd ions from entering the cytosol through the action of the plasma membranes, which could theoretically represent the best defence mechanism (Sanita di Toppi & Gabbrielli, 1999); (iii) active effluent, which could be another way of detoxification as observed by the increased asparagine in root exudates of *Lactuca sativa* (lettuce) and *Lupinus albus* (white lupin) under Cd stress (Costa et al., 1997); (iv) vacuolar compartmentalization, which prevents the free circulation of Cd ions in the cytosol and forces them into a limited area (Sanita di Toppi & Gabbrielli, 1999).

All of the above mentioned biophysical mechanisms for detoxification are actually based on biochemical processes of metal chelation. As reviewed by Rauser (1999) and Kotrba et al. (1999), there are three main groups of molecules that function as metal chelators: proteins (mainly metallothioneins), peptides (phytochelatins), and small molecules including organic acids, amino acids and phytin.

9

1.5.1 Metallothioneins and Other Metal-Binding Proteins

1.5.1.1 Metallothioneins

The most significant and fascinating mechanism for the detoxification of metal in organisms is through metallothioneins (MTs), which are a group of Cys-rich proteins or peptides that in their reduced state provide thiols for metal chelation. The discovery of a Cd-binding, Cys-rich protein from horse kidney by Margoshes and Vallee (1957) was the seminal finding that marked the birth of a field of research focused on the study of a low-molecular-weight polypeptide superfamily, the MTs. Since that discovery, MTs have been found to be broadly distributed among animals, eukaryotic microorganisms, certain prokaryotes and plants (Rauser, 1999).

MTs can be subdivided into classes on the basis of structural similarities. Class I MTs are defined as polypeptides whose primary structure is related to mammalian MTs, while class II MTs display no or only a very distant sequence similarity to mammalian MTs. The Cys-X-Cys (where X is an amino acid residue other than Cys) and Cys-Cys sequence motifs are characteristic of and invariant for both class I and class II MTs (Kotrba et al., 1999).

The amino acid sequences of MTs from many mammalian sources reveal that all contain approximately 61 amino acid residues of remarkably similar composition, lacking in aromatic amino acids and histidine, while one third of the residues are cysteines. Their molecular weights range from 6 to 7 kDa. More importantly, all contain 20 Cys residues that remain invariant along the amino acid sequence. All 20 Cys residues have been shown to participate in the coordination of 7 mol of Cd or Zn per mol of MT. Coordination of these Cys residues results in a high binding affinity for Zn and Cd. Detailed structural properties of the individual mammalian MT metal coordinating sites have been obtained from ^{113}Cd NMR.

The seven atoms of bound Cd are arranged into two separate polynuclear metal clusters, one containing three and the other four metal ions (Figure 1-2, Klaassen et al., 1999). This satisfies the $metal_7(Cys)_{20}$ stoichiometry for MT.

The three-metal cluster forms a cyclohexane-like six-membered ring requiring 9 Cys thiolate ligands, whereas the four-metal cluster forms a bicyclo [3:1:3] structure, requiring 11 Cys thiolate ligands. The four-metal cluster comprises amino acid residues 31-61 (COOH terminus). The binding of metals to each cluster is ordered, with initial binding to the four-metal cluster. After these sites are saturated, binding occurs at the three-metal cluster. Binding in each cluster is cooperative. Release of metals is also cooperative, with metal leaving the three-metal cluster first. Therefore, the three-metal cluster is more labile in the sense that it gives up bound Cd ions more readily, whereas Cd is bound more tightly to the four-metal cluster (Klaassen et al., 1999).

Plants produce class II MTs that differ from the archetypal mammalian MT-I in their location and number of Cys. The first MT from plant was wheat germ Ec protein (Hofmann et al., 1984), it was approved to be a Zn-containing class II MT (Lane et al., 1987). The MT from wheat embryos has Cys in three domains (17 Cys residues), binds 5 molecules of Zn, and disappears with seedling development. The first 59 amino acids of the wheat MT protein have been sequenced (Kawashima et al., 1992). Proteins with Cys in two domains have been predicted from fifty-eight genes for MT-IIs from a range of plants (but not flaxseed). Most of the predicted proteins have not been isolated, and their metal binding is poorly documented (Rauser, 1999). In durum wheat, a gene sequence encoding a class II MT has been identified and expressed in *Escherichia coli*, this recombinant protein binds 4 ± 1 mol of Cd per mol of protein, and has a high tendency to form stable oligomeric structure (Bilecen et al., 2005).

Studies have shown the multiple physiological functions of MT as: (a) a "storehouse" for Zn and Cu, (b) a free-radical scavenger, and (c) protection against Cd toxicity. It appears that during evolution, the ability of MT to protect against Cd toxicity might have taken a more pivotal role in the maintenance of life processes, as compared with its other proposed functions. MT-null mice are "normal" but highly susceptible to Cd toxicity (Klaassen et al., 1999).

1.5.1.2 Other metal-binding proteins

Cd binding proteins that are either lacking Cys or containing aromatic amino acids have also been reported (Kotrba et al., 1999) and are called MT-like proteins, because these proteins do show, at least on the primary structure level, some features distinct from those of MTs. Cd-binding glycoproteins from mushroom (Meisch et al., 1983) and bivalve (Nair & Robinson, 1999) were also reported as MT-like proteins.

Discovery of MT-like proteins originally resulted from screening of cDNA libraries in a search for ethylene-regulated genes, root-specific mRNAs, transcripts repressed by elevated Cu^{2+} or induced by ion depletion. The cDNA sequences corresponding to MT-like proteins have been found in many plants of both monocots and dicots (Kotrba et al., 1999). Most of the plant MT-like proteins consist of about 63-83 amino acids. Terminal Cys-rich domains of plant MT-like proteins are separated by a central region without Cys residues. The presence of this central region is the principal difference from mammalian and fungal MTs as well as from wheat Ec protein and its homologue from maize. The size of the central region usually varies around 40 amino acids and also contains aromatic amino acids, which are "forbidden" in MTs (Kotrba et al., 1999).

It is very possible that MT-like proteins protect plants from heavy metal toxicity, based on the studies on the transgenic tobacco (*Nicotiana tabacum*) against high level of Cd in the medium (Suh et al., 1998). Transgenic tobacco with constitutively expressed MT-like gene could grow on the medium containing 200 μM Cd^{2+}, but non-transgenic ones suffered chlorosis.

1.5.2 Phytochelatins

Plants and certain fungi exposed to excess Cd form Cys-rich peptides (Rauser, 2000). In contrast to MTs of class I and II, these peptides are not a direct transcription product of genes. However, they exhibit some features characteristic of MTs and they are thus sometimes referred to as class III MTs (Kotrba et al., 1999). These peptides possess γ-Glu-Cys motifs and are most often called phytochelatins (PCs), a name first introduced by Grill et al. (1985). PCs are also called cadystins, γ-glutamyl peptides, poly(γ-glutamylcysteinyl)$_n$glycine, or $\gamma(EC)_nG$ (Kotrba et al., 1999). Unlike Class I and II MTs, the γ-Glu-Cys peptides of the class III MT or PCs are atypical, because the γ-carboxyamide bonds in the molecules are not known to be synthesized in ribosomes (Rauser, 1999).

Five families of PCs are known (Figure 1-3) based on the C-terminal amino acid, which can be glycine (Gly), β-alanine (β-Ala), Cys, serine (Ser), or Glu (Rauser, 1995). In each family, the γ-Glu-Cys pair is repeated 2-7 times depending on the organism and growing medium. Non-defined repeats are denoted by the subscript n. The first Class III MTs to be characterized were the $(\gamma\text{-Glu-Cys})_n$-Gly family. PCs occur in all plants examined, following Cd exposure, ranging from vascular and nonvascular plants to mosses and algae (Rauser, 1995; 1999).

PCs are believed to function as detoxifying agents for Cd by virtue of their ability to bind Cd and thereby prevent it from reacting with the sulphydryl groups of vital enzymes and proteins. PCs appear to be ubiquitous in the plant kingdom (Grill et al., 1989) and have been isolated and purified from the roots of a number of crops including wheat (Gong et al., 1990), rice (Klapheck et al., 1994), and maize (Rauser, 2000). Much of the work on PCs is related to Cd, with fewer observations for Cu and Zn (Rauser, 1999). The first experiment that demonstrated the specific Cd complexes in cells was from fission yeast *Schizosaccharomyces pombe* (Murasugi et al., 1981). Two such complexes were purified from yeast exposed to medium containing 1000 μM Cd for 5 hours. They were high molecular weight (HMW) and low molecular weight (LMW) with apparent molecular weights of 4 and 1.8 kDa, respectively.

The HMW complex bound mostly Cd, and a small amount of Zn and Cu, and it consisted of (γ-Glu-Cys)$_2$-Gly and (γ-Glu-Cys)$_3$-Gly. The same peptides were also characterized independently by another research group (Grill et al., 1985; 1987) from several plant species, and this was the earliest experimental evidence that plants produce PCs. The general structure of these peptides is (γ-Glu-Cys)$_n$-Gly, where n as high as 11 has been reported, but is generally in the 2 to 5 range (Cobbett, 2000). According to Grill et al. (1987), the apparent molecular weight observed was drastically dependent on the ionic strength during gel filtration. At high ionic strength (>0.3M), the isolated Cd complexes had an apparent molecular weight of 3.6 kDa, but this value increased to 8 kDa at low ionic strength. Grill et al. (1987) concluded that the molecular weight of the native metal-containing PC complex is 2 to 4 kDa, rather than about 10 kDa observed at low ionic strength.

14

Studies showed that heavy metal binding peptides are synthesized enzymatically by glutathione and PC synthetases through sequential addition of γ-glutamylcysteine residues to glutathione (Grill et al., 1987). The participation of glutathione in PC synthesis is substantiated by the drastic reduction of PC formation following inhibition of the biosynthesis of glutathione (Grill et al., 1987).

Upon the elucidation of the structures of PCs and the discovery of their wide distribution in the plant kingdom, it was proposed that PCs were the functional equivalent of MTs in animals (Grill et al., 1987). Subsequently, numerous examples of MT-like genes, and in some cases MT proteins, have been isolated from various plant species and it is now apparent that plants express both of these Cys-containing metal-binding ligands. Furthermore, it is likely that the two play relatively independent functions in metal detoxification and /or metabolism. However, this can only be confirmed when a complete set of MT-deficient mutants has been identified using model plants such as Arabidopsis (Cobbett, 2000).

1.5.3   Metal Chelation by Small Molecules

The role of organic acids as ligands for Cd has been simulated by Wang et al. (1991). They calculated that in plant vacuoles at pH 5.0, about 15% of total Cd could be chelated by 17 mM malate, 3% by 0.5 mM oxalate, 60% by 6 mM citrate, and another 10% would be bound by peptides, while the free Cd would be 6%. Wang et al. (1991) also reported that vacuolar citrate was the main chelator when plant vegetative cells were grown under low Cd level condition; when the Cd concentration in the environment was high, Cd-binding peptides were the most effective chelator.

Normally the major storage form for minerals in mature seeds is phytate, a mixed salt of myoinositol hexaphosphoric acid or phytic acid. Phytate may be dispersed throughout the protein matrix, or localized into dense aggregates called globoids in the protein body, which are special vacuoles in the seeds (Rauser, 1999). Although Zn-phytate was easily identified, attempts to isolate and characterize Cd-phytate under Cd or Cd + Zn exposure have not been successful (Van Steveninck et al., 1994).

1.6   RESPONSE OF PLANTS TO Cd STRESS

Although PCs clearly have a role in Cd detoxification, their physiological or ecological relevance is questionable. Whereas most experimental studies use Cd concentration above 1 μM ($\approx$ 0.1 ppm) (Sanita di Toppi & Gabbrielli, 1999), it has been estimated that non-polluted soils contain Cd concentrations ranging up to 0.3 μM ($\approx$ 0.034 ppm) (Wagner, 1993). Wang et al (1991) have also argued that at low levels of Cd exposure, as represented by most soils, Cd would be largely complexed with vacuolar citrate in plant roots or leaves and only at high levels of Cd exposure (not generally found in natural environments) might PCs play a role in binding Cd. Contrary to this argument is the observation that a PC-deficient mutant of *Arabidopsis* is highly sensitive to Cd concentrations as low as 0.6 μM ($\approx$0.067 ppm) (Howden et al., 1995). Even at Cd concentrations where the mutant is not obviously sensitive, the wild type may nonetheless have a selective advantage. This suggests that PCs may have a role in heavy metal detoxification in an unpolluted environment. The absence of PC-deficient mutants of other species makes this question difficult to address (Cobbett, 2000).

Studies of Cd uptake mechanisms are usually performed with plants in solution culture. Most research on mechanisms of Cd accumulation by plants has been performed with high or toxic exposure to Cd. Such high Cd exposure must occur relatively infrequently under normal field crop production. Plants grown on uncontaminated soil generally have tissue Cd concentrations very much less than reported in solution culture studies. For example, durum wheat seedlings grown for 13 days in solution culture at 1 μM Cd had 75 μg Cd per gram of shoot dry matter (Jalil et al., 1994; Grant et al., 1998), whereas durum wheat grown to flowering in pots of uncontaminated silty clay loam fertilized with ammonium nitrate had 0.18 – 0.21 μg Cd per gram shoot dry matter (Choudhary et al., 1994; Grant et al., 1998). Although some aspects of Cd metabolism elucidated at high levels of Cd exposure may be relevant to normal crop production conditions, other results are likely to be misleading (Grant et al., 1998).

Most research with PCs has been conducted with intact plants or plant cells exposed to Cd at relatively high concentrations. However, Tukendorf and Rauser (1990) working with maize, found measurable PC in roots of seedlings grown in nutrient solution with no added Cd. They observed a 50% increase with 0.01 μM added Cd and a sixfold increase with 0.05 μM added Cd (Tukendorf & Rauser, 1990). Rice plants exposed to ^{35}S-Cys or 0.09 μM ^{109}Cd in solution for 24 hours accumulated ^{109}Cd and ^{35}S in the same low molecular weight soluble fraction of roots (Dabin et al., 1978), which might have contained PCs, glutathione or other sulphur compounds complexed with Cd. Ahner et al. (1994) have shown that concentrations of Cd as low as 10^{-6} μM induced the synthesis of PC in several species of marine phytoplankton. In the studies of Tukendorf and Rauser (1990) and Ahner et al. (1994) the

binding of Cd to the PCs was not demonstrated. The role of PCs in the accumulation of Cd in crops exposed to low Cd levels remains to be clarified (Grant et al., 1998).

Interestingly, when these researchers tested genotypes with and without effective PC biosynthesis, the outcome was a surprise in that the sensitive mutants (low PCs) had a significantly lower degree of transport of Cd to shoots than wild type (Howden et al., 1995). A similar result was observed in corn inbreds that differed substantially in shoot Cd; higher levels of PCs were associated with higher shoot Cd (Florijn et al., 1993; Chaney et al., 1997). To add to the complicated mechanism of plant Cd detoxification, studies showed that PC synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (Ebbs et al., 2000).

Chaney et al. (1997) pointed out that scientists should be more suspicious of the potential role of PCs in Cd tolerance in plants. It seems increasingly likely that Cd tolerance mechanisms are incidental biochemical phenomena. Although Cd-PCs can be found at low levels in plants in the environment, they account for only a small fraction of the tissue Cd (Schat & Kalff, 1992; Ahner et al., 1994; Rauser & Mouwly, 1995).

1.7 Cd TRANSPORT AND SUBCELLULAR STORAGE IN PLANTS

Generally plants regulate their metal uptake by mechanisms involving the root system, which selectively transports and distributes ions between tissues and cells. For example Cd was translocated into the seeds of linseed via the phloem with pericarps and leaves as Cd sources (Becher et al., 1997). The genotype differences concerning the Cd concentrations in the seeds may be explained in terms of differences in phloem translocation of Cd (Becher et al., 1997). Movement of Cd from roots to shoots is likely to occur via xylem

18

and to be driven by transpiration from the leaves (Salt et al., 1995). X-ray absorbance fine structure (XAFS) analysis showed that Cd in the xylem sap of *B. juncea* was chelated by oxygen or nitrogen atoms, suggesting the involvement of organic acids in Cd translocation. XAFS analysis produced no evidence for sulphur coordination of Cd, confirming that PCs and other thiol-containing ligands play no direct role in Cd transport in the xylem (Salt et al., 1995; Salt et al., 1998).

Little is known about the subcellular storage location of Cd in plants exposed to low levels of Cd. At high Cd concentrations in nutrient solutions, Cd-PC complexes accumulate in the vacuole (Vogeli-Lange & Wagner, 1990) and may be associated with CdS crystallites (Reese et al., 1992). Wagner (1979) found no evidence of vacuolar Cd storage in leaves of plants exposed to low levels of Cd. Instead, Cd was associated with soluble and organelle fractions of the cytosol. Vogeli-Lange and Wagner (1996) speculated on mechanisms of Cd accumulation at low levels of exposure and suggested that complexation in the cytosol with glutathione, transportation into the vacuole and complexation therein with organic acids may be important mechanisms. However, vacuolar storage at low levels of exposure is inconsistent with earlier results (Wagner, 1979; Krotz et al., 1989). Low molecular weight ^{109}Cd- and ^{35}S-labelled compound(s) observed in rice plant roots (Dabin et al., 1978) could have been Cd complexed with glutathione, which would support the role for glutathione proposed by Vogeli-Lange and Wagner (1996) as reviewed by Grant et al.(1998).

PC-Cd complexes are sequestered in vacuoles. In fission yeast this process has been most clearly demonstrated through studies of the Cd-sensitive mutant, *hmt1* (Ortiz et al., 1995). Sequestration of PCs to the vacuole has been observed in plants (Vogeli-Lange & Wagner, 1990; Cobbett, 2000).

In some plants and in yeasts, HMW PC-Cd complexes contain both Cd and acid-labile sulfide (Rauser, 1991). In general the ratio of S^{2-}:Cd is higher in the HMW complex compared with the LMW complex. Those complexes with a comparatively high ratio of S^{2-}:Cd consist of aggregates of CdS crystallite core coated with PCs. The incorporation of sulfide into the HMW complexes increases both the amount of Cd per molecule and the stability of the complex (Cobbett, 2000).

1.8   TECHNIQUES USED FOR STUDYING Cd BINDING COMPONENTS IN PLANTS

1.8.1   Isolation and Purification

The general techniques for isolation and purification of metal binding proteins/peptides from plant materials have been outlined in the reviews of Rauser (1990; 1991) and Steffens (1990). They are patterned from the research experience on animal MTs. Basically, there have been no significant changes to the isolation and purification protocols during the last fifteen years: Tris or potassium phosphate buffers have been used to extract proteins, followed by ion exchange and /or gel filtration chromatography to separate the metal binding fractions of interest.

Extraction buffers generally used are based on Tris or potassium phosphate ranging in pH from 7.2 to 8.6, and sometimes containing one or more additives such as sodium chloride, 2-mercaptoethanol or dithiothreitol, ascorbic acid, thiourea, and potassium cyanide (Rauser, 1990). The reducing agent used in the buffer is to protect Cd thiolate coordination. It is possible to find the most effective extraction buffer and purification protocol through a

combination of metal analysis and polypeptide measurement by HPLC and post-column quantitation of thiols (Rauser, 1990).

According to Rauser (1990), two groups of protocols are generally used in purification steps. The first group of protocols for obtaining Cd or Cu binding complexes is patterned after the isolation of animal MTs. The extract is first chromatographed on Sephadex G-75 or G-50, followed by anion exchange chromatography on DEAE-based columns. To meet the requirement of small volumes of extracts for gel filtration, tissues are usually lyophilized before extraction, or extracts are concentrated by partial or total lyophilization, ultrafiltration, dialysis against polyethylene glycol, or precipitation with $(NH_4)_2SO_4$. One disadvantage of lyophilizing tissue or solutions is the loss of some glutathione, a potential constituent of metal-binding complexes (Rauser, 1990).

The second group of protocols relies on chromatography of the crude extract first on diethylaminoethyl (DEAE)- or quaternary aminoethyl (QAE)-based anion exchangers, which is followed by gel filtration on Sephadex G-50, G-75, or Bio-Gel P6. By this means dilute extracts could be applied to the anion exchange column where the metal-binding complexes are effectively concentrated from solution (Rauser, 1990).

Several other purification methods have been used with varying degrees of success. A highly purified Cd-binding complex was obtained by covalent chromatography on thiopropyl Sepharose-6B following initial gel filtration (Jackson et al., 1987). Preparative polyacrylamide gel electrophoresis (PAGE) was used for isolating Cd-binding complexes from leaves of cabbage (Wagner, 1984) and tobbaco (Reese & Wagner, 1987). Isoelectric focusing and chromatofocusing column were not applicable because it was very difficult to separate the metal-binding complexes from the carrier ampholytes (Rauser, 1990). High-

performance liquid chromatography using anion exchange chromatography or an ion-pairing technique gave one major Cd-containing peak, with contaminants still evident when the complexes were dissociated (Rauser, 1990). Attempts to purify a 10 kDa complex by metal chelate affinity chromatography were unsuccessful according to Wagner and Trotter (1982) and mentioned by Rauser in his review (1990). Wagner and Trotter indicated that both Cd- and Zn-charged columns were prepared and tested using the conditions described by Porath et al. (1975). Transferrin was bound to and released from the Zn-loaded column essentially as described by Porath, but this column did not bind a substantial amount of 10 kDa complex. Free Cd was tightly bound and only eluted with EDTA. In our opinion, the unsuccessful experiment was not because of the method itself, but because the authors didn't realize that the column should be prepared by pre-equilibration with Cd^{2+} and that Cd^{2+} has to be removed from the 10 kDa complex first before loading the extract to the column. Theoretically, according to Porath et al. (1975), metal chelate affinity chromatography can work with not only Zn^{2+} and Cu^{2+} but also other transition elements such as Cd, which can form coordination compounds with histidine and Cys. Nair and Robinson (1999) purified a histidine rich glycoprotein that binds Cd from the blood plasma of the bivalve *Mytilus edulis* by metal chelate affinity chromatography.

The physiological functions of Cd-binding proteins and peptides are still elusive, and the only unequivocally acceptable function established is their role in protection against Cd toxicity (Klaassen et al., 1999; Rauser 1990; Cobbett 2000). However, a couple of applications using the isolated Cd-binding proteins/peptides were reported including micronutrient supplementation, environmental remediation and disease control (Kovacs-Nolan and Mine, 2006). For example, Cd-binding proteins could enhance the detoxification

of Cd (Takagi et al., 2002) and stimulate the growth and survival of cultured cells (West et al., 2005), and also used as an active ingredient in neuronal therapy for treatment of neuronally based disease (West et al., 2005).

1.8.2 Detection and Quantitation

Because of the metal-thiol coordination, metal binding peptides/proteins have characteristic UV absorption and circular dichroic spectra. The UV spectra exhibit absorbance at 255 nm, which is always higher than at 280 nm. For circular dichroic spectra, the positive cotton band is around 270 nm and the negative one around 255 nm. The peaks and cotton bands normally disappear when lowering pH to below 2, and can be restored to some extent by adjusting to the original pH (Plocke & Kagi, 1992; Prasad, 1995). Based on the review of Rauser (1990), the detection and quantitation methods that have been used are summarized in Table 1-1.

The techniques reviewed by Rauser (1990) have been used as the major methods for isolation and purification of metal binding protein/peptide since 1960. Since 1990, most of the work concerning metal binding complexes still use gel filtration and ion exchange chromatographies as the key techniques for capturing metal fractions, and atomic absorption spectroscopy as the "standard" technique in Cd detection. Mass spectrometry has been used for this research after 1990, and more recently, inductively coupled plasma – mass spectrometry (ICP/MS) (after 1995) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (after 2000) were used very successfully. All the literature reviews on plant metal binding complexes after 1990 have dealt with the structures and functions of the metal binding complexes, rather than isolation and separation

techniques. This also means that the gel filtration and ion exchange chromatographies combined with HPLC, PAGE, metal analysis (optical, CD, ICP/MS and $^{109/113}$Cd) and amino acid analysis have been developed as somewhat "standard protocols" for the research on metal binding complexes in plant materials.

1.9 THESIS RATIONALE: Cd ACCUMULATION IN FLAXSEED IS DIFFERENT FROM OTHER CROPS

1.9.1 Cd Accumulation in Crops

In most plants, the vacuole of the vegetative cells comprises more than 80 to 90% of the cell volume and acts as a central storage compartment for ions (Wink, 1993). If PC was the mechanism for Cd detoxification, this complex should be transported and stored in the cell vacuole (Brune et al., 1995; Gries & Wagner, 1998). The vacuole compartmentalization is indispensable in the fulfillment of detoxification by PC. In other words, PC is the specific high affinity vehicle inside the cell for transporting Cd into compartments (vacuoles) for detoxification, especially under excess Cd stress for plant vegetative cells such as roots, shoots, leaves and cultured cells.

Durum wheat grain contained up to 21 and 36% of the total amount of Cd in the whole plant when grown in soil containing 0.124 and 0.323 ppm Cd, respectively (Cieslinski et al., 1996). Studies with durum wheat (Hart et al., 1998), winter wheat (Herren & Feller, 1997), and peanut (Popelka et al., 1996) have indicated that Cd transport into grain is phloem-mediated (Hocking & McLaughlin, 2000). According to Wagner (1993) the most likely mechanism for Cd transport under this condition is via small organic acids. Studies have already shown that thiol-containing ligands do not play a role in Cd translocation from

roots to shoots (Salt et al., 1995; Salt et al., 1998). As a conclusion of the above information, the subcellular transportation and storage of Cd were fulfilled by PC; whereas, the in-body transportation from organ to organ, like roots to shoots, and then to seeds, were mostly furnished by small organic acids like citric acid.

According to this author's speculation based on this literature review, one of the likely mechanisms for Cd detoxification in flaxseed is through chelation with proteins, such as MTs, or other metal binding proteins. A possible mechanism may involve: Cd transportation by small organic acids to the seed, where the seed produces MT-like proteins that bind the Cd to reduce its mobility and toxicity. These MT-like proteins are coded by gene(s) determined by flaxseed genotype. It may be that a single gene is involved, as it has been shown that the difference in Cd accumulation in durum wheat lines is controlled by a single gene (Clarke, 1995; Penner et al., 1995; Hocking & McLaughlin, 2000). The genetics of Cd accumulation in flaxseed has not been studied and further work is needed in this area.

Because of the generally low level of Cd in seeds compared to vegetative tissues, until recently there has been no direct study reported on Cd binding proteins in seed. The Ec protein, Cd-binding and Cys-rich proteins in wheat and maize were discovered by investigation of the gene, and other similar MT proteins were discovered by screening of cDNA libraries. The route of discovery has been from gene to protein, then to metal binding characteristics (Kotrba et al., 1999).

Generally Cd has been found mainly in the outer parts of the seeds. For cereal grains, all or most of the Cd can be removed by the milling processes (Siebel et al., 1974; Hinesly et al., 1979; Muchova & Jaska, 1996; FAO/WHO, 1998). The concentration of Cd in wheat bran was twice that in wholemeal flour (Chaudri et al., 1995). Higher Cd concentration is

also found in the outer part of maize (Hinesly et al., 1979; Mullins & Sommers, 1986) and cocoa beans (Prugarova & Kovac, 1987), and Cd tends to accumulate in the germ portion of the corn grain rather than in other parts (Garcia et al., 1974; Hinesly et al., 1978). According to Braude et al. (1980) sludge-grown soybean contained 6.73 ppm of Cd in the hull and 1.81 ppm in other parts. If processed into oil and defatted flakes, most of the Cd was retained in the flakes (2.40 ppm), while oil contained relatively low level of Cd (0.8 ppm). Casterline and Yip (1975) found that Cd in soybeans (grown with Cd-containing sewage sludge at the University of Illinois experimental farm) was bound by protein fractions with molecular weights of over 50 kD (Braude et al., 1980). However, in rice the Cd has been found to be mostly bound to the major protein glutelin but not to the outer parts of the grains (Suzuki et al., 1997).

1.9.2 Cd Accumulation in Flaxseed

For most crop plants, Cd accumulation in generative organs is minor in comparison with the Cd accumulation in roots and shoots. However, this is not the case in flaxseed, known to accumulate high concentration of Cd in the seeds even when the Cd contamination of the soil is low. This can be of major importance if flaxseed is used as food in the diet (Becher et al., 1997). Another important phenomenon is that different genotypes of flaxseed accumulate quite different amounts of Cd in the seeds even when grown under the same conditions (Becher et al., 1997; Li et al., 1997). However, the underlying mechanism for this difference as well as the form of Cd existing in flaxseed is still unknown.

Generally, relative to cereal crops, flaxseed tends to accumulate higher concentrations of Cd in the seeds (Grant & Bailey, 1997). The three-year average content of Cd in the seeds

of flax grown in soil containing 0.124 ppm Cd was 45% of the total amount of Cd in the whole plant (Grant et al., 2000). Even with a low soil Cd concentration, the Cd content in flaxseed (0.5-0.6 ppm) exceeded permitted levels, while that in wheat grain was only about 0.1 ppm (Schubert, 1992).

To our knowledge, there is no data available for Cd distribution and storage mechanisms in flaxseed. It is believed that Cd is not present in the flaxseed oil but is bound to proteins (Koloziejczk & Fedec, 1995). It is not known how Cd binds to the protein fraction in flaxseed; it may bind to minor proteins, as in wheat (Wagner & Nulty 1984; Hanley-Bowdoin & Lane 1983), or to the major storage proteins as in rice (Suzuki et al 1997).

The information regarding Cd-binding proteins is much needed to understand the mechanism for Cd accumulation in flaxseed, and this knowledge can be used to develop a marker system for selection of the genotype with low Cd accumulation ability in the seeds. There is also a need to examine a far wider range of flaxseed germplasm to find breeding material that accumulates much lower levels of Cd accumulation in seed.

1.10 THESIS HYPOTHESES AND OBJECTIVES

The hypotheses of this research are as follows:

- Cd-binding components can be extracted and isolated from major components of flaxseed.
- The accumulation and distribution of Cd in flaxseed components are dependent on cultivar and growing location.
- Characterization of chemical, physical and biological properties of the Cd-binding components in flaxseed can provide structure and function information useful for genotypic or phenotypic manipulations.

The objectives of this research are as follows:

Long-term objectives

The long-term objectives of this research are to understand Cd accumulation and its major binding forms in flaxseed, and to establish a method of separating major Cd-binding components from major storage proteins, in order to provide useful information and guidance for the selection of flax genotypes with low Cd accumulation ability in their seeds, as well as to help to promote the increased utilization of flaxseed as a functional food.

Objectives for this thesis

The specific objectives for this thesis project are to:

- Establish a process for the isolation of the putative Cd-binding fractions, and determine the distribution of protein, Cd and other metals (Cu, Zn, Ca) in various protein fractions from flaxseed.

- Compare the distribution of protein, Cd and other metals in various protein fractions from flaxseed as a function of cultivar and growing location.

- Characterize selected properties of the major Cd-binding fraction including spectral characteristics (UV/Vis, circular dichroism), physical and chemical characteristics (MALDI-TOF MS, HPLC/MS, solubility, SDS-PAGE, IEF), and amino acid composition, and potential biological effects on cultured cells.

1.11 TABLES AND FIGURES

Table 1-1. Techniques used for detection and quantitation of Cd binding components, summarized from Rauser's review (1990)

Techniques	Application
Atomic absorption spectroscopy	Direct Cd detection during isolation and purification processes. Widely used as "standard" method before 1990.
Isotope ^{109}Cd	Detect and quantitate by gamma scintillation counting.
Differential pulse stripping voltametry	Used to measure Cd in gel samples from electrophoresis and isoelectric focusing.
UV absorption at 250 to 255 nm	Used as a guide for monitoring Cd binding proteins in column effluents; not very reliable.
pH shift	UV spectrum between 200 to 350 nm and its characteristics upon acidification and back titration to alkaline pH, used as preliminary diagnostic indicators of metal binding peptides. Reliability depends on sample purity.
Circular dichroism	Indicate Cd-thiolate coordination for Cd-peptides (a positive cotton band at 275-280 nm, and a negative one at 253-257 nm).
Differential pulse polarography	Applied to MTs, but received limited use with plant metal-binding complexes.
Ellman method [5,5-dithiobis(2-nitrobenzoic acid), DTNB]	Used to characterize and estimate the amounts of SH in metal-binding complexes; sulfide can also be measured following its liberation during HPLC in acidic conditions by reaction with DTNB.
Colorimetric procedure of King & Morris (1967)	To determine the amount of acid-labile sulfide in metal-binding complexes.
Immunological methods (e.g. ELISA)	Require antibodies against the plant polypeptides of metal binding complexes because antibodies against MTs do not react with PCs.
Analytical PAGE	Give limited information about metal-binding complexes.
Amino acid analysis	On purified metal-binding complexes, must be reviewed as preliminary information. Large differences in amino acid composition are mostly due to variations in purity of the preparations. If the analyses are interpreted in terms of PCs, they show that Cys, Glu, and Gly account for 45 to 97% of the amino acid found in Cd-binding complexes, while Cys ranged from 8 to 43%.
Reverse phase HPLC on C_{18} columns	Polypeptides forming the metal-binding complexes can be resolved.

29
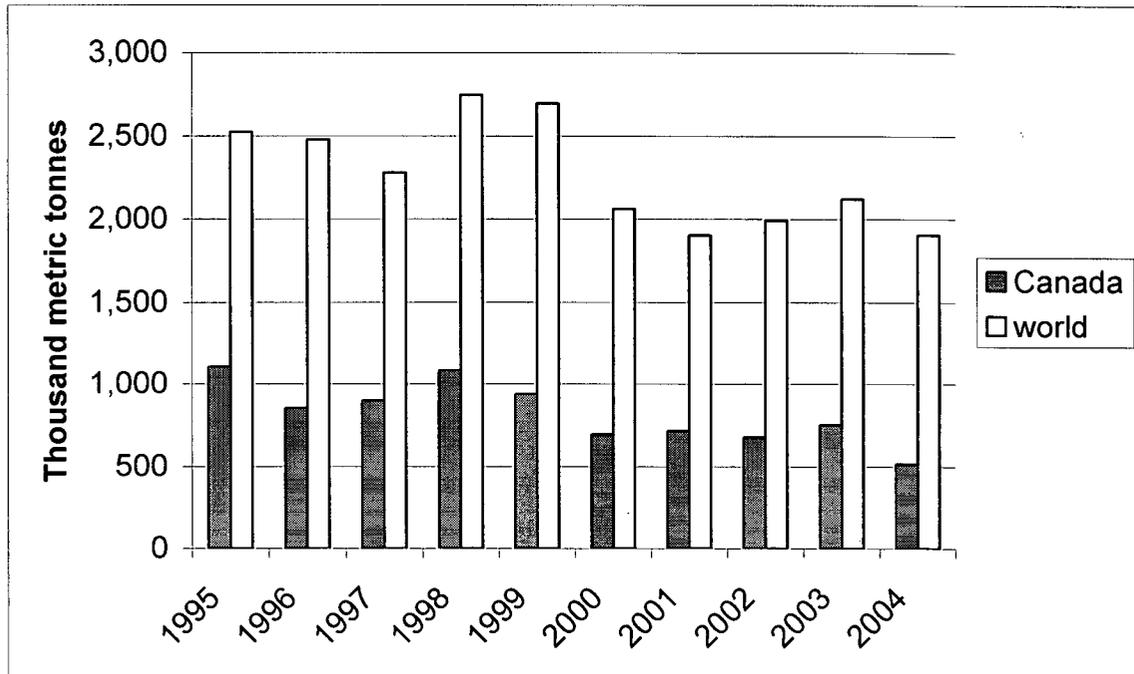
Figure 1-1. Canada and world flaxseed production for the past 10 years. Data used were from Agricultural Production Indices, FAO Statistical Databases, Food and Agriculture Organization of the United Nations (FAO), updated on July 14, 2005. (http://faostat.fao.org/). The lower production of year 2004 was due to frost in Canada, and production in Canada is forecast to rise sharply by about 75% next year (Agriculture and Agri-Food Canada, 2005).

Cd₃ CLUSTER

(β-domain)

Cd₄ CLUSTER
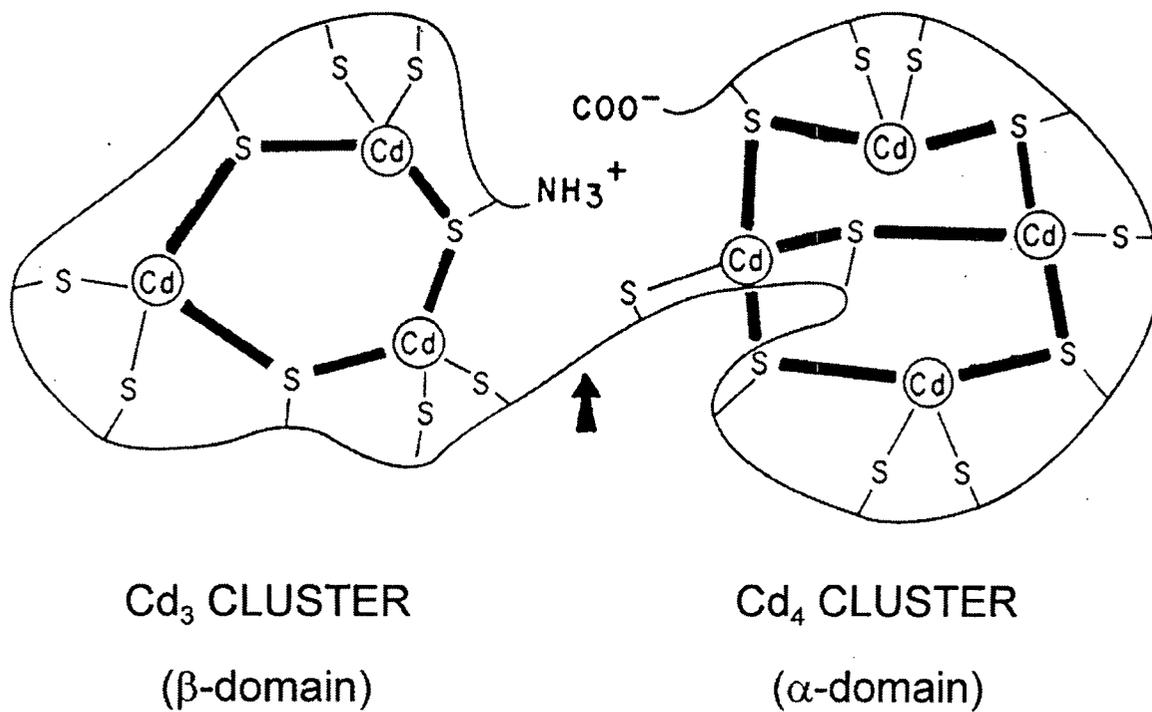
(α-domain)

Figure 1-2. Domain nature of metallothionein. Four and three atoms of Cd are coordinated in α- and β-domains of metallothionein, respectively (Klaassen et al., 1999).

γ-Glu-Cys

γ-Glu-Cys-Glu   γ-Glu-Cys-Gly γ-Glu-Cys-β-Ala γ-Glu-Cys-Ser

(γ-Glu-Cys)$_2$-Glu (γ-Glu-Cys)$_2$ (γ-Glu-Cys)$_2$-Gly (γ-Glu-Cys)$_2$-β-Ala (γ-Glu-Cys)$_2$-Ser

(γ-Glu-Cys)$_n$-Glu (γ-Glu-Cys)$_n$ (γ-Glu-Cys)$_n$-Gly (γ-Glu-Cys)$_n$-β-Ala (γ-Glu-Cys)$_n$-Ser
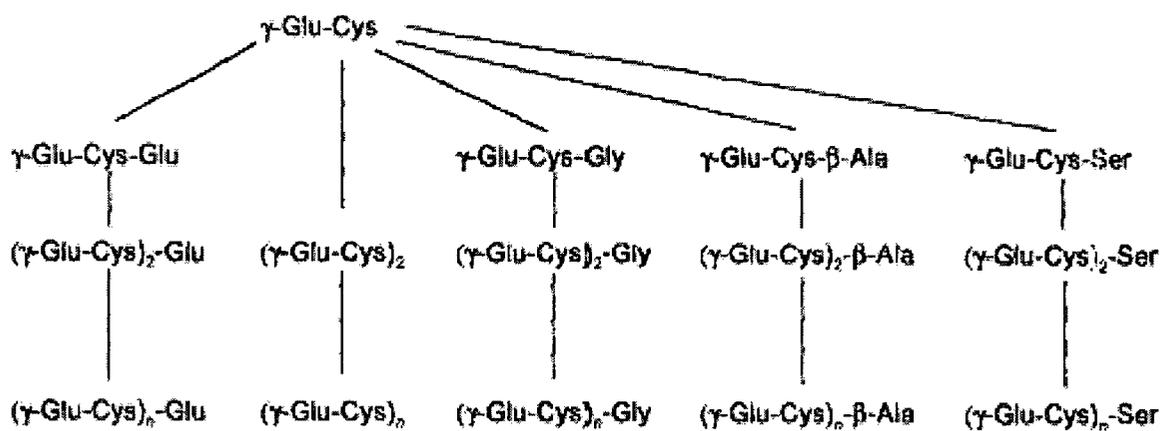
Figure 1-3. Structure of various γ-Glu-Cys peptides. A scheme summarizing the five families of γ-Glu-Cys peptides involved in metal sequestration in plants and certain yeasts. The lines connecting peptides refer to family relationships and do not necessarily specify biosynthetic sequence (Rauser, 1995).

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38

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CHAPTER 2 ISOLATION AND PURIFICATION OF CADMIUM-BINDING COMPONENTS AND THE DISTRIBUTION OF PROTEIN AND METALS IN DIFFERENT FRACTIONS OF FLAXSEED (CV NORMAN AND CV FLANDERS)[1]

2.1   INTRODUCTION

World flaxseed production has averaged about 2.0 million tonnes over the past five years, and Canada is by far the world's largest flaxseed producer, accounting for about one-third of the total world production and most of the export trade (Daun et al., 2003). In recent years, flaxseed has been used as a functional food ingredient in the world market based on its potential to reduce the risk of cardiovascular diseases (Bloedon & Szapary, 2004), various cancers (Thompson, 2003; Saarinen et al., 2003; Bougnoux & Chajes, 2003), kidney disease (Ogborn, 2003), and many other health-related functions.

However, flaxseed contains significant amounts of cadmium (Cd) that often exceed the dietary critical value or maximum level of 0.3 ppm (Marquard et al., 1990; Moraghan, 1993; Cieslinkski et al., 1996; Becher et al., 1997). Cd is generally considered as a toxic element, which can accumulate in the human kidney through the food chain, causing renal tubular dysfunction and pulmonary emphysema. In non-occupationally exposed populations, the major source of exposure to and intake of Cd are diet and smoking (Singh & McLaughlin, 1999, Burgat-Sacaze et al., 1996). Because the half-life of Cd in the human body is about 20 years, the consumption of foods containing higher levels of Cd may result in chronic toxicity (Jackson & Alloway, 1992).

Cd in flaxseed is not present in the oil, and is believed to be bound to proteins (Kolodziejczyk & Fedec, 1995). The protein contents of flaxseed cultivars grown in Canada generally are well above 36% (Oomah & Mazza 1993). Several attempts have been made to extract flaxseed protein with buffered salt solutions as reviewed by Oomah and Mazza (1993). The major proteins in flaxseed are 12S and 2S, accounting for 58 and 42%, respectively (Youle & Huang 1981). About 85% of the total nitrogen from degummed, defatted, and dehulled flaxseed meal was extracted with 1M NaCl at pH 7.0. Gel filtration on Sepharose 6B separated this protein into three fractions accounting for 3, 67, and 30% of the total content. Ion exchange chromatography on DEAE-Sephadex eluted the total protein between 0 and 0.5M NaCl into four peaks. The presence of these four proteins was also confirmed by the sedimentation-velocity patterns in 1M NaCl (Madhusudhan & Singh, 1983), with S_{20},w values of 1.4, 5.0, 9.0, and 14.0 S, and relative proportions of 20, 10, 66, and 4%, respectively. One of the major low molecular weight protein fractions was characterized as a protein with sedimentation coefficient of 1.6 S and a molecular weight of 15 to 18 kDa (Youle & Huang 1981). The N and C-terminal amino acids of this protein were alanine and lysine. The protein consisted of a single polypeptide chain characterized by high content of β-structure and helical conformation of 51 and 26-32%, respectively, and an amino acid composition high in lysine, cysteine, glutamic acid, and glycine contents. About 93% of the protein was soluble in water and 99% soluble in 0.05M NaCl (Oomah & Mazza, 1993). Proteins extracted from dehulled and defatted flaxseed cultivar NorMan by buffered 0.1M salt solution could be fractionated by anion exchange chromatography to yield a major fraction with molecular weight of 365 kDa, as determined by Sephacryl S-300 gel

permeation chromatography (Chung et al., 2005). The protein recovered by this method was 64% of the total proteins in flaxseed cultivar NorMan (Chung, 2001).

Since it was reported that the Cd in rice grains was bound to the major storage protein glutelin (Suzuki et al., 1997), the research group led by Dr. Eunice Li-Chan initiated studies to characterize the major storage protein in flaxseed (Chung, 2001), and to determine the presence of Cd-binding components in protein extracts of dehulled and defatted flaxseed (Li-Chan et al., 2002). Whereas the major storage protein of flaxseed was isolated in a 0.25 M NaCl fraction by DEAE Sephacel ion exchange chromatography (Chung, 2001), phytochelatin (PC) -like components were found in two fractions eluting at high salt concentrations of 0.45 and 0.50 M NaCl, corresponding to 35-40 mS/cm (Li-Chan et al., 2002).

Although this previous research determined the presence of Cd-binding components, it focused on the high salt-eluted fractions, present in only trace quantities, and did not provide any insight into the mechanism of the bulk Cd presence in flaxseed. Another limitation of this earlier research was that the ion exchange column was overloaded with protein to facilitate recovery of the trace amounts of high salt-eluted fractions. Under these conditions, the real Cd distribution in various protein fractions could not be determined. It has been suggested that the existence of multiple metal binding proteins having different anionic character is likely (Li-Chan et al., 2002). Furthermore, Cd may not only be bound to metallothioneins or PCs, but it also may be associated with different ligands, such as organic acids, amino acids, and phytin (Rauser, 1999).

Information regarding Cd binding components is much needed to understand the mechanism for Cd accumulation in flaxseed. This knowledge may be helpful in future

research to develop a marker system for the selection of genotypes with low tendency for Cd accumulation in the seeds. There is also a need to examine a far wider range of flaxseed germplasm to find potential breeding material with lower levels of Cd accumulation in seed. Therefore, intensive research is needed to identify the distribution and mechanism of Cd accumulation in flaxseed in order to provide the knowledge base to formulate strategies to reduce the risk of Cd toxicity and to ensure continued safety with the increased flaxseed utilization in food and medicinal industries.

The objectives of this research are to reveal the distribution of Cd in flaxseed proteins and to investigate the feasibility of isolating flaxseed proteins with low Cd content, which can then be used as food ingredients. As a long-term objective, the results from this research will also provide useful information on which to base further investigation of the mechanisms of Cd uptake and retention in flaxseed.

2.2   MATERIALS AND METHODS

2.2.1   Reagents

Tris Ultrapure (Tris-(hydroxymethyl)aminomethane) was from ICN Biomedicals, Inc., Costa Mesa, CA. Sodium chloride, sodium azide and hydrochloric acid certified A.C.S. were from Fisher Scientific, Nepean, ON. 2-Mercaptoethanol electrophoresis reagent, blue dextran, bovine α-chymotrypsin , and bovine α-lactalbumin were from Sigma-Aldrich Canada Ltd, Oakville, ON, Canada. Bicinchoninic acid (BCA) protein assay reagents A and B were from Pierce Chemical Company, Rockford, IL. The deionised distilled water (~ 18 megaohms) used for all experiments was produced by a Barnstead water purification system. DEAE-

Sephacel and Sephadex G50 were purchased from Amersham Pharmacia Biotech, Inc., Baie d'Urfe, PQ, Canada.

2.2.2 Flaxseed

Flaxseed cultivars NorMan (Figure 2-1) and Flanders were obtained from Agriculture and Agri-Food Canada Cereal Research Center (Morden, MB, Canada). The cultivar NorMan containing 0.53 ppm Cd in the seed was harvested in southern Manitoba, and Flanders (Cd levels of 1.54 and 0.38 ppm) was grown in Morden (FM) and Portage la Prairie (FP), respectively. To facilitate protein extraction and chromatographic separation, flaxseeds were first dehulled as described by Li-Chan et al. (2002) using a Strong Scott Barley Pearler fitted with a 2 mm screen (6.25 holes/cm^2, Figure 2-2) and an air aspirator (Oomah et al., 1996) to remove most of the mucilage from hulls and to reduce the seed stickiness. The dehulled seeds (Figure 2-3) were then ground (Thomas Wiley Mill, Philadelphia, PA) to pass a 1 mm screen. The ground seeds were defatted by two changes of hexane and one change of petroleum ether, one hour each with magnetic stirring, by using a seed to solvent ratio of 1:10 (w/v). The defatted material was then air dried under a fume hood for at least 4 hours to remove traces of solvents (Figure 2-4).

2.2.3 Protein Extraction

The defatted flaxseed powder (ca. 5 g from 10 g of dehulled seed) was extracted according to the method of Li-Chan et al. (2002) with 16 volumes of nitrogen purged extraction buffer (0.1 M Tris + 0.1 M NaCl + 10 mM mercaptoethanol, pH 8.6) at 4 °C for 16 h with constant magnetic stirring. The extract was centrifuged (10,000 g x 30 min, Sorvall

RC5B, Sorvall Instruments, Dupont, CT) at 8-10 °C and the supernatant further centrifuged at 27,000 g for 30 min.

2.2.4 Ion Exchange Chromatography

Ion exchange chromatography was carried out using a modified procedure of Li-Chan et al. (2002). The anion exchange resin DEAE-Sephacel was equilibrated with buffer consisting of 0.01 M Tris + 0.01 M NaCl at pH 8.6. The protein extract (starting from 10 g of dehulled seed for NorMan, and 2.5 to 3 g of dehulled seed for Flanders) was diluted 10-fold with distilled and deionized water , then mixed and incubated at ambient temperature with the pre-equilibrated DEAE-Sephacel resin (500 mL for protein extract from NorMan and 250 mL for protein extract from Flanders) for one hour with gentle manual stirring. The resin was then gently and slowly washed with equilibrating buffer to remove any unbound proteins. The resin with bound proteins was packed in a column (5.0 x 30 cm). The bound fractions were then eluted by step gradients containing increasing concentration of NaCl from 0.1 M to 1.0 M NaCl in 0.01 M Tris buffer at pH 8.6. The flowrate was 5 mL/min and the fraction size was 20 mL/tube for the column loaded with NorMan protein extract, and 2 mL/min and 10 mL/tube for the column loaded with Flanders protein extract. Fractions were collected and monitored for conductivity and absorbance at 280 nm.

2.2.5 Size Exclusion Chromatography

The high Cd-binding fraction eluting from ion exchange chromatography with 0.1 M NaCl was freeze-dried and then re-dissolved in a small volume of distilled and deionized water. The re-dissolved sample (ca. 80 mg freeze dried solids in 15 to 20 mL) was loaded on

a column (2.5 x 120 cm) of Sephadex G50 gel (500 mL) that was pre-equilibrated with distilled and deionized water. The protein was eluted with distilled and deionized water at a flowrate of 1 mL/min. Fractions of 10 mL/tube were collected and monitored by measuring conductivity and absorbance at 280 nm. This column was calibrated for molecular-weight estimation by using the following substances (molecular weight): blue dextran (~ 2000 kDa), α-chymotrypsin bovine (25 kDa), α-lactalbumin bovine (14 kDa) and sodium azide (65 Da).

2.2.6 Analyses of Column Fractions

2.2.6.1 Monitoring of column eluted fractions

The absorbance spectrum was measured by using an ATI Unicam UV/Vis Spectrometer (Unicam Limited, Cambridge, UK). Conductivity was measured with a MeterLab® CDM 210 conductivity meter (Radiometer Analytical SAS, Lyon, France).

2.2.6.2 Protein content

Protein content of triplicate samples was determined using a BCA (bicinchoninic acid) Protein Assay Kit (#23225, Pierce Chemical Company, Rockford, IL, USA) as well as by a nitrogen combustion method using a LECO FP-428 Nitrogen and Protein Determinator (LECO Instruments Ltd, Mississauga, ON, Canada) calibrated with ethylenediaminetetraacetic acid (9.58 % x N). Flaxseed protein content was calculated from nitrogen using a conversion factor of 5.41 according to Tkachuk (1969).

2.2.6.3 Metal content

The contents of Cd, Cu, Zn, and Ca in the ion exchange and G50 fractions were measured by ICP-MS with an ultrasonic nebulization pulse membrane desolvation inlet by Elemental Research Inc. (North Vancouver, BC, Canada). The detection limits for Cd, Cu, Zn, and Ca were 0.1, 1.0, 10, and 100 ppb, respectively. The concentrations of metals in the buffer blank were 3.97 ± 0.91 ppm Ca, 0.07 ± 0.02 ppm Cu, < 0.03 ppm Zn and < 0.3 ppb Cd.

2.3   RESULTS

2.3.1   Flaxseed Cultivar NorMan

2.3.1.1 Protein and Cd distribution in flaxseed after dehulling, defatting, and extraction

Ten grams of dehulled flaxseed cultivar NorMan containing 5260 ng Cd and 2390 mg proteins were defatted by hexane and petroleum ether. Over 95% of the Cd and proteins were recovered into the defatted powder, which was then extracted by 0.1 M Tris buffer + 0.1 M NaCl at pH 8.6. The extract recovered 82 and 56% of Cd and protein, respectively, based on dehulled seed (Table 2-1). The Cd content on a protein basis (Cd ng/protein mg) was not changed after defatting, but increased by 45% from 2.2 to 3.2 after extraction.

2.3.1.2 Protein and Cd distribution in fractions from ion exchange chromatography

The flaxseed protein extract was fractionated by ion exchange chromatography on a DEAE-Sephacel column (Figure 2-5). The major peak eluting at 0.25 M NaCl, was a sharp peak with the highest absorbance value. Two smaller peaks were observed – a broad peak eluted at 0.1 M NaCl and a narrow peak eluted at 0.45 M NaCl, which contained two

shoulders. Two minor peaks eluted at 0.5 M and 1.0 M NaCl, and sometimes they were absent in the profiles depending on the quantity of protein loaded onto the column. About 80% of the extracted proteins and 73% of the extracted Cd from flaxseed were bound to the ion exchange column.

Cd was present in the unbound fraction as well as each of the 0.1 to 0.45 M NaCl eluted fractions (Table 2-2). However, the 0.1 M NaCl eluted fraction was the major Cd binding peak, containing 66% of the eluted Cd in only 7% of the eluted proteins (or 43% of the Cd in only 3.6% protein, based on dehulled flaxseed). The 0.25 M NaCl fraction was the major protein peak, containing 72% of the eluted protein, but with only 25% of the eluted Cd (Table 2-2). The Cd content on protein basis was increased from 2.2 ppm in dehulled seeds to 26 ppm in the 0.1M NaCl fraction, but was reduced to 0.9 ppm in the 0.25M NaCl fraction.

2.3.1.3 Protein and Cd distribution in fractions from size exclusion chromatography

The major Cd binding fraction eluting at 0.1 M NaCl from DEAE-Sephacel was further separated into 3 peaks (Figure 2-6) by size exclusion chromatography on a Sephadex G50 column (fractionation range 1,500 to 30,000 Da). The first peak eluting just after V_0 (void volume) was termed the high molecular weight peak (HMW peak). The second peak (Peak A) eluted at 390 mL, corresponding to a molecular weight of ~14 kDa. The third peak (Peak B), eluting after V_t, probably represents small molecular weight components including peptides. Both Peak A and Peak B contained Cd, with Peak B being the major Cd binding fraction, having a high Cd: protein ratio of 184 ppm (Table 2-3) compared to that in dehulled seeds of 2.2 ppm (Table 2-1). Peak A contained 21% of the eluted Cd in 60% of the eluted

protein, while Peak B had 79% of the eluted Cd in only 14% of the eluted proteins. The HMW peak had 26% of the eluted protein with no detectable Cd.

2.3.1.4 Distribution of Ca, Cu and Zn in ion exchange and size exclusion chromatographic fractions

The results of metal analyses on Ca, Cu and Zn for fractions from ion exchange and size exclusion chromatography are shown in Table 2-4. Despite the limitation of the variability between repeated runs, certain trends were observed. Ca was distributed in every fraction of ion exchange chromatography with highest (51%) and lowest (11%) content found in the unbound and in 0.1 M NaCl fractions, respectively (Table 2-4). After G50 gel filtration chromatography of the 0.1 M NaCl fraction, the majority of Ca was found in Peak B, which had a Ca content four times higher than that in HMW peak and Peak A (Table 2-4). The Cu contents were approximately equally distributed ($\approx$ 41%) in the 0.1 M and 0.25 M NaCl fractions, and Peak A and B both contained large amounts of Cu (65 and 30% respectively). According to Table 2-4, most of the Zn was eluted at the intermediate salt fractions of 0.1 M (44%) and 0.25 M (36%) NaCl in ion exchange chromatography, while about 20% of the Zn was eluted with the unbound fraction. After size exclusion chromatography, Peak A and B had 31 and 68% of the eluted Zn, respectively (Table 2-4).

2.3.2 Flaxseed Cultivar Flanders

2.3.2.1 Protein and Cd distribution during ion exchange chromatography

Flaxseed cultivar Flanders grown at Morden (FM) and Portage la Prairie (FP) had Cd levels of 1.54 and 0.38 ppm, respectively. The protein extracts before loading to the ion

exchange chromatographic columns had Cd levels of 6.0 and 2.2 ppm for FM and FP, respectively (Table 2-5). The protein extracts fractionated by ion exchange chromatography on a DEAE-Sephacel column generated similar elution profiles for FM and FP (Figure 2-7). The major peak eluted at 0.25 M NaCl was a sharp peak with the highest absorbance value. Two smaller peaks were also observed at 0.1 M and 0.45 M NaCl. Two minor peaks eluted at 0.5 M and 1.0 M NaCl, and sometimes they were not observed in the profile depending on the quantity of protein loaded onto the column.

Cd and protein distributions in each of the ion exchange chromatographic fraction are also shown in Table 2-5. For high Cd level flaxseed FM, Cd was present in the unbound fraction, as well as in each of the 0.1 to 1.0 M NaCl fractions. In comparison, for the low level Cd flaxseed FP, Cd was present in unbound and 0.1 M and 0.25 M NaCl fractions, but not detectable in the high salt fractions eluted at 0.45, 0.50 and 1.0 M NaCl. However, the 0.1 M NaCl eluted fraction was the major Cd-binding peak, containing 49.5 and 51.7% of the eluted Cd and only 7.2 and 8.2% of the eluted proteins for FM and FP, respectively. The 0.25 M NaCl fraction was the major protein peak, containing 61.3 and 58.4% of the eluted proteins with about 14% of the eluted Cd in both cases for FM and FP (Table 2-5).

The Cd level on protein basis after ion exchange chromatography changed from 6.0 ppm in the loaded extract to 1.3 ppm in the 0.25 M NaCl eluted major protein fraction for FM, and from 2.2 ppm in the protein extract to 0.4 ppm in the 0.25 M NaCl eluted major protein fraction for FP (Table 2-5). At the same time, the Cd level on protein basis in the major Cd-binding fraction eluted by 0.1 M NaCl was increased to 39.8 ppm for FM and 9.7 ppm for FP, respectively.

2.3.2.2 Protein and Cd distribution during size exclusion chromatography

The major Cd binding fraction eluting at 0.1 M NaCl from DEAE-Sephacel was further separated into 3 peaks (Figure 2-8) by size exclusion chromatography on a Sephadex G50 column with similar results to those found for flaxseed NorMan experiment (Section 2.3.1.3). The elution profiles and protein distribution in their fractions were similar for FM and FP. The Cd distribution showed distinct differences, even though Peak B was the major Cd-binding fraction for both FM and FP (Table 2-6). No Cd was detectable in the HMW peak for either FM or FP. Peak A was the major protein fraction, comprising 62 and 67% of the eluted proteins of FM and FP, respectively (Table 2-6). Peak A contained 27% of the Cd in high Cd level seed FM, but no detectable Cd was found for the low Cd level seed FP. Peak B contained 38 and 32% of the eluted proteins for FM and FP, respectively (Table 2-6). After size exclusion chromatography, the Cd level on protein basis was doubled in Peak B compared with the loading sample (Table 2-6). For FM, Peak B had 58 ppm of Cd, while the original loaded sample contained 28 ppm. For FP, peak B had Cd at 25 ppm, while the original loaded sample contained 11 ppm (Table 2-6).

2.3.2.3 Ca, Cu and Zn distribution in chromatographic fractions

The Ca, Cu, and Zn distribution in each fractions from ion exchange and size exclusion chromatographies are summarized in Table 2-7 for flaxseed FM, and Table 2-8 for flaxseed FP. More than half of the Ca eluted into the unbound fraction from ion exchange chromatography for both FM and FP. Ca level decreased in each fraction eluted with increasing salt content. For both FM and FP, the high salt fractions of 0.45, 0.50 and 1.0 M contained less than 10% of the total eluted metals. The highest level of Cu was eluted in the

0.1 M NaCl fraction for both FM and FP. The overall trend of metal distribution was quite similar between FM and FP. However, the 0.25 M NaCl contained the highest level of Zn for FP, but the highest level of Zn for FM was the in 0.1 M NaCl fraction. Zn was under the detection limit for the 0.45 M NaCl fraction of FM, but 1.49% Zn was present in the 0.45 M NaCl fraction of FP. For size exclusion chromatography, the highest level of each metal studied was in the Peak B, with the exception of Cu in FM, which was highest in Peak A.

2.4   DISCUSSION

The studies on the Cd-binding fractions in flaxseed could only be carried out with sufficient supply of flaxseed sample; therefore, the commercial flaxseed cultivar NorMan with 0.53 ppm Cd was used for most of the experiments throughout this thesis project, and the protocols of protein extraction and purification were established based on the data from NorMan cultivar. The established protocols were further verified using two samples of another flaxseed cultivar Flanders; the Flanders sample grown in Morden had a high Cd level (1.54 ppm) while the other, grown in Portage la Prairie, contained a lower level of Cd (0.38 ppm), thereby positioning flaxseed NorMan at the intermediate level of Cd.

Dehulling of flaxseed prior to extraction alleviated the high viscosity generally associated with mucilage. This pre-treatment facilitated the protein extraction and subsequent chromatography to separate different protein fractions (Li-Chen et al., 2002). Based on dehulled seed, the Cd and protein recoveries after protein extraction using Tris buffer from the defatted powder were 82 and 56%, respectively (Table 2-1). Based on defatted powder, the Cd and protein recoveries were 86 and 58%, respectively. These recoveries were deemed appropriate since protein recovery greater than 50% from high globulin-containing defatted

powder has been reported to be very difficult to achieve (Marcone et al., 1998; Derbyshire et al., 1976).

The protein extracts fractionated by anion exchange chromatography on a DEAE-Sephacel column generated very similar profiles for flax cultivar Flanders (Figure 2-7) and NorMan (Figure 2-5). The major peak with the highest absorbance eluted at 0.25 M NaCl. Two smaller peaks eluted at 0.1 M and 0.45 M NaCl, and two minor peaks eluted at 0.5 and 1.0 M NaCl. The protein and Cd distributions in each fraction from ion exchange chromatography were also quite similar between flax cultivars NorMan and Flanders. For all three tested samples of NorMan, FM and FP, the major Cd-binding fraction eluted at 0.1 M NaCl, and the major protein peak eluted at 0.25 M NaCl. After ion exchange chromatography, about 50 to 66% of Cd was in the 0.1 M NaCl fraction with 7 to 8% of the protein, and 14 to 25% of Cd was in the 0.25 M NaCl fraction with 58 to 72% of the protein. The Cd level on protein basis were very low in the 0.25 M NaCl fraction and much higher in the 0.1 M NaCl fraction, and the levels were consistent with the Cd level in the seeds. The Cd level on protein basis for the 0.25 M NaCl fraction were 0.4, 0.9 and 1.3 ppm for FP, NorMan and FM, respectively (Table 2-9), compared to Cd levels (expressed on solids basis) in the corresponding seeds of 0.38, 0.53, and 1.54 ppm, respectively.

Even though over half of the Cd was eluted into the major Cd-binding fraction of 0.1 M NaCl, Cd was also present in the unbound fraction as well as several fractions eluted at different NaCl concentrations from ion exchange chromatography, suggesting multiplicity of Cd binding factors (Tables 2-2 & 2-5). In the present study, the FM sample contained the highest level of Cd (1.54 ppm) compared with the NorMan (0.53 ppm) and FP (0.38 ppm) samples. Cd was present in all the fractions from FM, but was not detectable in the high salt

fractions of 0.45 M to 1.0 M NaCl for FP. For flaxseed FM, the high salt fractions of 0.5 M and 1.0 M NaCl contained very high Cd level on a protein basis, which implied that components with very high affinity for Cd, which are buffer extractable and anionic in nature, may exist or be induced for detoxification purpose when very high amount of Cd accumulate in flaxseeds. Nevertheless, the high affinity components did not constitute the major Cd binding fractions because these peaks contained less than 0.2% of the eluted proteins from the chromatographic column (Table 2-5).

The present fractionation protocol successfully separated the major Cd-binding fraction (0.1 M NaCl) from the major protein fraction (0.25 M NaCl). The major protein fraction had much lower Cd level compared with the original seeds. The Cd content expressed on a protein basis was reduced in the 0.25 M NaCl fraction to 0.9 ppm for NorMan, 1.3 ppm for FM and 0.4 ppm for FP, compared to the corresponding whole protein extracts containing 3.2, 6.0, and 2.2 ppm Cd for NorMan, FM and FP, respectively (Table 2-9). The Cd content in the 0.25 M NaCl fraction, which contains the flaxseed major storage globulins, is lower than the critical value of 1.3 ppm Cd on protein basis (0.3 ppm Cd in the whole seeds represents 1.3 ppm Cd in the protein, based on 24% protein in whole seeds). Results from this experiment indicate the potential to isolate the major storage protein from flaxseed with lower Cd content. In contrast, Suzuki et al. (1997) reported that Cd in rice grain grown in Cd contaminated fields is present in a form bound to the major glutelin storage protein.

The major Cd-binding fraction of 0.1 M NaCl was further separated by G50 size exclusion chromatography. The pattern of the eluted column profiles was similar for the three NorMan, FM and FP samples (Figure 2-6 & 2-8), each containing three peaks: the high molecular weight peak, peak A and B. However, the actual size of each peak differed,

probably a result of different loading quantities (80, 46, and 40 mg protein loaded for NorMan, FM, and FP, respectively, on a 500-mL column). Peak A, with apparent molecular weight of 14 kDa, could be an aggregate of low molecular weight complexes, because size exclusion chromatography was performed at low ionic strength where, according to Grill et al. (1987), metal-containing PC complexes of 2-4 kDa may aggregate and increase in molecular weight to 10 kDa. The HMW peaks for NorMan, FM and FP had no detectable Cd. Based on BCA protein assay, the Peak A was the major protein peak containing 58 to 62% proteins with 0 to 21% Cd; Peak B was the major Cd-binding fraction contained 73 to 100% Cd with 14 to 39% proteins. For the lowest Cd-containing flaxseed FP, Cd was not detectable in the Peak A.

Based on BCA protein assay, the protein contents of HMW peak, Peak A, and Peak B were 26, 60, and 14% of the total eluted protein, respectively (Table 2-3); however, Peak B was the peak eluting from size exclusion chromatography with largest area and highest peak value as measured by the absorbance at 280 nm (Figure 2-6). BCA protein assay estimates protein content by measuring the color response of peptide bands (αNH - αCO amide bond) (Smith et al., 1985), while protein determination by absorbance at 280 nm is mainly a function of the aromatic rings of tryptophan and tyrosine residues, as well as the cysteine content of the protein (Gill and van Hippel, 1989). Considering the low values determined by BCA assay and the high absorbance at 280 nm, Peak B probably contained unusual amide bonds and/or an abundance of aromatic ring or conjugated double bond structures. BCA protein assay is based on the biuret reaction (Smith et al., 1985); single amino acids and dipeptides do not participate in the biuret reaction, and tripeptides and larger polypeptides or proteins are needed for the biuret reaction. Hence, the structure of PC with repeated unit of γ-

Glu-Cys will not give a proper color reaction with BCA. Furthermore, the reaction that leads to BCA color formation as a result of the reduction of Cu^{2+} is also strongly influenced by the presence of any of four particular amino acids (cysteine, cystine, tryptophan, and tyrosine) in the amino acid sequence of the protein (Wiedelman et al., 1988). Therefore, the concentration of Peak B determined by the BCA assay may be incorrect if this peak contains PCs, dipeptides, unusual amino acids or aromatic or double-bonded structures other than tryptophan and tyrosine.

The high absorbance value at 280 nm for Peak B is not likely attributed to phenolics. Flaxseed has been reported to contain high phenolics, but the bound phenolics levels are very low (Oomah et al., 1995). Most of the phenolics in flaxseed have been removed by dehulling process, and phenolics can not be extracted by aqueous tris buffer used for protein extraction. Furthermore, the low protein content by BCA protein assay on Peak B with high A280 value indicated that the Peak B components were not composed of phenolics, because BCA protein assay is highly sensitive to phenolics and would yield a high instead of low protein content if phenolics were in the Peak B. On a weight basis, gallic acid, tannic acid, pyrogallic acid, and pyrocatechol yield 2.1-, 9.3-, 86.0-, and 106-fold more absorption in this method compared to bovine serum albumin (Kamath & Pattabiraman, 1988, Siebert & Lynn, 2005).

The chromatographic profiles and patterns of protein and Cd distribution in each column fraction were similar between the two flaxseed cultivars NorMan and Flanders. This indicated that the established protocol was successful for separating major Cd-binding proteins and major storage proteins, yielding representative and valuable results of Cd and protein distribution data. However, the variations among the three samples of NorMan, FM and FP were obvious, too. As summarized in Table 2-9, for all three samples, the major Cd-

binding fraction eluted at 0.1 M NaCl contained only about 7% of the total proteins eluted from ion exchange chromatography, but 50% (FM and FP) to 66% (NorMan) of the Cd. As for the major protein fraction eluted at 0.25 M NaCl, both FM and FP contained about 14% of the Cd with about 60% of the proteins, but NorMan contained 25% of the Cd with 72% of the protein. The data for samples FM and FP were similar to each other, but differed from NorMan, which could be attributed to a cultivar effect. Greater variation was observed for Cd and protein distribution in the size exclusion chromatographic fractions. For the lowest Cd level flaxseed sample FP, Peak A contained no Cd, and all the detectable Cd was present in peak B. Comparing the same cultivar grown in 2 locations FM and FP, Peak B of FM had a Cd level that was double the Cd level for Peak B of FP on protein basis. The medium Cd level cultivar NorMan had a very high Cd level in peak B, which may again reflect cultivar differences. High Cd level on protein basis apparently was related to higher seed Cd level in the same cultivar (FM and FP), but not between different cultivars (NorMan and Flanders).

Calcium, copper and zinc also were monitored along with Cd in each fraction from ion exchange and G50 chromatographic columns since the toxic effects of Cd in plants have been shown to be modified by essential elements like Ca, Cu and Zn (Das et al., 1997). It has also been hypothesized that elements with similar physical and chemical properties may act antagonistically to each other biologically, competing for the same transport and storage sites in the cell and displacing each other from reactive enzymatic and receptor proteins (Das et al., 1997).

The metal distribution in each chromatographic fraction of NorMan, FM and FP are shown in Tables 2-4, 2-7 and 2-8, respectively. Over 50% of the Ca was eluted into the unbound fraction from ion exchange chromatography, and 55-87% was eluted into Peak B

from size exclusion chromatography. More than 65% of the Cu was eluted into the 0.1 M and 0.25 M NaCl fractions, and Cu was almost evenly distributed into these two fractions. The major Cu binding fraction after size exclusion chromatography was Peak A for NorMan and FM, and Peak B for FP, all of them containing about 60% of the eluted Cu. The highest Zn binding fraction was the 0.1 M NaCl fraction for both NorMan and FM, and the 0.25 M NaCl for FP. In the eluted fractions from size exclusion chromatography, the major binding peak for Zn was Peak B for all three samples of NorMan, FM and FP with about 70 to 80% of the eluted Zn was in Peak B.

In most cases, Zn reduces the uptake of Cd by plants (Kabata-Pendias & Pendias, 1991). Zn and Cd are chemically similar and may compete for binding sites in the plant (Grant et al., 1998). Studies from Grant and Bailey (1997) showed a negative relationship between Zn and Cd concentrations in the whole flaxseed. This implies that an active competitive interaction may exist in the seed between Cd and Zn, and may suggest the competition for specific carriers. In contrast, in animal studies, a highly positive correlation between Cd and Zn concentrations in liver and kidneys has been noted (Oishi et al., 2000). The Cd-induced retention of Zn in the liver and/or kidney is due to Cd accumulation and metallothionein induction in these organs (Brzoska & Moniuszko-Jakoniuk, 2001).

The results of metal analyses showed considerable variability between replicated runs (Table 2-4). Although the same starting material of dehulled flaxseed was used for these experiments, the dehulled flaxseed was separately defatted and extracted for protein for each run of ion exchange column chromatography. Thus, some of the variability in the chromatographic profiles may be attributed to slight differences in the concentration and composition of the extracts used for chromatography (Table 2-1). A second reason for the

variability was that the protein extracts were first mixed with the resin in a batch mode; after incubation and washing, the resin with bound protein was packed into the column for elution. This method greatly decreased the time for sample loading, but may have resulted in loss of some protein and bound metals during washing and packing.

2.5   CONCLUSIONS

The techniques of ion exchange and size exclusion chromatography effectively separated the Cd-binding components from the major storage proteins of flaxseed (Figure 2-9). The major Cd binding fraction was eluted by 0.1 M NaCl from ion exchange chromatography, containing 50 to 66% of the Cd but only 7 to 8% of the total protein eluted from the column.   This major Cd-binding fraction of 0.1 M NaCl was further fractionated by G50 size exclusion chromatography into three peaks: non Cd binding fraction of HMW peak (MW higher than 30 kDa), and two Cd binding fractions of Peak A (MW around 14 kDa) and Peak B (MW no more than 1.5 kDa), where Peak B was the major Cd binding fraction with 73 to 100% of the eluted Cd in 14 to 39% of the eluted proteins as determined by BCA protein assay. However, it should be noted that Peak B may contain some non-peptide components that could not be quantified by BCA protein assay.

The major storage protein fraction eluted from ion exchange chromatography at 0.25 M NaCl contained 58 to 72% of the eluted proteins with only 14 to 25% of the eluted Cd. Expressed on a protein basis, this fraction had 0.4 to 1.3 ppm Cd, a value not higher than the critical value of 1.3 ppm Cd in the original seeds. This result indicates the potential to isolate the major storage protein from flaxseed with lower Cd content that can be safely used as

functional food ingredient. This laboratory-scale protocol therefore provides a basis for developing the potential industrial scale process.

The Cd distribution characteristics in each chromatographic fraction suggested the multiplicity of Cd binding factors. The 0.1 M NaCl is the major Cd binding fraction, but Cd also eluted into the unbound fraction. As well, Cd concentration increased in the high salt fractions of 0.45 to 1.0 M NaCl when seeds contained higher Cd, suggesting that the high salt fraction may contain high affinity Cd binding factors. There were no significant differences in the chromatographic profiles and the pattern of protein and Cd distribution in each fraction between NorMan and Flanders flax cultivars. However, cultivar effects may influence the quantity of protein and Cd eluted in the fractions of ion exchange chromatography.

2.6   TABLES AND FIGURES

Table 2-1. Cd and protein contents in dehulled seeds, defatted powder and protein extract from flaxseed NorMan cultivar[a]

Samples	Cd		Protein		Cd/protein
	ng	%[b]	mg	%	ppm
dehulled seeds[c]	5260	100.0	2390	100.0	2.2
defatted powder	5010±430	95.2	2297±150	96.1	2.2
protein extract	4320±980	82.1	1330±280	55.6	3.2

[a] Data are the average values ± standard deviation of three separate experiments starting with 10 g of dehulled flaxseed of cultivar NorMan.

[b] The % value represents % recovery based on dehulled seed.

[c] Data is from Agriculture and Agri-food Canada Pacific Agri-Food Research Center

Table 2-2. Cd and protein contents in each fraction from ion exchange chromatography of flaxseed NorMan cultivar[a]

Samples	Cd		Protein		Cd/protein
	ng	%[b]	mg	%	ppm
Unbound	285±53	8.3	239±118	18.5	1.2
0.1 M NaCl	2260±830	65.6	86±30	6.7	26
0.25 M NaCl	876±841	25.4	932±154	72.4	0.9
0.45 M NaCl	25±44	0.7	31±31	2.4	0.8
Column total	3450	100.0	1290	100.0	

[a] Data were from the average values ± standard deviation of three separate experiments loaded with protein extract from 10 g of dehulled flaxseed.
[b] % calculations were based on the total amount eluted from the column.

Table 2-3. Cadmium and protein contents in each fraction from G50 size exclusion chromatography of flaxseed NorMan cultivar[a]

Samples	Cd		Protein		Cd/protein
	ng	%[b]	mg	%	ppm
HMW peak	ud[c]	N/A	17.3	25.8	N/A
Peak A	473	21.3	40.2	60.1	11.8
Peak B	1750	78.7	9.50	14.1	184
Column total	2220	100.0	67.0	100.0	

[a] Data were from the average values of two separate experiments loaded with major Cd-binding fraction of 0.1 M NaCl from ion exchange chromatography starting with 10 g of dehulled flaxseed.

[b] % calculations were based on the total amount eluted from the column.

[c] ud is under the detection limit of 0.1 ppb for Cd.

Table 2-4. Contents of Ca, Cu, and Zn in ion exchange and size exclusion (G50) chromatographic fractions for flaxseed NorMan cultivar

	Ca		Cu		Zn	
	μg	%[c]	μg	%	μg	%
Ion exchange fractions[a]						
Unbound	1010±160	50.7	23.3±20.6	12.8	23.5±9.3	19.6
0.1M	217±317	10.9	75.1±60.2	41.2	53.0±22.2	44.1
0.25M	333±174	16.7	76.4±20.4	41.9	43.0±5.9	35.8
0.45M	433±279	21.7	7.65±2.18	4.2	0.558±0.966	0.5
Total	1990	100.0	182	100.1	120	100.0
G50 fractions[b]						
HMW peak	64.3	15.6	20.3	4.8	1.69	2.0
Peak A	70.0	16.9	276	65.2	26.5	30.5
Peak B	279	67.5	127	30.0	58.7	67.6
Total	413	100.0	423	100.0	86.9	100.1

[a] Data were from the average values ± standard deviation of three separate experiments loaded with protein extract from 10 g of dehulled flaxseed.

[b] Data were from the average values of two separate experiments loaded with major Cd-binding fraction of 0.1 M NaCl from ion exchange chromatography starting with 10 g of dehulled flaxseed.

[c] % calculations were based on the total amount eluted from the column.

Table 2-5. Protein and Cd distributions in ion exchange chromatographic fractions of flaxseed cultivar Flanders grown in Morden (FM) and Portage la Prairie (FP)[a]

Fractions	Cd				Protein				Cd/protein	
	FM		FP		FM		FP		FM	FP
	ng	%[b]	ng	%	mg	%	mg	%	ppm	
Extract	7450		3030		1250		1390		6.0	2.2
Unbound	1490	20.8	665	34.7	331	26.6	356	28.5	4.5	1.9
0.1M	3550	49.5	990	51.7	89.3	7.18	102	8.2	39.8	9.7
0.25M	1010	14.1	261	13.6	763	61.3	729	58.4	1.3	0.4
0.45M	838	11.7	ud[c]	0	57.6	4.63	56	4.5	14.5	N/A[d]
0.50M	122	1.7	ud	0	1.44	0.12	4.43	0.4	84.7	N/A
1.0M	160	2.23	ud	0	2.05	0.16	1.81	0.1	78.0	N/A
Column total	7170		1916		1244		1249			

[a] Data were the average from two separate experiments calculated based on 10 g of dehulled flaxseed as starting material.

[b] % calculated based on column total eluted amount as 100%.

[c] ud means under detection limit of 0.1 ppb for Cd.

[d] N/A means not applicable.

Table 2-6. Protein and Cd distributions in size exclusion chromatographic fractions of flaxseed cultivar Flanders grown in Morden (FM) and Portage la Prairie (FP)[a]

Fractions	Cd				Protein				Cd/protein	
	FM		FP		FM		FP		FM	FP
	ng	%[b]	ng	%	mg	%	mg	%	ppm	
0.1M	2590		718		93.8		62.9		27.6	11.4
HMW peak	ud[c]	N/A[d]	ud	N/A	< 0.1	N/A	< 0.25	0.4	N/A	N/A
Peak A	609	27.2	ud	N/A	45.4	61.5	42.1	67.4	13.4	N/A
Peak B	1630	72.8	506	100	28.3	38.4	20.1	32.2	57.6	25.2
Column total	2239		506		73.8		62.5			

[a] Data were the average values of two separate experiments loaded with major Cd-binding fraction of 0.1M NaCl from ion exchange chromatography starting with 10 g of dehulled flaxseed.

[b] % calculated based on column total eluted amount as 100%.

[c] ud means under detection limit.

[d] N/A means not applicable.

Table 2-7. Contents of Ca, Cu, and Zn in ion exchange and size exclusion (G50) chromatographic fractions of flaxseed Flanders grown in Morden (FM)[a]

	Ca		Cu		Zn	
	µg	%	µg	%	µg	%
Ion exchange fractions						
Unbound	1790	69.6	19.9	15.0	49.7	33.0
0.1M	257	10.0	44.9	33.9	56.3	37.4
0.25M	273	10.6	40.6	30.7	40.7	27.0
0.45M	171	6.7	18.9	14.3	ud[b]	0.0
0.50M	37.6	1.5	3.98	3.0	2.28	1.5
1.0M	41.6	1.6	4.01	3.0	1.52	1.0
Total	2570.2	100.0	132.3	100.0	150.5	100.0
G50 fractions ·						
HMW peak	ud[b]	0.0	0.61	0.8	ud[b]	0.0
Peak A	14.5	12.6	45.7	61.8	8.7	23.6
Peak B	101	87.4	27.7	37.4	28.1	76.4
Total	115.5	100.0	74.0	100.0	36.8	100.0

[a] Data were the values from two separate experiments loaded with protein extract from 10 g of dehulled flaxseed.

[b] ud means under detection limit.

Table 2-8. Contents of Ca, Cu, and Zn in ion exchange and size exclusion (G50) chromatographic fractions of flaxseed Flanders grown in Portage la Prairie (FP)[a]

	Ca		Cu		Zn	
	μg	%	μg	%	μg	%
Ion exchange fractions						
Unbound	2270	53.8	27.8	20.1	33.0	32.8
0.1M	898	21.3	59.3	43.0	20.4	20.3
0.25M	665	15.7	40.9	29.6	41.9	41.7
0.45M	316	7.5	8.07	5.8	1.49	1.5
0.50M	55	1.3	1.38	1.0	2.75	2.7
1.0M	18.4	0.4	0.55	0.4	0.92	0.9
Total	4222.4	100.0	138.0	100.0	100.5	100.0
G50 fractions						
HMW peak	170	15.9	1.31	1.2	0.00	0.0
Peak A	303	28.3	43.7	38.9	5.23	18.0
Peak B	598	55.8	67.2	59.9	23.9	82.0
Total	1071	100.0	112.2	100.0	29.1	100.0

[a] Data were the values from two separate experiment loaded with protein extract from 10 g of dehulled flaxseed.

Table 2-9. Comparison of Cd and protein contents in the major Cd binding fractions from ion exchange and size exclusion chromatography of three different flaxseed samples NorMan, Flanders grown in Morden (FM) and in Portage la Prairie (FP).

		NorMan	FM	FP
Protein extract	Cd/protein (ppm)	3.2	6.8	1.7
Ion exchange column fractions				
0.1M	Cd (%[a])	66	50	52
	Protein (%)	7	7	8
	Cd/protein (ppm)	26	40	9.7
0.25M	Cd (%)	25	14	14
	Protein (%)	72	61	58
	Cd/protein (ppm)	0.9	1.3	0.4
Gel filtration column fractions				
Peak A	Cd (%)	21	27	0
	Protein (%)	60	62	58
	Cd/protein (ppm)	12	13	0
Peak B	Cd (%)	79	73	100
	Protein (%)	14	39	28
	Cd/protein (ppm)	184	54	25

[a] each percentage amount is based on the total eluted amount as 100%.
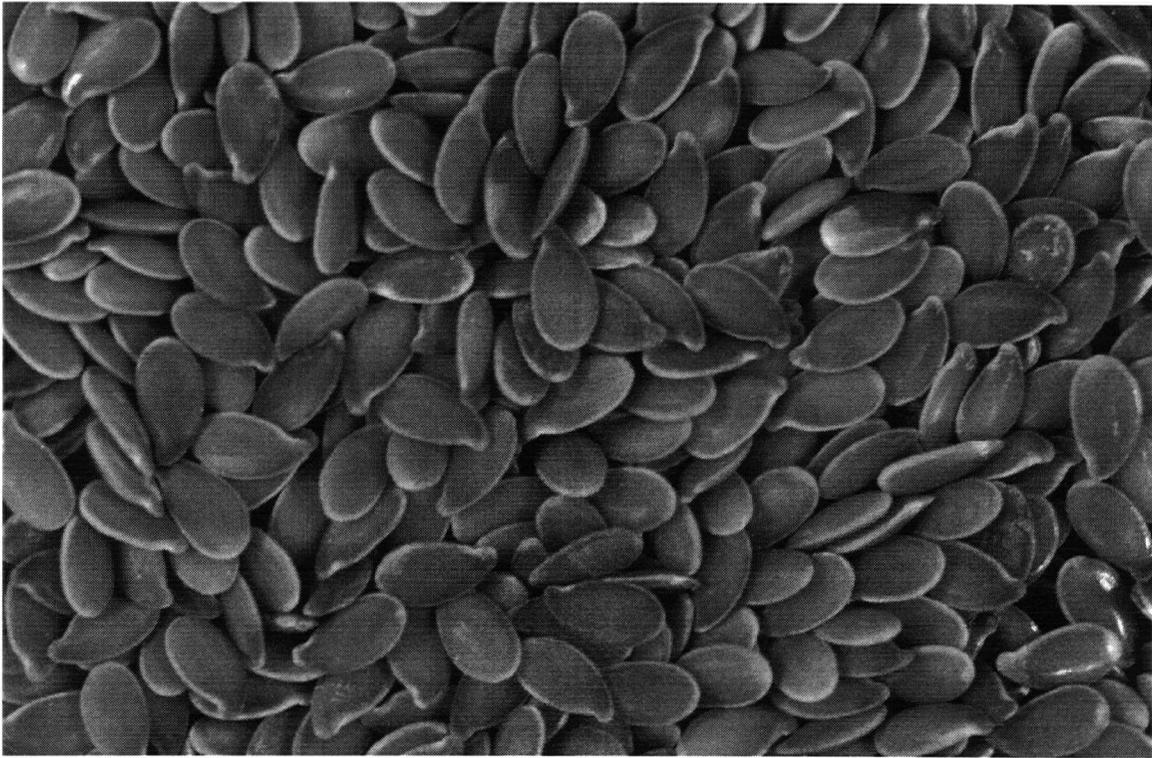
Figure 2-1. Whole flaxseed of NorMan cultivar.

Figure 2-2. Flaxseeds of NorMan cultivar after processing by a Strong Scott barley perler with the hulls (darker shade) separated from the seeds (lighter shade).
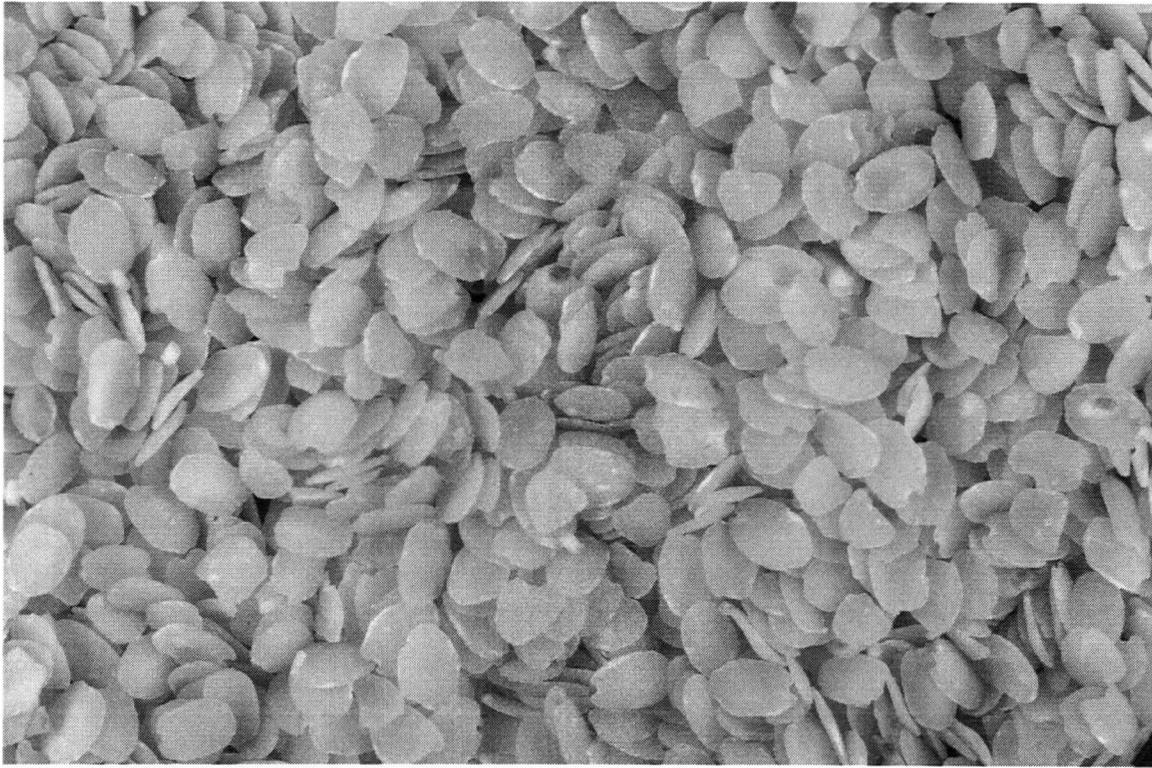
Figure 2-3. Dehulled flaxseeds of NorMan cultivar after processing to remove the hulls by an air aspirator and manual sorting.

Figure 2-4. Defatted dehulled flaxseeds of NorMan cultivar ground by a Wiley Mill and then defatted by hexane and petroleum ether.
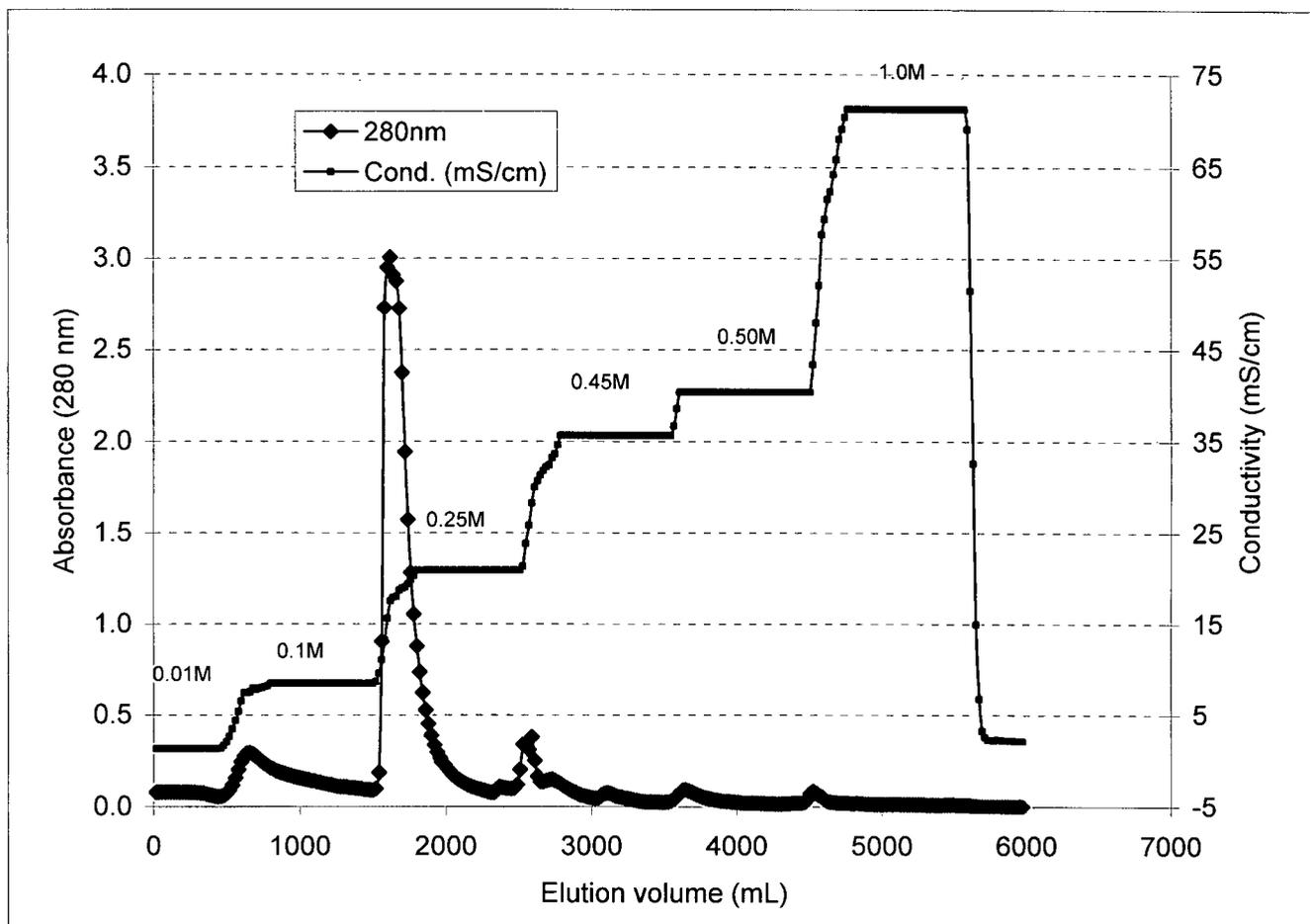
Figure 2-5. DEAE-Sephacel ion exchange chromatography of protein extract from 10 grams of dehulled seeds of NorMan cultivar. Proteins were eluted with 0.1 - 1.0 M NaCl in 0.01 M Tris buffer at pH8.6.
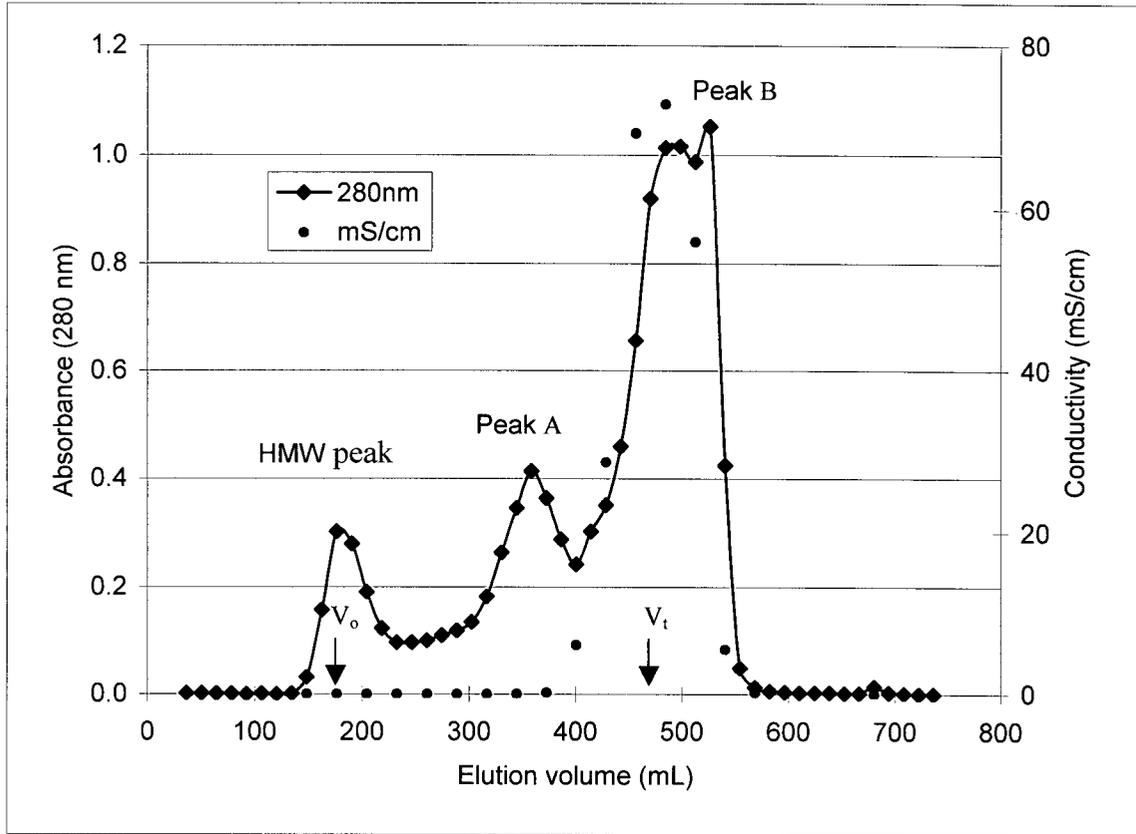
Figure 2-6. Sephadex G50 size exclusion chromatography of 0.1 M NaCl fraction eluted in deionised and distilled water. The 0.1M NaCl fraction was collected from ion exchange chromatography of flaxseed NorMan cultivar (Figure 2-5).

(a): Flanders grown in Morden
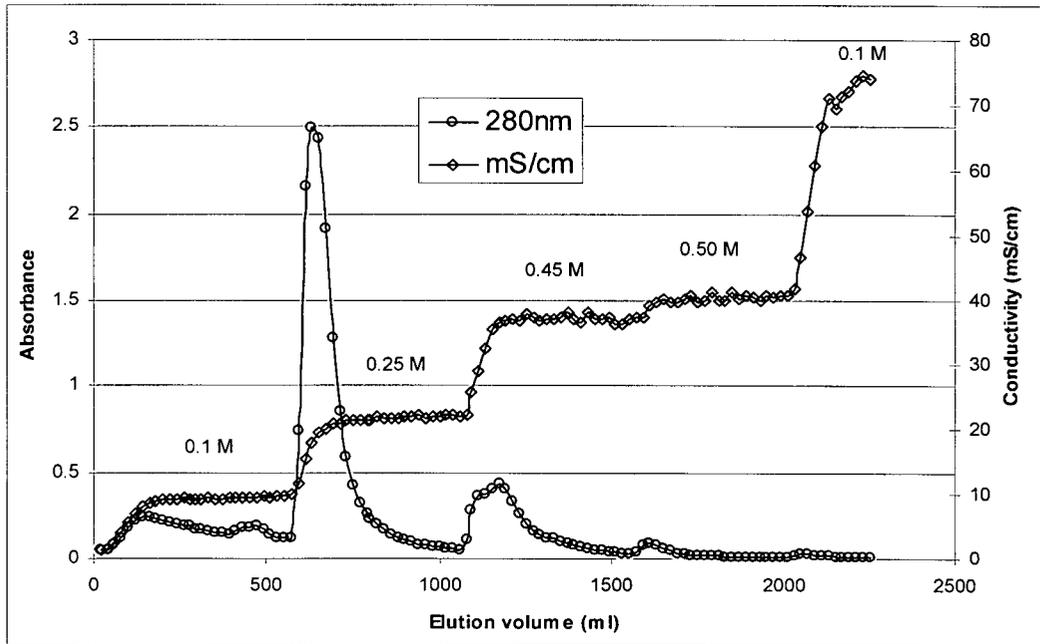
(b): Flanders grown in Portage la Prairie
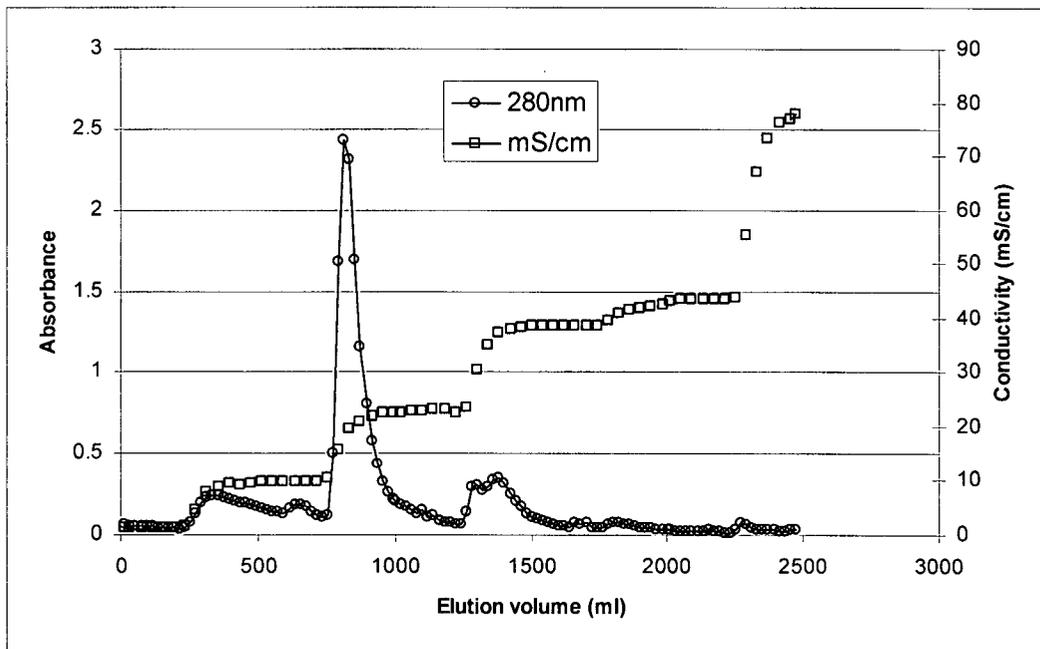
Figure 2-7. DEAE-Sephacel ion exchange chromatography of protein extract from dehulled seeds of Flanders cultivar. (a): Flanders grown in Morden, (b): Flanders grown in Portage la Prairie. Proteins were eluted with 0.01 to 1.0 M NaCl in 0.01M Tris buffer at pH8.6.

82

(a): Flanders grown in Morden
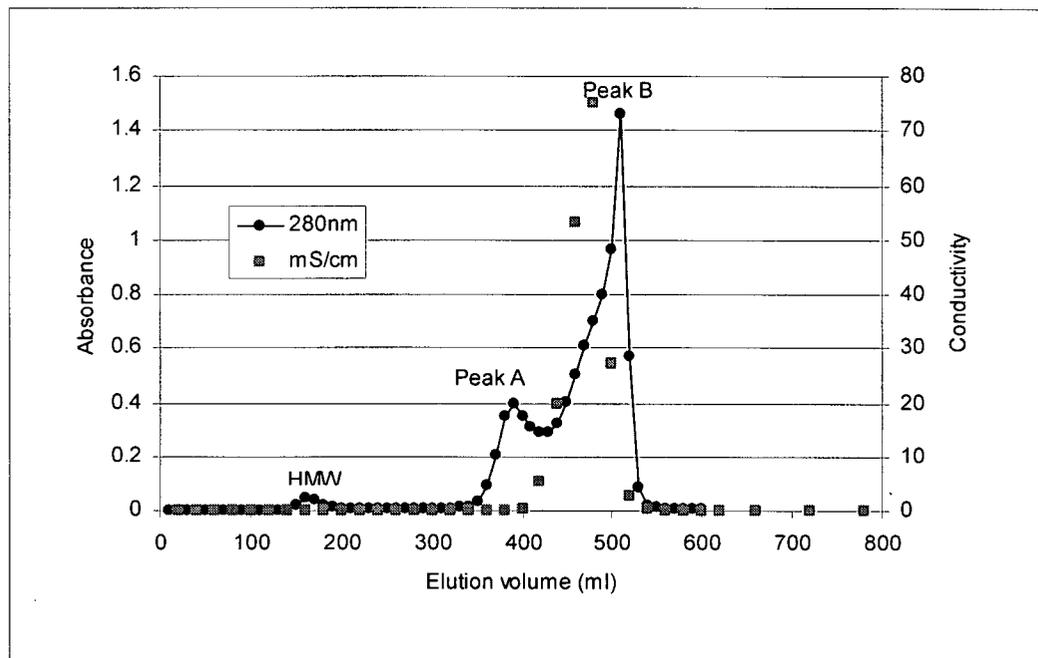
(b): Flanders grown in Portage la Prairie
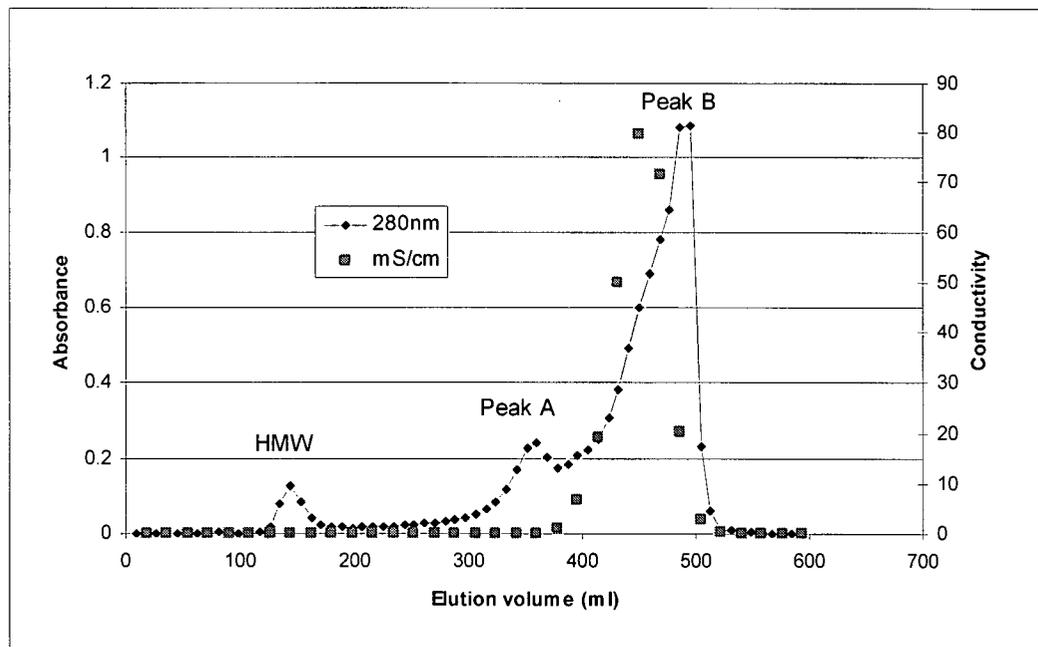
Figure 2-8. Sephadex G50 size exclusion chromatography of 0.1M NaCl fraction eluted in deionized distilled water. The 0.1M NaCl fraction was collected from ion exchange chromatography loaded with (a) Flanders grown in Morden, (b) Flanders grown in Portage la Prairie.
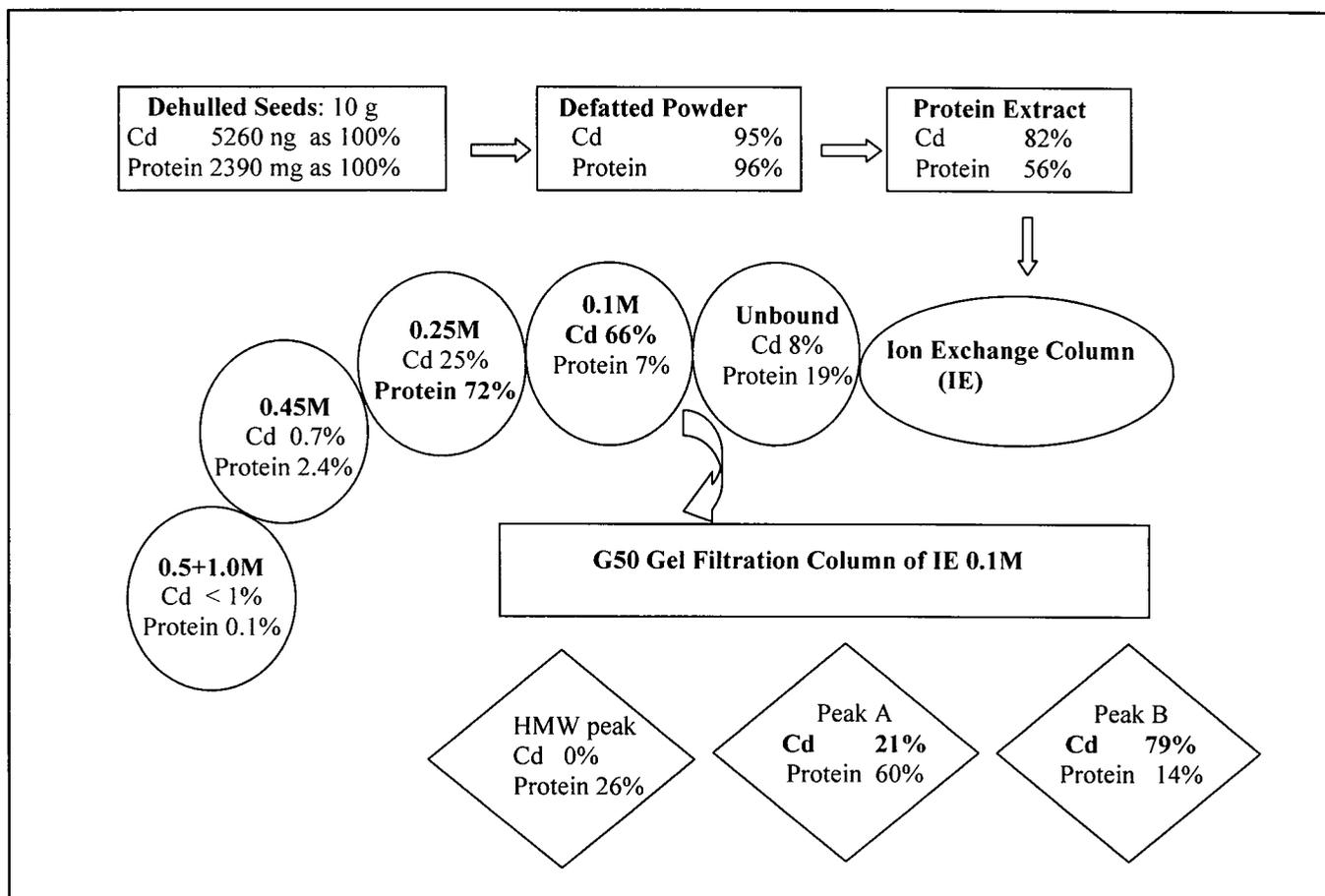
Figure 2-9. Summary of Cd and protein recoveries from each step and fraction of isolation and extraction. Percentage recoveries in boxes are expressed on the basis of the total amount in the starting material of dehulled flaxseed of NorMan cultivar; percentages of column fractions are based on the total eluted amount.

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CHAPTER 3 Cd-BINDING COMPONENTS IN FLAXSEEDS AS A FUNCTION OF GROWING LOCATION AND CULTIVAR

3.1 INTRODUCTION

The heavy metal Cd is a toxic element naturally existing in the earth's crust (Tahvonen, 1996). The flax plant absorbs Cd from soil and accumulates most of the absorbed Cd in the seeds, leading to a Cd level that exceeds the dietary critical value or maximum level of 0.3 ppm (Becher et al., 1997). Cd in flaxseed is further transferred to the food chain (Marquard et al., 1990; Moraghan, 1993; Cieslinski et al., 1996) and finally poses a health problem by accumulating in the human kidney after consumption. The accumulation of Cd in the renal cortex may lead to renal tubular dysfunction, disturbances in calcium metabolism, hypercalciuria, and the formation of renal stones (Tahvonen, 1996).

Cd contents have been determined in 16 genotypes of flaxseed grown at 5 different locations in Germany (Marquard et al., 1990) with a range of Cd contents between 0.1 to 1.7 ppm. This study found that genotypic differences in uptake and accumulation of Cd do exist in flaxseed. The results of those trials also demonstrated that flaxseed low in Cd-content could be produced only at suitable locations. Another study demonstrated that 74 flax lines grown on uncontaminated, alkaline soils showed large variations in seed Cd level, which ranged from 0.14 to 1.37 ppm (Li et al., 1997), and it was speculated that breeding for low seed Cd in flax should be feasible. A study by Hammond et al. (2000) supported the hypothesis that Cd accumulation in flaxseed is genotype dependent. They analyzed 2,700 accessions of flaxseed grown in a relatively high Cd soil in 1994, and found that the mean Cd level of all accessions was 1.04 ppm, ranging from 0.27 to 3.60 ppm. A multiple linear

regression model fitting numerous characteristics indicated a contribution from plant genotype, oil percentage, country of origin, and maturity. All three accessions with Cd level less than < 0.3 ppm were from Pakistan. In Canada, low Cd concentration has been used as a criterion in breeding of oilseed flax (Grant et al., 1998).

The average content of Cd in soils lies between 0.06 and 1.1 ppm worldwide, and the calculated worldwide mean is 0.53 ppm Cd in surface soils. The Cd level of loamy and clay soils in Canada ranges from 0.12 to 1.60 ppm with a mean of 0.64 ppm (Kabata-Pendias and Pendias, 1991). The Cd content in Manitoba soils ranges from 0.1 to 7.9 ppm, although 95% of the samples fall below the average of 0.53 ppm observed in soils world-wide (Haluschak et al., 1998). The Canadian Grain Commission carried out a trace element survey in 2003 on flaxseed samples, and a map of Cd levels in flax was produced for the 1992-2000 crops. The map has been released to the Flax Council for distribution to flax growers and marketers as an aid in making production and sourcing decisions (Canadian Grain Commission, 2004). According to this map, Cd accumulation in flax is apparently location dependent, and the Cd levels ranged from 0.1 to 1.13 ppm based on the mean values for crops from 1992 to 2000 in Alberta, Saskatchewan, and Manitoba.

Cd concentration in crops varies considerably from location to location as reviewed by Grant et al. (1998). Geographical differences in Cd level in flaxseed may relate to variation in soil characteristics such as parent material, Cd concentration, pH, salinity, organic matter content and soil texture, to atmospheric deposition, or to patterns of agronomic management such as fertilizer use or cultivar selection (Grant et al., 1998). Crops grown on soils derived from black shales in the Canadian prairies had higher levels of Cd than plants grown on till soils (Malegus & Goh, 1995). A field study at three locations over 3

years demonstrated that location had a large effect on Cd concentration in flaxseed, and year to year variations were also large, with higher seed Cd concentrations occurring in years with greater precipitation (Grant et al., 2000). A study with 3 flaxseed cultivars in two different types of soils containing 0.124 and 0.323 ppm Cd, respectively, showed that Cd bioaccumulation and distribution within the crops were strongly affected by both soil type and plant cultivar (Cieslinski et al., 1996).

Accumulation of Cd in flaxseed, as influenced by cultivars and locations, has been studied extensively during last decade, but no data are available regarding the influence of flaxseed cultivars and locations on the distribution of Cd within the components of the flaxseed. These data are essential if flaxseed is to be used as a functional food ingredient. Even though the overall Cd content in a certain flaxseed cultivar may be high, the real concern lies in the Cd which is present in the fractions of flaxseed to be used for food purpose. Systematic research has been carried out in Dr. Eunice Li-Chan's laboratory to understand the mechanisms of Cd accumulation and distribution in flaxseed. Cd is not present in the flaxseed oil and hull and is believed to be bound to proteins (Kolodziejczyk & Fedec, 1995). Proteins of flaxseed cultivar NorMan, with a Cd level at 0.526 ppm, were extracted from dehulled and defatted powder, and fractionated by DEAE-Sephacel anion exchange chromatography under column overloading condition. It was found that phytochelatin-like complexes existed in the high salt fractions eluted at concentrations of 0.45 and 0.50 M NaCl (Li-Chan et al., 2002). Further investigation indicated that the majority of the Cd in the seeds could be separated into a low salt fraction containing 66% of total eluted Cd in only 7% of the total eluted proteins, while the major storage proteins were eluted by 0.25 M NaCl in a later peak containing 72% of the eluted protein with only 25% of

the eluted Cd. This result indicated the potential to isolate the major storage protein in flaxseed with a low Cd content (Lei et al., 2003). Another flaxseed cultivar Flanders was used to verify the results from cultivar NorMan. Flaxseed of Flanders cultivar grown in Morden was found to have a Cd level at 1.54 ppm, while that grown in Portage la Prairie had a Cd level at 0.38 ppm. Even though the Cd contents in the different flaxseed samples were quite different, similar results were observed with respect to Cd distribution, where about 50 to 66% of the total flaxseed Cd was in the 0.1 M NaCl fraction composed of only 7 to 8% of the total protein, while only 14 to 25% of the total Cd was in the 0.25 M NaCl fraction containing 58 to 72% of the total protein.

In order to verify the relevance of the above experimental results and the established protocols to other flaxseed cultivars, and also to reveal the possible relationships of the Cd distribution pattern in flaxseed as influenced by different cultivars and locations, further experiments on a larger sample size were conducted, which involved five different flaxseed cultivars growing in three different locations.

The five cultivars selected for this study were all bred in Canada. AC Linora, AC McDuff, and NorLin were developed by the AAFC Research Station in Morden, Manitoba, while CDC Normandy and Flanders were developed at the Crop Development Centre at The University of Saskatchewan in Saskatoon, Saskatchewan. AC Linora is a medium early-maturing oilseed flax, with high yield potential in both early and late seeding. It is widely adapted in the prairie provinces (Kenaschuk & Rashid, 1993). AC McDuff is late maturing and has very high oil content, very good lodging resistance and high yield in the Black Soil zone of the prairies (Kenaschuk & Rashid, 1994). CDC Normandy is a medium-early maturing cultivar, with medium oil content, good oil quality, fair lodging resistance and high

yields when seeded early in the Black and the Brown Soil zones of the prairies (Rowland et al., 2002). Flanders is a late-maturing and high-yielding oilseed flax that is very high yielding in all areas of western Canada. It is also high in oil content and oil quality (Rowland et al., 1990). NorLin is a medium-early, high-yielding oilseed flax, and is widely adapted in the prairies (Kenaschuk & Hoes, 1986).

The objectives of the study were therefore to compare the distribution of proteins, Cd, and other metals in various fractions from flaxseed as a function of cultivar and location.

3.2   MATERIALS AND METHODS

3.2.1   Reagents

Reagents used were the same as reported in Section 2.2.1., Chapter 2.

3.2.2   Flaxseeds

The five cultivars grown in Morden (49° 12′ N, 98° 5′ 59″ W), Portage la Prairie (49° 57′ N, 98° 19′ 59″ W), and Rosebank (49° 57′ N, 98° 19′ 59″ W) were harvested in 1998 by the AAFC Research Station in Morden, Manitoba. The samples used in this experiment were obtained through Dr. G. Mazza and Dr. D. Oomah at the AAFC, Pacific Agri-Food Research Centre, Summerland, British Columbia. The dehulled and defatted flaxseed powders were prepared according to the procedures described in Section 2.2.2., Chapter 2.

3.2.3 Protein Extraction

The whole protein extracts from 3 g of dehulled and defatted powder of 15 flaxseed samples were prepared by using the procedures previously described in Section 2.2.3., Chapter 2.

3.2.4 Ion Exchange Chromatography

The method for separation of whole protein extracts was a modification of the procedures described in Section 2.2.4., Chapter 2. The modification included the use of 350 mL instead of 500 mL of DEAE-Sephacel. The whole extracts from 3 g of dehulled and defatted powder were also diluted 10 times before loading to the column, which was eluted at a flowrate of 10 mL/min.

3.2.5 Analysis of Column Fractions

Absorbance spectra, protein content and metal (Ca, Cu, Zn, and Cd) analyses of each fraction from ion exchange chromatography were determined by the methods described in Section 2.2.6., Chapter 2.

3.2.6 SDS-PAGE

The 15 protein extracts of five flaxseed cultivars grown at three locations were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All extracts were adjusted to a protein concentration of 1.1 mg/mL. To 80 μL of each protein sample were added 20 μL of 10% (w/v) SDS and 1 μL of 1% bromophenol blue; to some samples 2 μL of 2-mercaptoethanol were added for reducing conditions. The prepared

samples were boiled for 20 minutes in Eppendorf tubes, and then centrifuged for 5 minutes at 16,000 g in a benchtop Eppendorf centrifuge (Fisher Scientific, Nepean, On), and the supernatants were used for electrophoresis. Proteins (3 µL/lane) were electrophoresed on PhastGel® gradient 10-15 according to the PhastSystem™ Separation Technique (File No. 110—SDS-PAGE; Amersham Pharmacia Biotech Inc., Quebec, PQ). Molecular weight markers (Sigma Marker™ Wide Molecular Weight Range, M-4038; Sigma, St. Louis, MO) were used. The PhastGels® were stained with Coomassie blue according to the PhastSystem™ Development Technique (File No. 200—Fast Coomassie staining). Ferguson plots for molecular weight estimation were prepared by plotting the logarithms of the protein standard molecular weights against their corresponding relative mobility. The relative mobility was calculated as the distance of migration at the centre of the protein band in the resolving gel divided by the distance of migration of the tracking dye. Molecular weights of the samples were determined from their relative mobility using the Ferguson plot (Smith, 1994).

3.2.7 Statistical Analysis

One-way and two-way ANOVA (analysis of variance) followed by Tukey's Pairwise Comparisons were used to analyze the experimental data using MINITAB Release 13.30.

3.3 RESULTS AND DISCUSSION

3.3.1 Influence of Locations and Cultivars on Cd Levels in Whole Flaxseed

In order to investigate the possible influeces of location and cultivar on Cd level in flaxseed, five flaxseed cultivars grown in three different locations were obtained for the

experiment. The Cd levels ranged from 0.25 to 1.54 ppm as outlined in Table 3-1. A significant influence of growing location on Cd content was observed, with ranges of 0.964-1.543, 0.246-0.380 and 0.310-0.413 ppm for cultivars grown at Morden, Portage La Prairie and Rosebank, respectively. The Cd level of the cultivars grown in Morden was three to four times higher than those grown in Portage la Prairie and Rosebank. According to two-way ANOVA, using cultivar and location as two factors, and assuming that the interactions were not significant, there was a highly significant difference among the Cd levels in the three locations ($p < 0.001$), but not among cultivars ($0.1 < p < 0.5$). Similar location effect was also observed for Cd level on protein basis among cultivars, but there was no location or cultivar effect on flaxseed protein contents.

The strong effect of location was primarily attributed to the much higher seed Cd of flax grown in Morden, which tended to predominate over other potential effects such as that from cultivar. In order to investigate whether a potential cultivar effect would be detected for the two locations having more similar Cd levels, a two-way ANOVA was done on a subset of the data excluding the 5 samples from the Morden location (Table 3-1). The results showed that in fact there was a slight cultivar effect ($p = 0.059$) as well as location effect ($p = 0.019$) when the ANOVA was performed excluding the Morden data. Flaxseed of cultivar Flanders grown in Rosebank contained the highest Cd. Thus, our results support the conclusion of Grant et al (2000) that the growing location had a great impact on Cd level in flaxseed. The dependence of Cd level on flaxseed genotype had been reported earlier (Becher et al, 1997; Li et al., 1997), but in the present study, the predominant effect of location, particularly Morden, shadowed possible effects of cultivar on Cd.

3.3.2 Influence of Locations and Cultivars on Cd and Protein Distribution in Chromatographic Fractions

3.3.2.1 Percentage distribution in each chromatographic fraction

The protein extracts of the 15 flaxseed samples were loaded to the DEAE-Sephacel column to separate the major Cd binding and major protein peaks. The chromatographic profiles are shown in Figure 3-1a to 3-1e. The profiles were very similar to the profiles of flaxseed NorMan and Flanders (Figure 2-5 & 2-7). The major protein peak was eluted in the 0.25 M NaCl fraction, and two smaller peaks were observed by elution with 0.1 and 0.5 M NaCl.

The pattern of Cd and protein distributions in each ion exchange chromatographic fraction of the 5 cultivars at three different locations (Table 3-2) was also similar to the pattern for flaxseed NorMan (Table 2-2). The highest concentration of Cd was found in the 0.1 M NaCl fraction, and highest concentration of protein was in the 0.25 M NaCl fraction. However, some exceptions are noted. For AC McDuff grown in Morden and for Flanders grown in Rosebank, the 0.25 M NaCl fraction contained the highest amount of Cd (Table 3-2). Another significant phenomenon can be noticed in Table 3-2 is that the Cd content of the 0.1 and 0.25 M NaCl fraction of each flaxseed cultivar grown at the Morden location is two to four times higher than Cd content in each corresponding fraction of that cultivar grown at other locations. This is apparently a reflection of the location effect on the Cd contents in the whole seeds, because the two fractions of 0.1 and 0.25 M NaCl contained about 80% of the total proteins in the protein extract from whole flaxseeds.

In order to facilitate the comparison of the Cd and protein in each fraction among different cultivars and locations, the Cd and protein distribution percentages (i.e., the

amounts in each fraction expressed as a percentage of the total eluted amount from column) were calculated for each fraction. A general trend in the distribution of Cd and protein in each fraction can be observed for the different cultivars and the cultivars at different locations (Table 3-3). The average Cd contents ($\pm$SD) in each fraction of the 15 samples were 3.4 ($\pm$2.0), 51.7 ($\pm$6.4), 39.2 ($\pm$8.2), and 5.7% ($\pm$4.7%) for unbound, 0.1, 0.25, and 0.5 M NaCl fractions, respectively; the average protein contents in each fraction were 15.8 ($\pm$1.3), 12.3 ($\pm$2.2), 65.5 ($\pm$3.4), and 6.4% ($\pm$2.2%) for unbound, 0.1, 0.25, and 0.5 M NaCl fractions, respectively. The major protein fraction of 0.25 M NaCl contained 65.5% of the total column-eluted protein with 39.2% of the eluted Cd, whereas the major Cd fraction contained 12.3% of the protein with 51.7% of the column eluted Cd. This means that half of the Cd in protein extract can be removed in the 0.1 M NaCl fraction, allowing the recovery of a major protein fraction with a much lower Cd content on a protein basis than in the starting flaxseed.

There was a significant difference among cultivars, at the 99% confidence interval (p = 0.005), in the % Cd distributed into the unbound fractions , with the cultivars AC Linora and NorLin being higher than other three cultivars (Table 3-3). In contrast, the % Cd distribution in the unbound fractions showed no significant difference among different locations. There were also no significant differences in Cd distribution in the 0.1, 0.25 and 0.50 M NaCl fractions among different cultivars or locations based on two-way ANOVA analysis.

The analysis of Cd content by ICP-MS only detects the total level of Cd in each fraction, and does not provide information on the form of Cd existing in that fraction. The higher Cd distribution in the unbound fractions of AC Linora and NorLin could have resulted from the dissociation of Cd from the Cd-binding components that bound to the column,

which could imply that the Cd-binding components in flaxseed cultivars of AC Linora and NorLin might be less stable compared with Cd-binding components in flaxseed cultivars AC McDuff, CDC Normandy, and Flanders. The dissociation of Cd from Cd-binding components may be related to intrinsic characteristics of the cultivars. Among the five cultivars, AC Linora (Kenaschuk & Rashid, 1993) and NorLin (Kenaschuk & Hoes, 1986) share 50% parental relationship, as one of their parent cultivars is Linott. These two cultivars differ from the other cultivars in parental family, and the other three cultivars themselves do not share any common parental family (Kenaschuk & Rashid, 1994; Rowland et al., 2002; Rowland et al., 1990). The insignificant difference in the % Cd distribution in each NaCl eluted fraction (0.1, 0.25 and 0.5 M) among different cultivars and locations indicated that the percentage distribution of the various Cd-binding molecules separated by anion exchange chromatography is independent of cultivar and location. Similarly, there is no significant difference in percentage protein distribution in each fraction among different cultivars and locations based on two-way ANOVA analyses.

3.3.2.2 Cd distribution on protein basis in each chromatographic fraction

As mentioned above (Section 3.3.2.1), the major Cd-binding fraction was eluted at 0.1 M NaCl and the major protein fraction was eluted at 0.25 M NaCl for 13 of the analyzed samples, but exceptions were noted for AC McDuff at Morden and Flanders at Rosebank, where the major Cd-binding fraction was eluted at 0.25 M NaCl. However, if the Cd contents were compared based on proteins, that is the ratio of ng Cd /mg protein is to be compared, it is found that the 0.1 M NaCl fraction was without exception the major Cd-binding fraction in all 15 samples including AC McDuff at Morden and Flanders at Rosebank (Table 3-4). The

Cd level on protein basis in the whole seeds and protein extracts were analyzed by two-way ANOVA. The results showed that Cd level were highly significant (p = 0.000) among different locations, but not among cultivars (Table 3-4).

The statistical analysis also showed that the Cd concentration (Cd/protein) in the unbound fraction and the high salt fraction of 0.50 M NaCl showed no significant difference among cultivars and locations. However, there were very highly significant differences (P = 0.000) in the Cd levels in 0.1 and 0.25 M NaCl fractions among different locations but not cultivars (Table 3-4). Table 3-5 is the summary of the Cd level protein basis in each fraction at different locations. Fractions of the flaxseed in Morden contained the highest ratios of Cd/proteins compared with those in Portage la Prairie and Rosebank. The Cd concentrations on a protein basis were 1.47, 35.73, 4.20, and 3.79 (ng/mg protein) for unbound, 0.1, 0.25 and 0.50 M NaCl for Morden; these concentrations were all 2 to 3 times higher than those for Portage la Prairie and Rosebank. Compared with Cd levels in the whole seeds, the major Cd binding fractions of 0.1 M NaCl were around 5 to 9 times higher in Cd concentration after fractionation by ion exchange chromatography (Table 3-5). These results strongly support that the 0.1 M NaCl fraction contained the major Cd binding components compared with the other fractions.

The Cd level in the soil of Morden is higher than 0.3 ppm (Haluschak et al., 1998), while the Cd levels in Portage la Prairie and Rosebank are less than 0.3 ppm (Haluschak et al., 1998; Grant et al., 2000). This suggests that Cd accumulation in flaxseed is positively related to the soil Cd level. However, regardless of the Cd level in the soil, the 0.1 M NaCl fraction remained the major Cd binding fraction. The grand average of Cd/protein ratios for the 15 samples regardless of cultivars and locations were 0.92, 19.36, 2.55, and 3.53 ng

Cd/mg proteins for the unbound, 0.1, 0.25 and 0.50 M NaCl fractions. The Cd concentration in the 0.1 M NaCl fraction was much higher than all other fractions, being 21, 7.6, and 5.5 times of the Cd concentration in unbound, 0.25 and 0.50 M NaCl fractions, respectively, and 4.6 and 6.4 times higher than the Cd level in the unfractionated protein extract and whole seeds, respectively. In contrast, the Cd level in the 0.25 M NaCl fraction was 40 and 16% less than the protein extract and whole seeds, respectively.

3.3.3 Ca, Cu, and Zn Distribution

The Ca, Cu and Zn contents of each ion exchange chromatographic fraction of the 15 flaxseed samples were also measured along with Cd, and the results are summarized in Table 3-6. In order to facilitate the comparison, the total amount of metals in each fraction was calculated as percentage of the total amount eluted from the column, and the results are shown in Table 3-7. Most of the Ca (30 to 56%) was eluted into the unbound fraction in all the 15 samples and in all the locations, except in the case of the Flanders, which had a similar Ca content (30 to 34%) in both unbound and 0.25 M NaCl fraction for locations of Morden and Portage la Prairie. The Cu distribution was more complicated than Ca distribution. The major Cu binding fraction was the 0.25 M NaCl fraction (around 50 to 75%) in most cases, but sometimes it was the 0.5 M NaCl fraction, such as for AC Linora in Morden (73.5%), CDC Normandy in Morden (68.1%) and Rosebank (55.4%), and NorLin in Morden (65.4%) and Portage la Prairie (69.8%). The Zn distribution was quite different from Ca and Cu, being similar in the 0.1 and 0.25 M NaCl fractions, which contained 35 to 60% of the eluted Zn. Less than 10% of Zn eluted into the unbound and 0.5 M NaCl fractions.

The ratio of metal/protein (µg/mg) can indicate which is the major metal binding fraction on a protein basis. Table 3-8 shows the metal distribution based on unit protein. The major Ca binding fraction was the 0.1 M NaCl fraction for all cultivars grown in three locations, while the major Cu and Zn binding fractions were 0.25 M NaCl, except for NorLin grown in Morden and Portage la Prairie, where the major Cu binding fraction was the 0.5 M NaCl fraction. The ANOVA analysis failed to reveal significant difference of metal/protein in each fraction regarding cultivar and location effect.

As a summary, Tables 3-9 and 3-10 show the average values for the percentage and metal (on protein basis) distributions in each ion exchange chromatographic fraction of the five flaxseed cultivars. The results in Table 3-9 and 3-10 were calculated based on those in Table 3-7 and 3-8, respectively, by taking the average values of the three locations. According to the percentage distribution (Table 3-9), the unbound fraction for all the five cultivars contained highest amount of Ca ranging from 36to 51%. The majority of the Cu was bound to the higher salt fractions while the unbound fraction only contained 1.5 to 5.6% eluted Cu. Over 90% of the Zn was eluted into the 0.1 and 0.25 M NaCl fractions. The analysis from the ratios of µg metal/mg protein (Table 3-10) revealed that the major Ca-binding fraction was eluted at 0.1 M NaCl rather than the unbound fraction; this was because the unbound fraction contained higher amount of protein. The major Cu binding fraction was eluted at 0.25 M NaCl, and the Zn binding componentss were almost equally distributed in the 0.1 and 0.25 M NaCl fractions.

3.3.4. SDS-PAGE Profiles of the Whole Protein Extracts

The whole protein extracts of the 5 flaxseed cultivars grown in three different locations were analyzed by SDS-PAGE on 10-15% gradient PhastGel®, as shown in Figures 3-2a to 3-2e. In each figure, lane 5 contains the molecular weight standard (Sigma Marker Wide Range, MW range 6.5 - 205 kDa), lanes 1 to 4 are the samples not treated with 2-mercaptoethanol (ME), and lanes 6 to 8 are the samples treated with ME. CDC Normandy grown at Morden was loaded in lane 4 on each gel, except in Figure3-2c, which contained AC McDuff grown at Morden in lane 4. The change in SDS-PAGE profiles of the protein extracts under non-reducing and reducing conditions indicated the presence of disulfide linkages in flaxseed proteins. Table 3-11 summarizes the calculated molecular weights of the bands on SDS-PAGE under non-reducing (without ME) and reducing conditions (with ME). The molecular weight of each band was calculated based on the position of the centre point of the bandwidth.

The SDS-PAGE profiles presented in Figures 3-2a to 3-2e showed differences in band intensity for the same cultivar as a function of different locations, even though the position and numbers of bands showed no obvious differences. The bands of AC Linora grown at Morden under non-reducing and reducing condition (lanes 3 and 8) were darker than the bands for AC Linora at other locations (Figure 3-2a). For AC McDuff (Figure 3-2b), the bands representing this cultivar grown at Morden (lane 3) were darker than those grown at the other two locations (lane 1 & 2) under non-reducing condition, but lighter (lane 8) than other two locations (lane 6 & 7) under reducing condition. Figure 3-2c shows that under reducing conditions the bands representing CDC Normandy grown at Morden (lane 8) were much darker than this cultivar grown at other locations, but there was no difference among

CDC Normandy at three locations under non-reducing condition. For Flanders (Figure 3-2d), under non-reducing conditions the bands for this cultivar grown at Morden (lane 3) were darker than those from the cultivar grown at the other locations (lane 1 & 2), while Flanders grown at Rosebank under reducing condition (lane 7) showed lighter bands than those for the other locations (lane 6 & 8). The biggest differences were observed for NorLin flaxseed. The samples of NorLin showed increasing intensity of the bands in each lane from Portage la Prairie, to Rosebank and Morden under both non-reducing and reducing conditions (Figure 3-2e).

There were clear differences among the 5 cultivars in both the number and position of bands in their SDS-PAGE profiles. Four darker bands between 5-14 and 40-55 kDa under non-reducing conditions and two to three darker bands between 5-14, 21-24, and 33-35 kDa under reducing conditions were observed. Figure 3-3 is a summary of the molecular weights and band positions of the five cultivars averaged on three locations of Morden, Portage la Prairie, and Rosebank. Under non-reducing conditions, cultivars AC Linora, AC McDuff, and Flanders (x-axis numbers 1, 2 and 4) are very similar in the molecular weight and band position distribution; under reducing conditions, some difference were noted, with 6, 7 and 8 bands being observed for AC Linora, AC McDuff and Flanders (x-axis numbers 6, 7 and 9), respectively. Both CDC Normandy and NorLin contained more bands at the higher molecular weight positions under non-reducing conditions than AC Linora, AC McDuff, and Flanders, with a total of 13 bands for NorLin, and 10 bands for CDC Normandy.. Under reducing conditions, the SDS-PAGE profiles of CDC Normandy and NorLin are quite similar (x-axis 8 and 10), with more bands between 20 and 30 kDa than the other 3 cultivars.

3.4   CONCLUSIONS

The techniques of protein extraction and fractionation by ion-exchange chromatography, which were established through experiments on flaxseed cultivar NorMan and Flanders, were applied to 15 flaxseed samples including 5 different cultivars grown at three separate locations. These techniques can effectively separate major Cd-binding fraction from major storage protein fraction and produce a flaxseed protein preparation with low level of Cd. The results from these 15 samples suggest that the protocols established should be applicable as a general guideline for production of low Cd level flaxseed proteins as food ingredients.

The major Cd-binding fraction was eluted by 0.1 M NaCl, which contained 51.7% of the total eluted Cd in 12.3% of the total eluted proteins; the major storage protein fraction was the 0.25 M NaCl fraction with 39.8% of eluted Cd in 65.5% of total eluted proteins. Studies of Cd distribution on protein basis in each chromatographic fraction confirmed that, without exception, the 0.1 M NaCl fraction contained the major Cd-binding components for all the 15 samples, regardless of cultivar or growing location.

Metal analysis also indicated that the major Ca binding fraction was eluted at 0.1 M NaCl (30 to 60% based on total eluted as 100%), and the major Cu binding fraction was eluted at 0.25 M NaCl (20 to 70%), while 0.1 and 0.25M NaCl fractions contained almost equal amount of Zn (33 to 59%).

The Cd accumulation in the whole seeds was very much dependent on location, which may reflect the soil Cd content in different locations. The location effect on Cd accumulation was highly significant at the 99% confidence interval. The Cd level of the cultivars grown in Morden was three to four times higher than those grown in Portage la

Prairie and Rosebank. There was also a slight cultivar effect as well as location effect when ANOVA was performed after excluding the data from Morden, which had much higher Cd levels and may thus shadow possible effect of cultivar on Cd. There was no effect of location or cultivar on protein contents of the flaxseed samples. This geographical dependence of Cd accumulation by flaxseed regardless of cultivars implies that flaxseed is sensitive to soil Cd and can easily accumulate Cd in the seeds.

Similar significant effects of location on the Cd level on protein basis in protein extracts, 0.1 and 0.25 M NaCl fractions were observed, with higher levels from cultivars grown in Morden than from cultivars grown in Portage la Prairie and Rosebank. The statistical analysis on the Cd/protein ratios confirmed that there were significant differences in the Cd levels expressed on a protein basis in the 0.1 and 0.25 M NaCl fractions as a function of location, but not cultivar. However, the % Cd and protein distributions in each NaCl eluted fraction (0.1, 0.25 and 0.50 M) were not significantly different among locations or cultivars. However, there was a significant difference at the 99% confidence interval in % Cd distribution among different cultivars in the unbound fractions only, with no difference among locations.

Gel electrophoretic profiles showed difference in relative intensity rather than in the position or number of bands among the samples of the same cultivar grown at different locations. This implies that the growing location may affect the quantity of the Cd binding components in flaxseeds, but may not change the possible mechanisms of Cd accumulation in the seeds. In contrast, differences in the SDS-PAGE profiles among different cultivars were observed in the band position and/or number, which suggest the possibility of different Cd binding components among different cultivars. The changes in SDS-PAGE profiles of the

protein extracts under non-reducing and reducing conditions indicated the presence of disulfide linkages in the flaxseed proteins.

3.5 ACKNOWLEDGEMENTS

3.6 SPECIAL NOTES

After the second draft of this chapter was written, it was brought to the author's attention that the data collected and used for the analyses described in this chapter had also been the basis for a paper entitled "Cd-binding protein components of flaxseed: Influence of cultivar and location (Oomah et al., in press; available online 28 November 2005). The raw data for that paper and the present chapter were collected by Mr. Brian Berekoff, a research assistant employed by the author's thesis supervisor Dr. Li-Chan to conduct this research as part of a collaborative research project on flaxseed with Dr Li-Chan as principal investigator, and two co-investigators Dr. G Mazza and Dr. B. D. Oomah at the Pacific Agri-Food Research Centre in Summerland, BC. Mr. Berekoff received training from the author of this thesis on the experimental protocols used in fractionating the flax components, and then conducted the research on the 15 samples (5 cultivars, 3 locations) in the AAFC Research station at Summerland under the supervision of Drs. Oomah and Mazza.

Even though the same set of raw data was used, the analysis of data and writing of this chapter and the paper mentioned above were conducted completely independently. The author of this thesis, as well as Dr. Li-Chan and Dr. G. Mazza were not aware of the mentioned paper (although they are listed as co-authors), until it was "in press" as the online version of the paper, at which time the author's supervisor immediately contacted Dr. Oomah. The latter provided verbal consent and approval to retain this chapter in this thesis.

Based on the policies stated in the "INTELLECTUAL PROPERTY GUIDE: Publication and the Thesis" [1] of the University of British Columbia, the author of this thesis has the right to retain this chapter as his own original work, because the "data themselves are not intellectual property"; only the ways of compiling and interpreting data constitute the intellectual property. Although the same set of data was used for this chapter and for the paper by Oomah et al. (in press), the compiling, analysis and interpretation of the data were carried out independently and differently. The published paper conducted data analyses based on the values of five flaxseed samples of different cultivars (averaged on three locations) or three flaxseed samples of different locations (averaged on five cultivars), while this thesis chapter conducted the analysis mainly based on the values of all 15 samples which were the 5 cultivars grown in three locations. While the overall conclusions between the paper and the thesis chapter are similar regarding Cd and protein distribution in each fraction and the effect of flaxseed growing location, there are some minor differences in conclusions regarding cultivar and location effects on metal distribution.

[1] **Excerpt from Intellectual Property Guide: Publication and the Thesis**

(http://www.grad.ubc.ca/students/ipguide/index.asp?menu=007,001,000,000)

"Thesis Data, Going Public

Joint ownership of data does not limit your ability to incorporate the data into your thesis with permission of the co-owners, and you will own copyright to your thesis as a whole as a result. This does not give you the right to use the data for other purposes without permission if it arose from a joint project. As a

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In legal terms, it is important to remember that data themselves are not intellectual property. They are neither an invention (i.e. patentable) nor an expression of an idea (i.e. a work protected by copyright). Nevertheless, data can be important and a potentially valuable outcome of academic research. If you compile or interpret data in a unique way, you may have an intellectual property interest in the result. The University also has an interest in the resulting intellectual property, if the University has provided resources or facilities that allowed you to compile or interpret the data."

3.7   TABLES AND FIGURES

Table 3-1 Cd and protein levels in the whole seeds of five flaxseed cultivars in three different locations*

Cultivar	Location	Cd/seeds (µg/g or ppm)	Cd/protein (ng/mg or ppm)	Protein (%)
AC Linora	Morden	0.964^a	4.55	21.2
AC McDuff	Morden	1.204^a	6.05	19.9
CDC Normandy	Morden	1.006^a	4.79	21.0
Flanders	Morden	1.543^a	7.18	21.5
NorLin	Morden	1.262^a	6.19	20.4
AC Linora	Portage la Prairie	0.311^{bc}	1.52	20.5
AC McDuff	Portage la Prairie	0.246^{bc}	1.18	20.9
CDC Normandy	Portage la Prairie	0.254^{bc}	1.18	21.5
Flanders	Portage la Prairie	0.380^{bd}	1.78	21.4
NorLin	Portage la Prairie	0.279^{bc}	1.51	18.5
AC Linora	Rosebank	0.350^{be}	1.93	18.1
AC McDuff	Rosebank	0.370^{be}	1.88	19.7
CDC Normandy	Rosebank	0.310^{be}	1.68	18.5
Flanders	Rosebank	0.413^{bf}	2.21	18.7
NorLin	Rosebank	0.335^{be}	1.75	19.1

* The Cd data were measured by ICP-MS by Elemental Research Inc.; the protein data were measured using a BCA protein assay kit.

Data of Cd/seeds were analyzed by two-way ANOVA (P<0.001). The superscripts containing the same letters are not significantly different and those with the different letters are significantly different based on Tukey's pairwise comparisons (P < 0.05). No significant differences were observed for protein levels

[a, b] Indicate if Cd level is statistically different among the 3 different locations.

[c, d, e, f] Indicate if Cd level is statistically different among the two locations (excluding data from flaxseed grown in Morden).

Table 3-2. Cd and protein distributions in each fraction of ion exchange chromatography of flaxseed cultivars AC Linora, AC McDuff, CDC Normandy, Flanders, and NorLin grown in Morden, Portage la Prairie, and Rosebank

Cultivar	Fraction	Cadmium (ng)			Protein (mg)		
		Morden	Portage la Prairie	Rosebank	Morden	Portage la Prairie	Rosebank
AC Linora	Unbound	169.0	69.3	74.3	64.6	83.9	67.5
	0.10 M	**2028.6[a]**	**728.0**	**638.6**	49.9	57.1	57.7
	0.25M	1543.8	463.1	375.9	**339.1**	**305.8**	**257.6**
	0.50M	66.8	106.7	41.8	33.7	32.4	16.7
	Column total	*3808.1*	*1367.1*	*1130.5*	*487.2*	*479.2*	*399.5*
AC McDuff	Unbound	55.0	36.0	59.4	94.2	94.3	100.5
	0.10 M	1773.0	**925.2**	**744.2**	66.8	75.9	77.7
	0.25M	**2067.2**	807.7	549.8	**381.9**	**404.5**	**398.8**
	0.50M	87.0	26.7	261.5	76.3	58.3	41.5
	Column total	*3982.2*	*1795.6*	*1614.9*	*619.2*	*633.1*	*618.5*
CDC Normandy	Unbound	34.3	34.7	79.2	67.5	71.9	98.4
	0.10 M	**1255.8**	**730.3**	**892.8**	28.0	66.0	80.5
	0.25M	950.4	602.7	426.3	**335.8**	**364.2**	**363.8**
	0.50M	21.6	196.0	31.5	18.9	34.8	25.3
	Column total	*2262.1*	*1563.6*	*1429.8*	*450.2*	*536.8*	*568.0*
Flanders	Unbound	74.3	19.8	10.8	92.2	85.2	94.7
	0.10 M	**2311.9**	**888.0**	581.4	85.6	74.7	68.5
	0.25M	1608.2	770.0	**936.2**	**373.9**	**332.0**	**334.3**
	0.50M	368.5	165.6	32.3	37.2	26.1	41.4
	Column total	*4362.8*	*1843.4*	*1560.7*	*588.8*	*518.1*	*538.9*
NorLin	Unbound	230.1	89.1	64.4	81.8	79.9	82.9
	0.10 M	**2453.5**	**1016.4**	**636.7**	62.0	69.6	70.7
	0.25M	1452.0	545.6	430.4	**372.6**	**308.0**	**351.4**
	0.50M	118.5	67.6	135.2	24.8	31.3	25.8
	Column total	*4254.1*	*1718.7*	*1266.6*	*541.2*	*488.8*	*530.8*

[a] Starting material was 10 g dehulled seed for each cultivar. The numbers in bold represent the highest Cd or protein amount was eluted from the column in that fraction.

Table 3-3. Percentage of Cd and protein distributions in each fraction of ion exchange chromatography of flaxseed cultivars AC Linora, AC McDuff, CDC Normandy, Flanders, and NorLin grown in Morden, Portage la Prairie, and Rosebank

Cultivar	Fraction	Cadmium (%)			Protein (%)		
		Morden	Portage la Prairie	Rosebank	Morden	Portage la Prairie	Rosebank
AC Linora	Unbound[a]	4.4	5.1	6.6	13.3	17.5	16.9
	0.10 M	53.3	53.3	56.5	10.2	11.9	14.4
	0.25M	40.5	33.9	33.2	69.6	63.8	64.5
	0.50M	1.8	7.8	3.7	6.9	6.8	4.2
	Column total	*100*	*100*	*100*	*100*	*100*	*100*
AC McDuff	Unbound[b]	1.4	2.0	3.7	15.2	14.9	16.3
	0.10 M	44.5	51.5	46.1	10.8	12.0	12.6
	0.25M	51.9	45.0	34.0	61.7	63.9	64.5
	0.50M	2.2	1.5	16.2	12.3	9.2	6.7
	Column total	*100*	*100*	*100*	*100*	*100*	*100*
CDC Normandy	Unbound[b]	1.5	2.2	5.5	15.0	13.4	17.3
	0.10 M	55.5	46.7	62.4	6.2	12.3	14.2
	0.25M	42.0	38.5	29.8	74.6	67.8	64.1
	0.50M	1.0	12.5	2.2	4.2	6.5	4.5
	Column total	*100*	*100*	*100*	*100*	*100*	*100*
Flanders	Unbound[b]	1.7	1.1	0.7	15.7	16.5	17.6
	0.10 M	53.0	48.2	37.3	14.5	14.4	12.7
	0.25M	36.9	41.8	60.0	63.5	64.1	62.0
	0.50M	8.4	9.0	2.1	6.3	5.0	7.7
	Column total	*100*	*100*	*100*	*100*	*100*	*100*
NorLin	Unbound[a]	5.4	5.2	5.1	15.1	16.3	15.6
	0.10 M	57.7	59.1	50.3	11.5	14.2	13.3
	0.25M	34.1	31.7	34.0	68.9	63.0	66.2
	0.50M	2.8	3.9	10.7	4.6	6.4	4.9
	Column total	*100*	*100*	*100*	*100*	*100*	*100*

Data for each fraction were analyzed by two-way ANOVA ($P<0.005$). The superscripts containing the same letters are not significantly different and those with different letters are significantly different based on Tukey's pairwise comparisons ($P < 0.05$). No significant differences were noted for % protein.

[a,b] Indicate if % Cd is statistically different among different cultivars in the unbound fractions.

Table 3-4. Ratios of Cd/protein in the five flaxseed cultivars grown in three different locations

Cultivar	Fractions	Cd/protein (ng/mg or ppm)		
		Morden	Portage la Prairie	Rosebank
AC Linora	Whole seeds	4.55^a	1.52^b	1.93^b
	Protein extract	7.82^c	2.85^d	2.83^d
	Unbound	2.62^e	0.83^e	1.10^e
	0.10 M NaCl	40.69^f	12.74^g	11.08^g
	0.25M NaCl	4.55^h	1.51^i	1.46^i
	0.50M NaCl	1.99^j	3.29^j	2.50^j
AC McDuff	Whole seeds	6.05^a	1.18^b	1.88^b
	Protein extract	6.43^c	2.84^d	2.61^d
	Unbound	0.58^e	0.38^e	0.59^e
	0.10 M NaCl	26.55^f	12.19^g	9.58^g
	0.25M NaCl	5.41^h	2.00^i	1.38^i
	0.50M NaCl	1.14^j	0.46^j	6.30^j
CDC Normandy	Whole seeds	4.79^a	1.18^b	1.68^b
	Protein extract	5.02^c	2.91^d	2.52^d
	Unbound	0.51^e	0.48^e	0.81^e
	0.10 M NaCl	44.83^f	11.07^g	11.09^g
	0.25M NaCl	2.83^h	1.65^i	1.17^i
	0.50M NaCl	1.15^j	5.64^j	1.24^j
Flanders	Whole seeds	7.18^a	1.78^b	2.21^b
	Protein extract	7.41^c	3.56^d	2.90^d
	Unbound	0.81^e	0.23^e	0.11^e
	0.10 M NaCl	27.01^f	11.89^g	8.49^g
	0.25M NaCl	4.30^h	2.32^i	2.80^i
	0.50M NaCl	9.91^j	6.34^j	0.78^j
NorLin	Whole seeds	6.19^a	1.51^b	1.75^b
	Protein extract	7.86^c	3.52^d	2.39^d
	Unbound	2.81^e	1.12^e	0.78^e
	0.10 M NaCl	39.58^f	14.60^g	9.01^g
	0.25M NaCl	3.90^h	1.77^i	1.22^i
	0.50M NaCl	4.78^j	2.16^j	5.24^j

Data were analyzed by two-way ANOVA ($P<0.001$). Numbers superscripted with the same letter are not significantly different and that with different letters are significantly different based on Tukey's pairwise comparisons ($P < 0.05$).

[a, b, c, d, e, f, g, h, i, j] Indicate if Cd/protein is statistically different within each fraction among different locations of the same cultivar or among different cultivars at the same location.

Table 3-5. Average ratios of Cd/proteins (ng/mg or ppm) in the whole seed, protein extract and each fraction from ion exchange chromatography as a function of location, averaged over five cultivars

	Morden	Portage la Prairie	Rosebank
Whole seeds	5.75 (±1.08)	1.43 (±0.26)	1.89 (±0.20)
Protein extract	6.91 (±1.20)	3.14 (±0.37)	2.65 (±0.21)
Unbound	1.47 (±1.15)	0.61 (±0.36)	0.68 (±0.37)
0.1M NaCl	35.73 (±8.40)	12.50 (±1.32)	9.85 (±1.19)
0.25M NaCl	4.20 (±0.94)	1.85 (±0.32)	1.61 (±0.68)
0.50M NaCl	3.79 (±3.73)	3.58 (±2.43)	3.21 (±2.45)

Note: numbers in brackets are the standard deviations based on n=5 cultivars.

Table 3-6. Ca, Cu, and Zn distributions in ion exchange chromatographic fractions of the 5 flaxseed cultivars grown at three different locations

Cultivars	Fractions	Ca (µg)			Cu (µg)			Zn (µg)		
		Morden	Portage la Prairie	Rosebank	Morden	Portage la Prairie	Rosebank	Morden	Portage la Prairie	Rosebank
AC Linora	Unbound	581.49	386.10	509.85	4.62	8.17	2.48	2.49	4.95	2.97
	0.10 M NaCl	134.40	149.50	105.40	16.67	22.23	19.59	70.98	71.50	44.64
	0.25M NaCl	156.25	264.60	169.51	107.50	35.43	33.98	41.88	53.66	33.17
	0.50M NaCl	359.05	160.05	120.06	57.38	4.46	2.56	4.18	3.88	0.52
	Column total	*1231.19*	*960.25*	*904.82*	*486.18*	*70.29*	*58.60*	*119.52*	*133.99*	*81.30*
AC McDuff	Unbound	500.00	480.00	420.75	2.95	1.68	2.18	2.00	3.00	3.47
	0.10 M NaCl	117.00	170.40	128.10	7.83	8.20	11.47	37.50	35.7	44.53
	0.25M NaCl	108.80	206.09	153.93	46.40	39.27	44.93	51.84	35.09	43.25
	0.50M NaCl	149.40	213.76	115.06	5.18	4.07	5.75	2.49	4.68	4.71
	Column total	*875.20*	*1070.25*	*817.84*	*62.36*	*53.23*	*64.33*	*93.83*	*78.47*	*95.95*
CDD Normandy	Unbound	254.80	465.30	519.75	2.50	1.09	7.77	2.45	3.47	2.48
	0.10 M NaCl	71.40	442.75	142.60	12.52	8.51	33.60	39.06	46.58	39.06
	0.25M NaCl	151.80	271.95	139.65	99.00	33.30	118.34	33.66	41.16	22.05
	0.50M NaCl	187.20	207.20	162.75	243.36	2.41	198.45	3.60	2.24	2.63
	Column total	*665.20*	*1387.20*	*964.75*	*357.38*	*45.30*	*358.16*	*78.77*	*93.44*	*66.21*
Flanders	Unbound	420.75	292.05	334.80	5.20	1.49	10.91	6.93	3.47	2.70
	0.10 M NaCl	366.00	102.00	64.60	15.07	13.50	21.62	73.20	40.20	30.94
	0.25M NaCl	407.70	331.10	136.40	32.24	37.11	106.02	55.12	53.90	34.10
	0.50M NaCl	129.48	230.00	215.00	3.74	2.76	517.08	2.49	12.88	5.38
	Column total	*1323.93*	*955.15*	*750.80*	*56.24*	*54.86*	*655.63*	*137.74*	*110.45*	*73.12*
NorLin	Unbound	433.35	277.20	366.30	7.54	4.21	1.19	2.68	2.48	1.49
	0.10 M NaCl	87.50	75.60	124.95	26.01	17.85	12.97	73.85	52.50	42.25
	0.25M NaCl	138.60	93.00	181.20	158.40	107.26	41.22	42.90	39.06	40.77
	0.50M NaCl	284.40	304.20	239.20	363.40	298.29	3.22	3.95	4.22	5.72
	Column total	*943.85*	*750.00*	*911.65*	*555.35*	*427.61*	*58.61*	*123.38*	*98.26*	*90.22*

Note: the data shown in each column was the total amount of metal in each fraction. The column total was the sum of the amounts in all the fractions of unbound, 0.1, 0.25, and 0.50M NaCl.

116

Table 3-7. Ca, Cu, and Zn percentage distributions in ion exchange chromatographic fractions of the 5 flaxseed cultivars grown at three different locations

Cultivars	Fractions	Ca (%)			Cu (%)			Zn (%)		
		Morden	Portage la Prairie	Rosebank	Morden	Portage la Prairie	Rosebank	Morden	Portage la Prairie	Rosebank
AC Linora	Unbound	47.2	40.2	56.3	1.0	11.6	4.2	2.1	3.7	3.7
	0.10 M NaCl	10.9	15.6	11.6	3.4	31.6	33.4	59.4	53.4	54.9
	0.25M NaCl	12.7	27.6	18.7	22.1	50.4	58.0	35.0	40.0	40.8
	0.50M NaCl	29.2	16.7	13.3	73.5	6.3	4.4	3.5	2.9	0.6
	Column total	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
AC McDuff	Unbound	57.1	44.8	51.4	4.7	3.2	3.4	2.1	3.8	3.6
	0.10 M NaCl	13.4	15.9	15.7	12.6	15.4	17.8	40.0	45.5	46.4
	0.25M NaCl	12.4	19.3	18.8	74.4	73.8	69.8	55.2	44.7	45.1
	0.50M NaCl	17.1	20.0	14.1	8.3	7.7	8.9	2.7	6.0	4.9
	Column total	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
CDD Normandy	Unbound	38.3	·33.5	53.9	0.7	2.4	2.2	3.1	3.7	3.7
	0.10 M NaCl	10.7	31.9	14.8	3.5	18.8	9.4	49.6	49.8	59.0
	0.25M NaCl	22.8	19.6	14.5	27.7	73.5	33.0	42.7	44.0	33.3
	0.50M NaCl	28.1	14.9	16.9	68.1	5.3	55.4	4.6	2.4	4.0
	Column total	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
Flanders	Unbound	31.8	30.6	44.6	9.2	2.7	1.7	5.0	3.1	3.7
	0.10 M NaCl	27.6	10.7	8.6	26.8	24.6	3.3	53.1	36.4	42.3
	0.25M NaCl	30.8	34.7	18.2	57.3	67.7	16.2	40.0	48.8	46.6
	0.50M NaCl	9.8	24.1	28.6	6.6	5.0	78.9	1.8	11.7	7.4
	Column total	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
NorLin	Unbound	45.9	37.0	40.2	1.4	1.0	2.0	2.2	2.5	1.6
	0.10 M NaCl	9.3	10.1	13.7	4.7	4.2	22.1	59.9	53.4	46.8
	0.25M NaCl	14.7	12.4	19.9	28.5	25.1	70.3	34.8	39.8	45.2
	0.50M NaCl	30.1	40.6	26.2	65.4	69.8	5.5	3.2	4.3	6.3
	Column total	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*

Table 3-8. Ratios of Ca/protein, Cu/protein, and Zn/protein in each fraction of ion exchange chromatography of the 5 flaxseed cultivars grown at three different locations

Cultivars	Fractions	Ca/protein (μg/mg or ppm)			Cu/protein (μg/mg or ppm)			Zn/protein (μg/mg or ppm)		
		Morden	Portage la Prairie	Rosebank	Morden	Portage la Prairie	Rosebank	Morden	Portage la Prairie	Rosebank
AC Linora	Unbound	1.82	2.21	1.43	0.09	0.18	0.08	0.24	0.33	0.16
	0.10 M NaCl	9.01	4.60	7.55	0.07	0.10	0.04	0.04	0.06	0.04
	0.25M NaCl	2.70	2.62	1.83	0.33	0.39	0.34	1.42	1.25	0.77
	0.50M NaCl	0.46	0.87	0.66	0.32	0.12	0.13	0.12	0.18	0.13
	Column total	*10.67*	*4.94*	*7.19*	*10.62*	*0.14*	*0.15*	*0.12*	*0.12*	*0.03*
AC McDuff	Unbound	1.30	0.94	1.04	0.11	0.08	0.08	0.22	0.14	0.18
	0.10 M NaCl	5.31	5.09	4.19	0.03	0.02	0.02	0.02	0.03	0.03
	0.25M NaCl	1.75	2.24	1.65	0.12	0.11	0.15	0.56	0.47	0.57
	0.50M NaCl	0.28	0.51	0.39	0.12	0.10	0.11	0.14	0.09	0.11
	Column total	*1.96*	*3.67*	*2.77*	*0.07*	*0.07*	*0.14*	*0.03*	*0.08*	*0.11*
CDD Normandy	Unbound	0.79	0.98	1.43	0.10	0.07	0.10	0.23	0.18	0.18
	0.10 M NaCl	3.78	6.47	5.28	0.04	0.02	0.08	0.04	0.05	0.03
	0.25M NaCl	2.55	6.71	1.77	0.45	0.13	0.42	1.39	0.71	0.49
	0.50M NaCl	0.45	0.75	0.38	0.29	0.09	0.33	0.10	0.11	0.06
	Column total	*9.92*	*5.96*	*6.43*	*12.90*	*0.07*	*7.84*	*0.19*	*0.06*	*0.10*
Flanders	Unbound	1.24	1.31	1.06	0.07	0.15	0.11	0.22	0.30	0.17
	0.10 M NaCl	4.56	3.43	3.53	0.06	0.02	0.12	0.08	0.04	0.03
	0.25M NaCl	4.28	1.37	0.94	0.18	0.18	0.32	0.86	0.54	0.45
	0.50M NaCl	1.09	1.00	0.41	0.09	0.11	0.32	0.15	0.16	0.10
	Column total	*3.48*	*8.80*	*5.19*	*0.10*	*0.11*	*12.49*	*0.07*	*0.49*	*0.13*
NorLin	Unbound	2.00	1.01	1.23	0.14	0.12	0.09	0.34	0.24	0.20
	0.10 M NaCl	5.30	3.47	4.42	0.09	0.05	0.01	0.03	0.03	0.02
	0.25M NaCl	1.41	1.09	1.77	0.42	0.26	0.18	1.19	0.75	0.60
	0.50M NaCl	0.37	0.30	0.52	0.43	0.35	0.12	0.12	0.13	0.12
	Column total	*11.47*	*9.70*	*9.27*	*14.66*	*9.51*	*0.13*	*0.16*	*0.13*	*0.22*

Note: the data shown in each column was metal amount relative to protein amount in each fraction, indicating metal level based on unit protein in each fraction. The column total is therefore the reflection of average metal concentration based on unit protein.

Table 3-9. Ca, Cu, and Zn percentage distributions in ion exchange chromatographic fractions of the 5 flaxseed cultivars averaged on three locations

Cultivar	Fraction	Ca %	Ca Standard deviation	Cu %	Cu Standard deviation	Zn %	Zn Standard deviation
AC Linora	Unbound	47.9	8.1	5.6	5.4	3.2	0.9
	0.10 M NaCl	12.7	2.5	22.8	16.8	55.9	3.1
	0.25M NaCl	19.7	7.5	43.5	18.9	38.6	3.1
	0.50M NaCl	19.7	8.4	28.1	39.4	2.3	1.5
	Column total	100.0	0.0	100.0	0.0	100.0	0.0
AC McDuff	Unbound	51.1	6.2	3.8	0.8	3.2	0.9
	0.10 M NaCl	15.0	1.4	15.3	2.6	44.0	3.5
	0.25M NaCl	16.8	3.8	72.7	2.5	48.3	6.0
	0.50M NaCl	17.1	3.0	8.3	0.6	4.5	1.7
	Column total	100.0	0.0	100.0	0.0	100.0	0.0
CDD Normandy	Unbound	41.9	10.7	1.8	0.9	3.5	0.3
	0.10 M NaCl	19.1	11.2	10.6	7.7	52.8	5.4
	0.25M NaCl	19.0	4.2	44.7	25.1	40.0	5.8
	0.50M NaCl	20.0	7.1	42.9	33.2	3.7	1.1
	Column total	100.0	0.0	100.0	0.0	100.0	0.0
Flanders	Unbound	35.7	7.8	4.5	4.1	3.9	1.0
	0.10 M NaCl	15.6	10.4	18.2	13.0	43.9	8.5
	0.25M NaCl	27.9	8.6	47.1	27.2	45.1	4.6
	0.50M NaCl	20.8	9.8	30.2	42.2	7.0	5.0
	Column total	100.0	0.0	100.0	0.0	100.0	0.0
NorLin	Unbound	41.0	4.5	1.5	0.5	2.1	0.5
	0.10 M NaCl	11.0	2.3	10.3	10.2	53.4	6.6
	0.25M NaCl	15.7	3.8	41.3	25.2	39.9	5.2
	0.50M NaCl	32.3	7.4	46.9	35.9	4.6	1.6
	Column total	100.0	0.0	100.0	0.0	100.0	0.0

Table 3-10. Ratios of Ca/protein, Cu/protein, and Zn/protein in ion exchange chromatographic fractions of the 5 flaxseed cultivars averaged over three locations

Cultivar	Fraction	Ca/protein		Cu/protein		Zn/protein	
		µg/mg (ppm)	Standard deviation	µg/mg (ppm)	Standard deviation	µg/mg (ppm)	Standard deviation
AC Linora	Unbound	1.82	0.39	0.12	0.06	0.24	0.09
	0.10 M NaCl	7.05	2.25	0.07	0.03	0.05	0.01
	0.25M NaCl	2.38	0.48	0.35	0.03	1.15	0.34
	0.50M NaCl	0.66	0.21	0.19	0.11	0.14	0.03
	Column total	7.60	2.89	3.64	6.05	0.09	0.05
AC McDuff	Unbound	1.09	0.19	0.09	0.02	0.18	0.04
	0.10 M NaCl	4.86	0.59	0.02	0.01	0.03	0.01
	0.25M NaCl	1.88	0.32	0.13	0.02	0.53	0.06
	0.50M NaCl	0.39	0.12	0.11	0.01	0.11	0.03
	Column total	2.80	0.86	0.09	0.04	0.07	0.04
CDD Normandy	Unbound	1.07	0.33	0.09	0.02	0.20	0.03
	0.10 M NaCl	5.18	1.35	0.05	0.03	0.04	0.01
	0.25M NaCl	3.68	2.66	0.33	0.18	0.86	0.47
	0.50M NaCl	0.53	0.20	0.24	0.13	0.09	0.03
	Column total	7.44	2.16	6.94	6.46	0.12	0.07
Flanders	Unbound	1.20	0.13	0.11	0.04	0.23	0.07
	0.10 M NaCl	3.84	0.63	0.07	0.05	0.05	0.03
	0.25M NaCl	2.20	1.82	0.23	0.08	0.62	0.22
	0.50M NaCl	0.83	0.37	0.17	0.13	0.14	0.03
	Column total	5.82	2.72	4.23	7.15	0.23	0.23
NorLin	Unbound	1.41	0.52	0.12	0.03	0.26	0.07
	0.10 M NaCl	4.40	0.92	0.05	0.04	0.03	0.01
	0.25M NaCl	1.42	0.34	0.29	0.12	0.85	0.31
	0.50M NaCl	0.40	0.11	0.30	0.16	0.12	0.01
	Column total	10.15	1.17	8.10	7.37	0.17	0.05

120

Table 3-11. Summary of the molecular weight (kDa) of the bands in the SDS-PAGE profiles of the five flaxseed cultivars at three different locations

	Band1	Band2	Band3	Band4	Band5	Band6	Band7	Band8	Band9	Band10	Band11	Band12	Band13
ACL M w/o ME	11.25	12.62	20.62	21.96	24.63	30.99	44.66	47.55	53.33				
ACL P w/o ME	11.25	12.36	21.06	21.28	23.62	30.03	44.66	47.05	53.33				
ACL R w/o ME	11.25	12.36	20.62	21.73	23.87	30.35	44.66	47.05	52.23				
Average	11.25	12.45	20.77	21.66	24.04	30.46	44.66	47.22	52.96				
Standard deviation	0.00	0.15	0.25	0.35	0.53	0.49	0.00	0.29	0.64				
ACL M w ME	12.62	21.28	22.65	25.41	32.99	51.69							
ACL P w ME	11.73	20.41	21.96	24.37	31.97	50.09							
ACL R w ME	12.36	21.06	22.42	25.15	33.34	50.09							
Average	12.24	20.92	22.34	24.98	32.77	50.62							
Standard deviation	0.46	0.45	0.35	0.54	0.71	0.92							
ACM M w/o ME	11.61	12.99	20.53	21.82	22.96	31.47	44.48	46.81	53.43				
ACM P w/o ME	11.61	12.99	20.12	21.60	22.96	31.47	44.03	46.33	52.35				
ACM R w/o ME	11.73	12.99	20.12	21.17	22.96	30.84	44.03	46.33	52.35				
Average	11.65	12.99	20.26	21.53	22.96	31.26	44.18	46.49	52.71				
Standard deviation	0.07	0.00	0.24	0.33	0.00	0.36	0.26	0.28	0.62				
ACM M w ME	12.34	17.27	21.17	22.96	24.91	33.46	52.35						
ACM P w ME	12.22	17.09	21.17	22.73	25.16	32.45	51.82						
ACM R w ME	12.34	17.27	21.17	22.73	24.91	33.12	51.82						
Average	12.30	17.21	21.17	22.81	24.99	33.01	52.00						
Standard deviation	0.07	0.10	0.00	0.13	0.14	0.51	0.31						
CDC M w/o ME	12.66	13.85	22.32	23.46	32.57	35.62	40.54	47.06	50.96	56.29			
CDC P w/o ME	12.54	13.98	22.77	23.93	33.89	36.70	40.54	47.06	50.46	56.29			
CDC R w/o ME	12.79	13.71	22.77	23.93	33.89	35.62	40.14	46.60	49.46	56.29			
Average	12.66	13.85	22.62	23.77	33.45	35.98	40.41	46.91	50.29	56.29			
Standard deviation	0.13	0.14	0.26	0.27	0.76	0.62	0.23	0.27	0.76	0.00			
CDC M w ME	13.44	17.93	23.00	24.41	26.43	28.34	30.08	34.57	35.27	53.03			
CDC P w ME	13.17	17.58	22.10	23.93	25.65	26.70	29.49	32.57	34.92	51.98			
CDC R w ME	13.17	17.76	22.77	23.93	25.91	27.23	29.78	33.23	34.92	51.98			
Average	13.26	17.76	22.62	24.09	26.00	27.42	29.78	33.46	35.04	52.33			
Standard deviation	0.16	0.18	0.47	0.28	0.40	0.84	0.30	1.02	0.20	0.61			

Table 3-11. Summary of the molecular weight (kDa) of the bands in the SDS-PAGE profiles of the five flaxseed cultivars at three different locations (Continued)

	Band1	Band2	Band3	Band4	Band5	Band6	Band7	Band8	Band9	Band10	Band11	Band12	Band13
FLD M w/o ME	**11.56**	**12.39**	21.19	22.49	23.64	32.16	43.76	**46.91**	**53.91**				
FLD P w/o ME	**11.79**	**13.02**	21.83	23.64	24.84	34.14	49.30	**51.30**	**56.66**				
FLD R w/o ME	**11.56**	**12.77**	21.83	23.17	24.60	33.13	46.91	**49.30**	**54.99**				
Average	**11.64**	**12.73**	21.62	23.10	24.36	33.14	46.66	**49.17**	**55.19**				
Standard deviation	0.13	0.32	0.37	0.58	0.63	0.99	2.78	2.20	1.39				
FLD M w ME	**12.39**	21.83	**23.40**	25.85	27.44	**35.52**	38.08	53.91					
FLD P w ME	**12.15**	21.19	**22.72**	24.84	26.63	**34.14**	36.96	51.81					
FLD R w ME	**12.03**	21.83	**23.17**	25.09	26.90	**34.48**	36.96	51.81					
Average	**12.19**	21.62	**23.10**	25.26	26.99	**34.71**	37.33	52.51					
Standard deviation	0.18	0.37	0.35	0.53	0.41	0.72	0.65	1.21					
NLN M w/o ME	**12.59**	**13.78**	21.66	23.01	32.38	35.44	38.80	43.77	45.11	47.43	**49.38**	**54.05**	58.57
NLN P w/o ME	**12.47**	**13.24**	21.23	22.78	32.06	34.05	38.41	42.90	46.02	47.43	**48.88**	**54.60**	57.99
NLN R w/o ME	**12.59**	**13.65**	21.88	23.01	31.73	35.09	38.80	42.90	46.02	47.43	**48.88**	**54.60**	58.57
Average	**12.55**	**13.56**	21.59	22.93	32.06	34.86	38.67	43.19	45.72	47.43	**49.05**	**54.42**	58.38
Standard deviation	0.07	0.28	0.33	0.13	0.33	0.72	0.23	0.50	0.53	0.00	**0.29**	**0.32**	0.33
NLN M w ME	**13.11**	18.26	22.78	**24.19**	26.48	27.85	29.58	33.37	**34.74**	53.51			
NLN P w ME	**12.59**	17.19	20.81	**22.10**	23.71	25.44	26.75	30.18	**31.73**	48.39			
NLN R w ME	**12.85**	17.54	21.66	**23.24**	24.94	26.48	28.13	31.73	**33.37**	50.89			
Average	**12.85**	17.66	21.75	**23.18**	25.04	26.59	28.15	31.76	**33.28**	50.93			
Standard deviation	0.26	0.55	0.99	1.05	1.39	1.21	1.42	1.60	1.51	2.56			

Note: ACL: AC Linora; ACM: AC McDuff; CDC: CDC Normandy; FLD: Flanders; NLN: NorLin; M: Morden; P: Portage la Prairie; R: Rosebank; w/o: without; w: with; ME: 2-mercaptoethanol. Band numbers in bold represent darker bands observed from the gel.
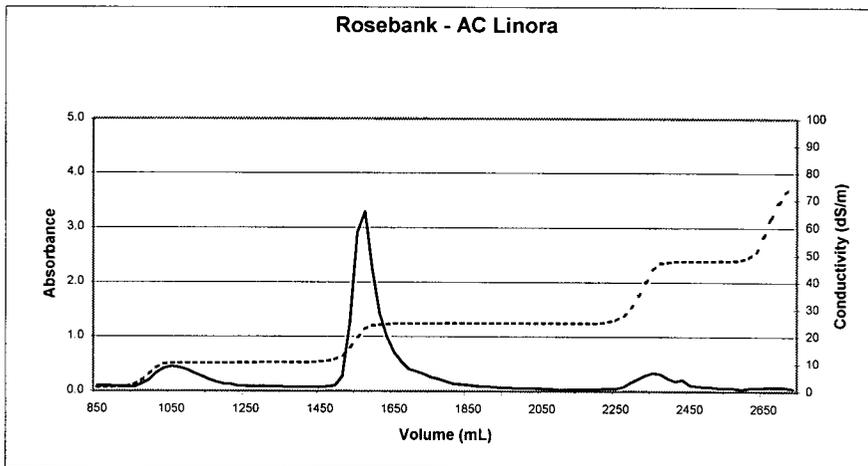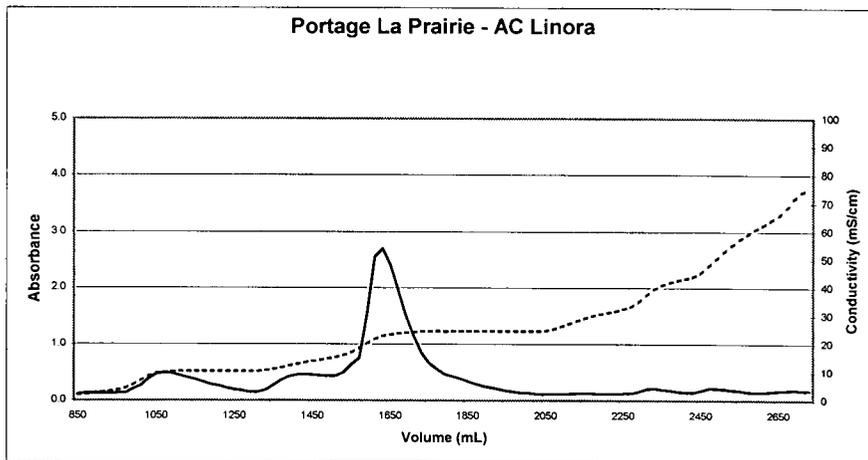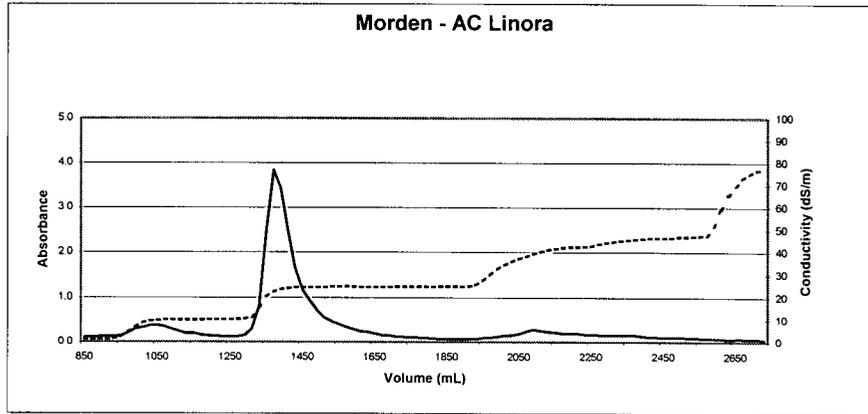
Figure 3-1a. Ion exchange chromatographic profiles of protein extracts from flaxseed AC Linora grown at three different locations.
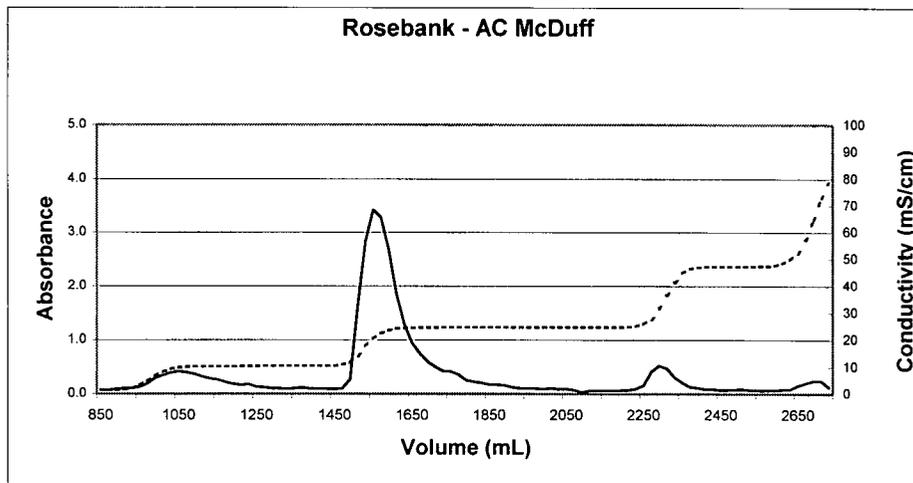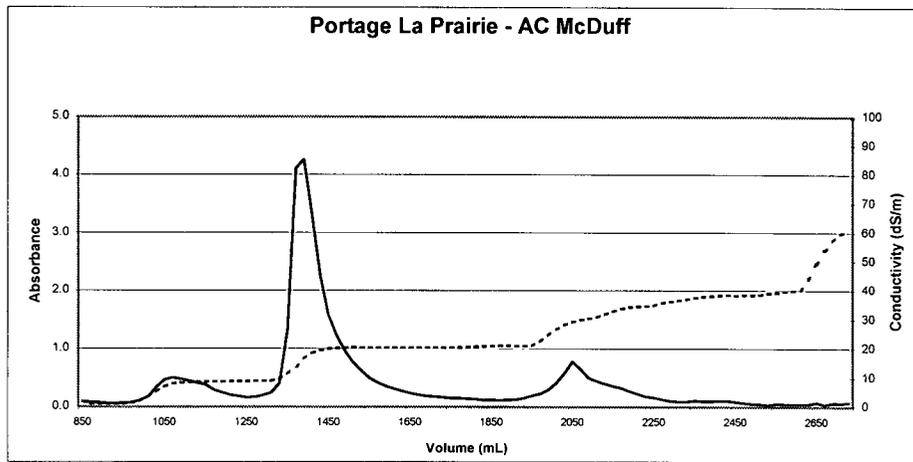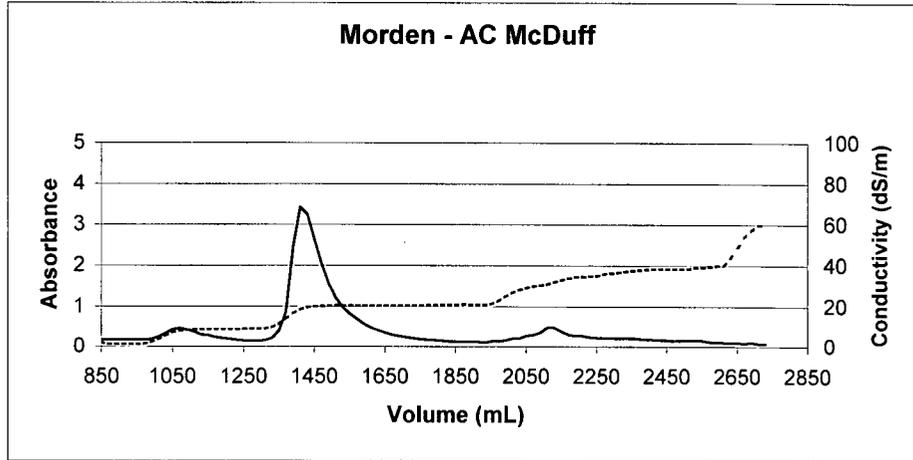
Figure 3-1b. Ion exchange chromatographic profiles of protein extracts from flaxseed AC McDuff grown at three different locations.
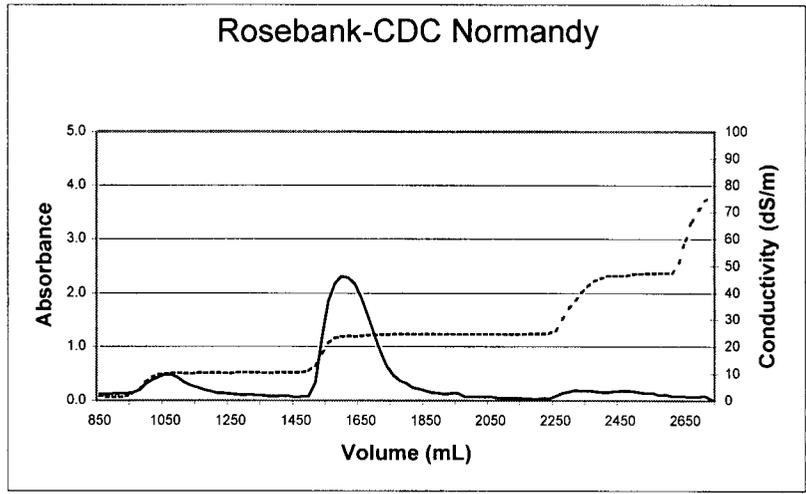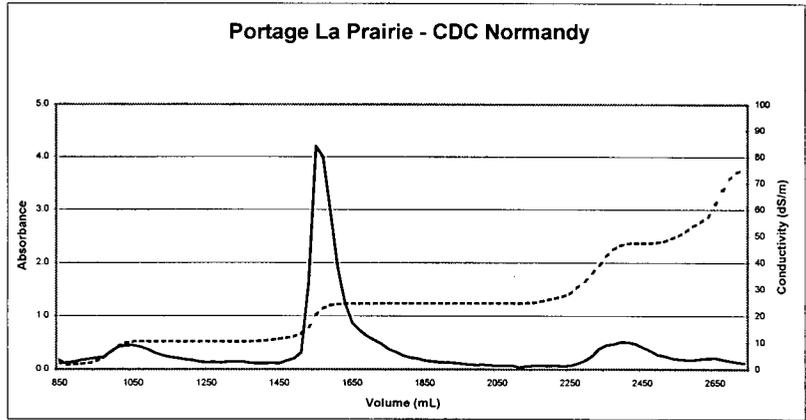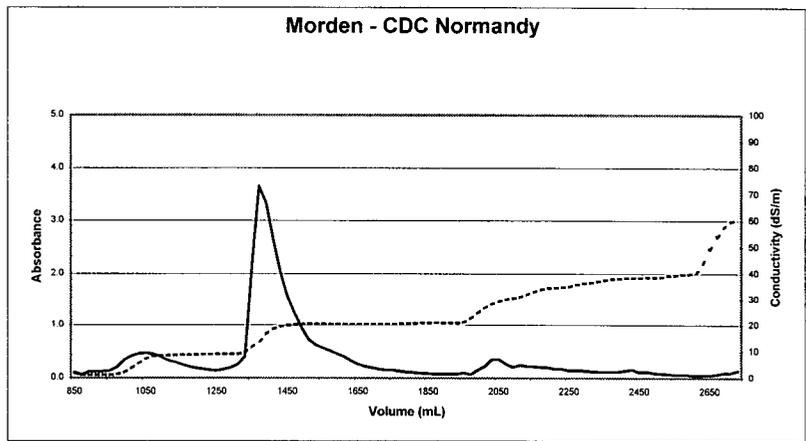
Figure 3-1c. Ion exchange chromatographic profiles of protein extracts from flaxseed CDC Normandy grown at three different locations.

125
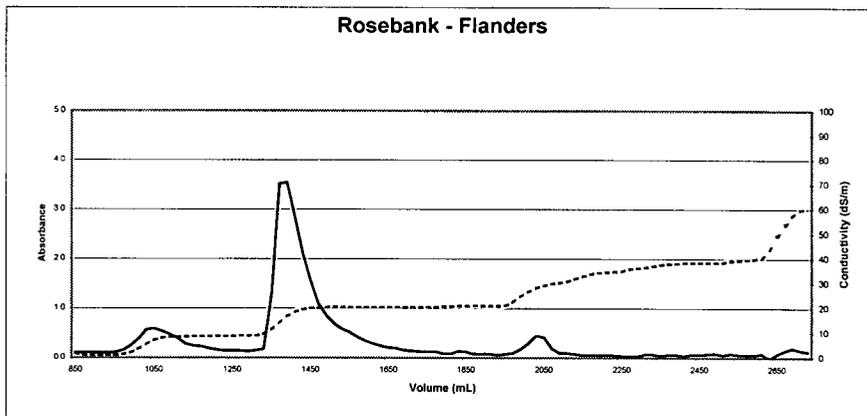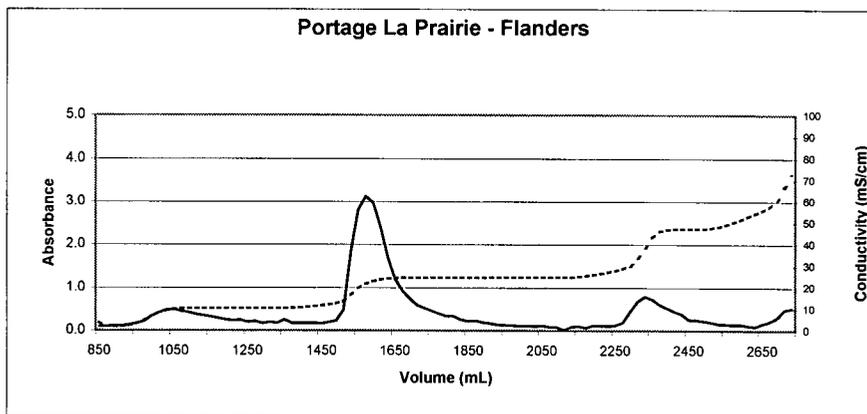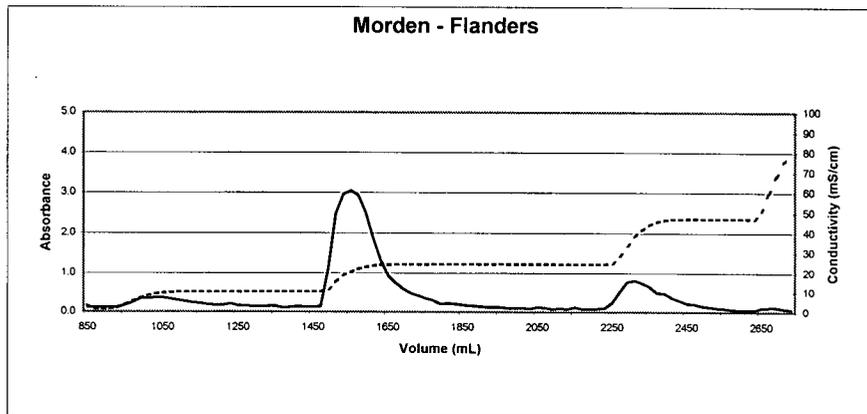
Figure 3-1d. Ion exchange chromatographic profiles of protein extracts from flaxseed Flanders grown at three different locations.
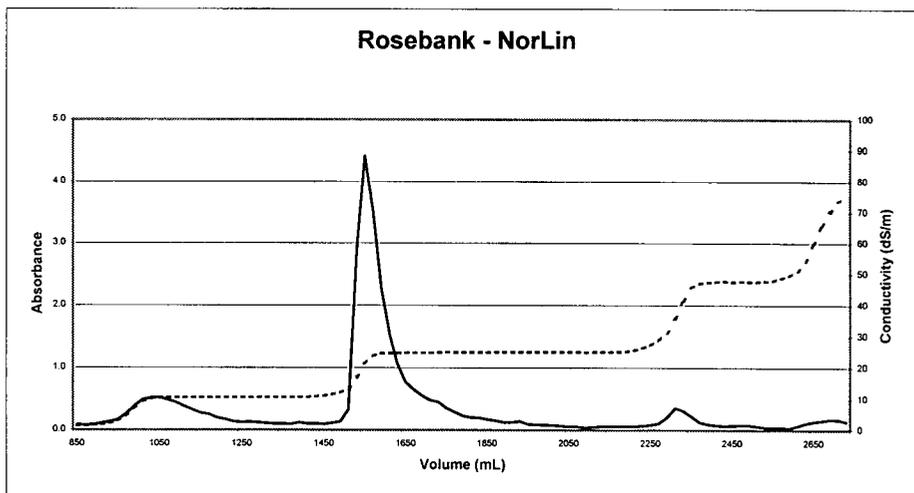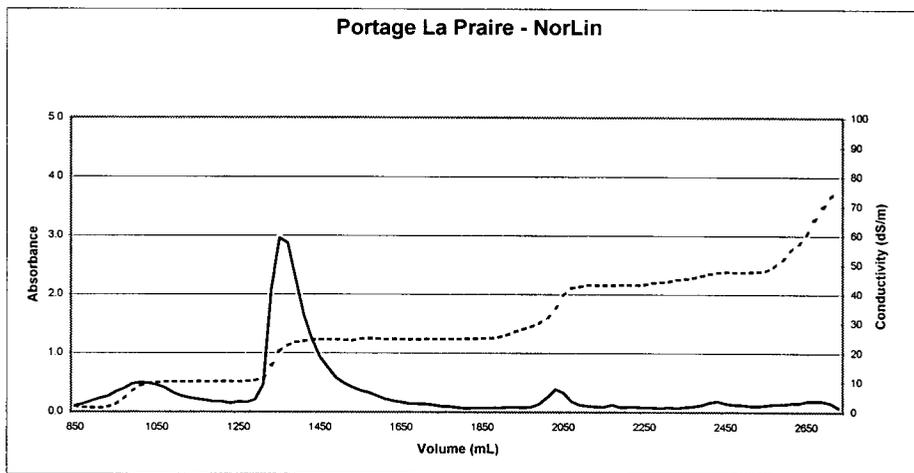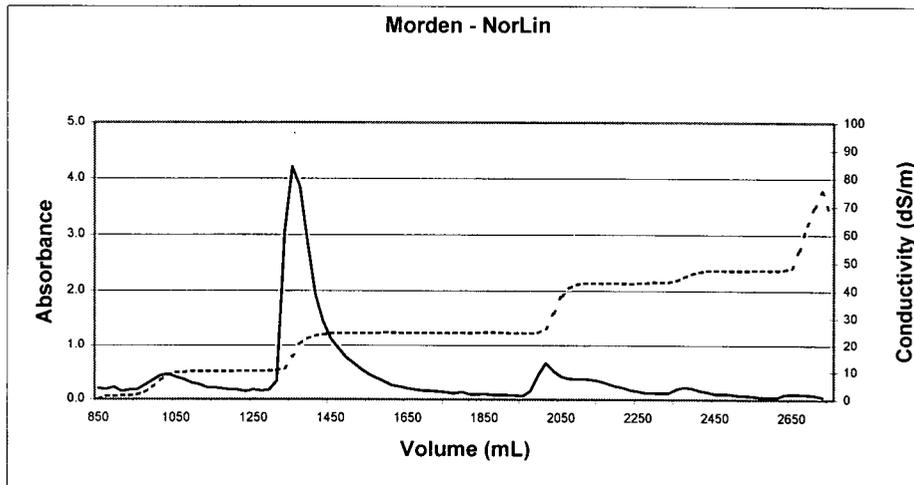
Figure 3-1e. Ion exchange chromatographic profiles of protein extracts from flaxseed Norlin grown at three different locations.
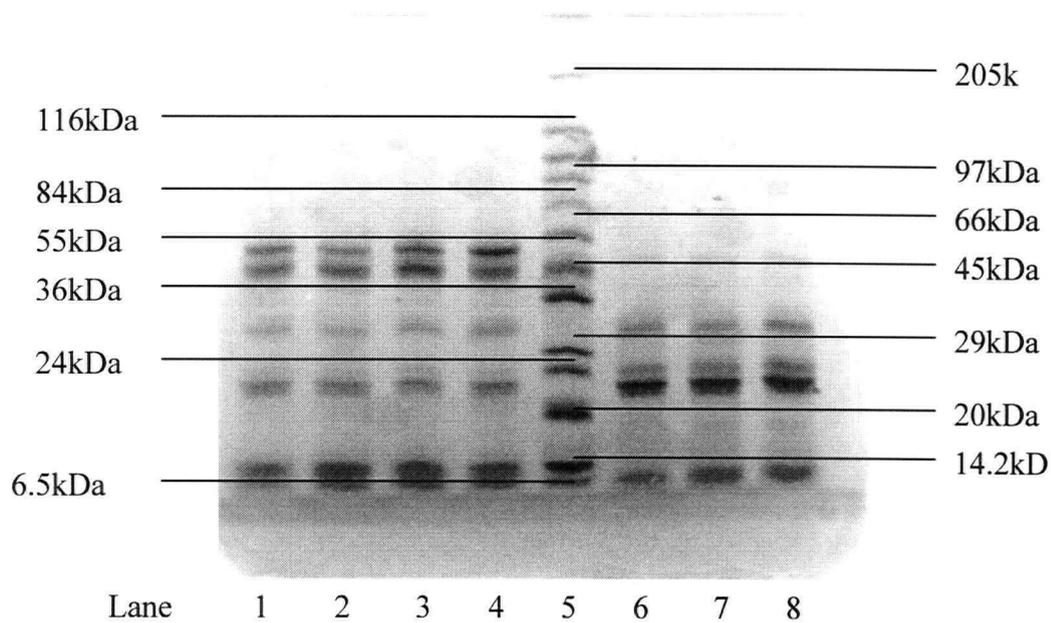
Figure 3-2a. SDS-PAGE of whole protein extracts from flaxseed AC Linora on 10-15 gradient PhastGel®.

Lane 1: AC Linora at Portage La Prairie without ME (2-mercaptoethanol)
Lane 2: AC Linora at Rosebank without ME
Lane 3: AC Linora at Morden without ME
Lane 4: CDC Normandy at Morden without ME
Lane 5: Sigma Marker Wide Range (MW range 6.5 - 205 kDa)
Lane 6: AC Linora at Portage La Prairie with ME
Lane 7: AC Linora at Rosebank with ME
Lane 8: AC Linora at Morden with ME
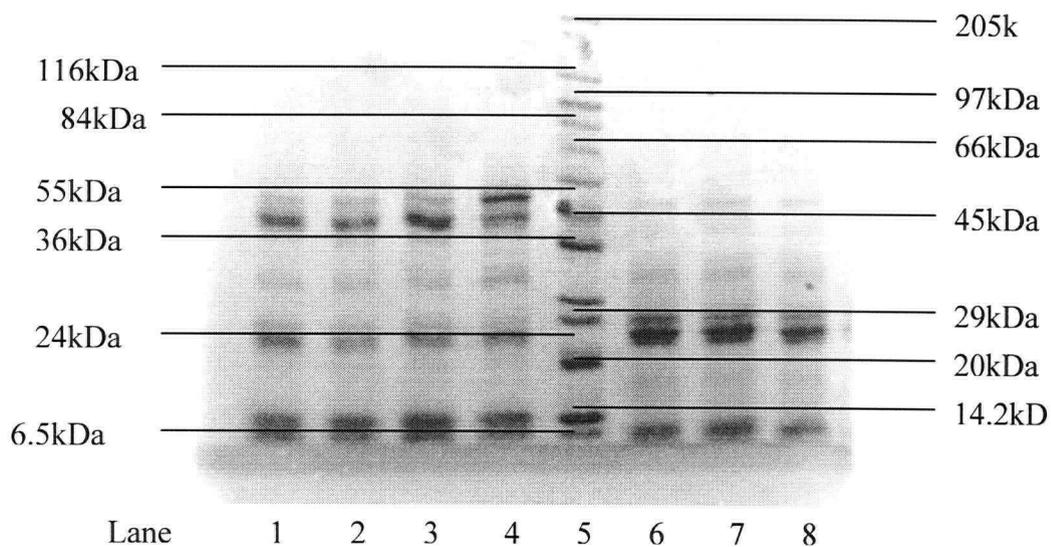
Figure 3-2b. SDS-PAGE of whole protein extracts from flaxseed AC McDuff on 10-15 gradient PhastGel®.

Lane 1: AC McDuff at Portage La Prairie without ME
Lane 2: AC McDuff at Rosebank without ME
Lane 3: AC McDuff at Morden without ME
Lane 4: CDC Normandy at Morden without ME
Lane 5: Sigma Marker Wide Range (MW range 6.5 - 205 kDa)
Lane 6: AC McDuff at Portage La Prairie with ME
Lane 7: AC McDuff at Rosebank with ME
Lane 8: AC McDuff at Morden with ME
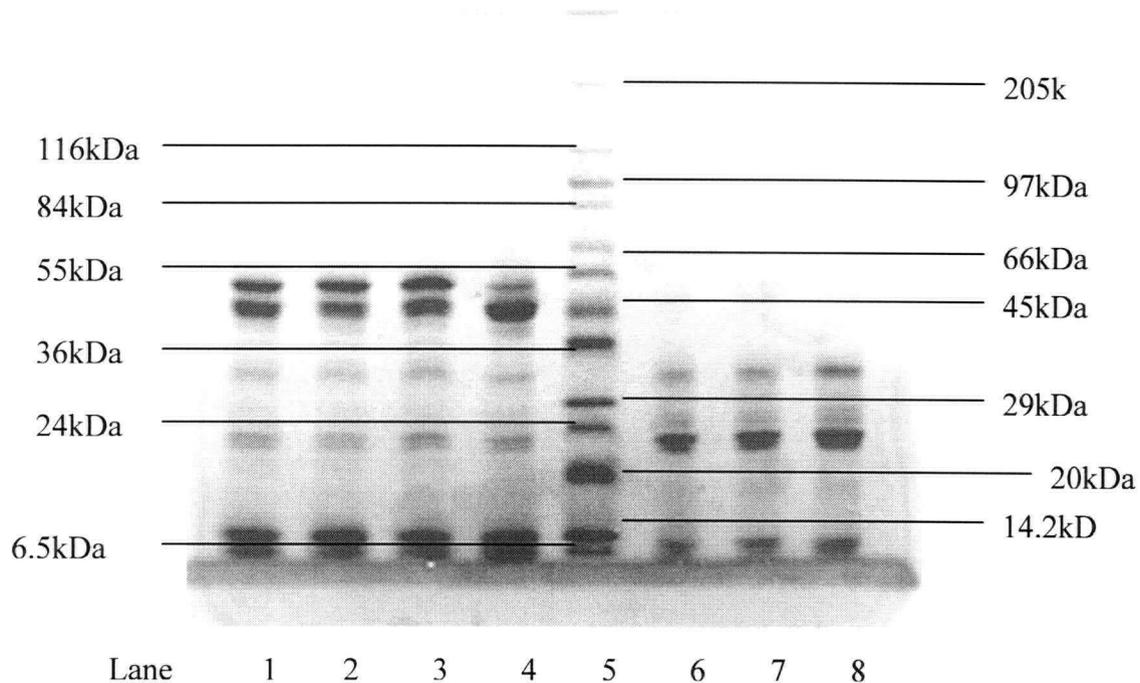
Figure 3-2c. SDS-PAGE of whole protein extracts from flaxseed CDC Normandy on 10-15 gradient PhastGel®.

Lane 1: CDC Normandy at Portage La Prairie without ME
Lane 2: CDC Normandy at Rosebank without ME
Lane 3: CDC Normandy at Morden without ME
Lane 4: AC McDuff at Morden without ME
Lane 5: Sigma Marker Wide Range (MW range 6.5 - 205 kDa)
Lane 6: CDC Normandy at Portage La Prairie with ME
Lane 7: CDC Normandy at Rosebank with ME
Lane 8: CDC Normandy at Morden with ME
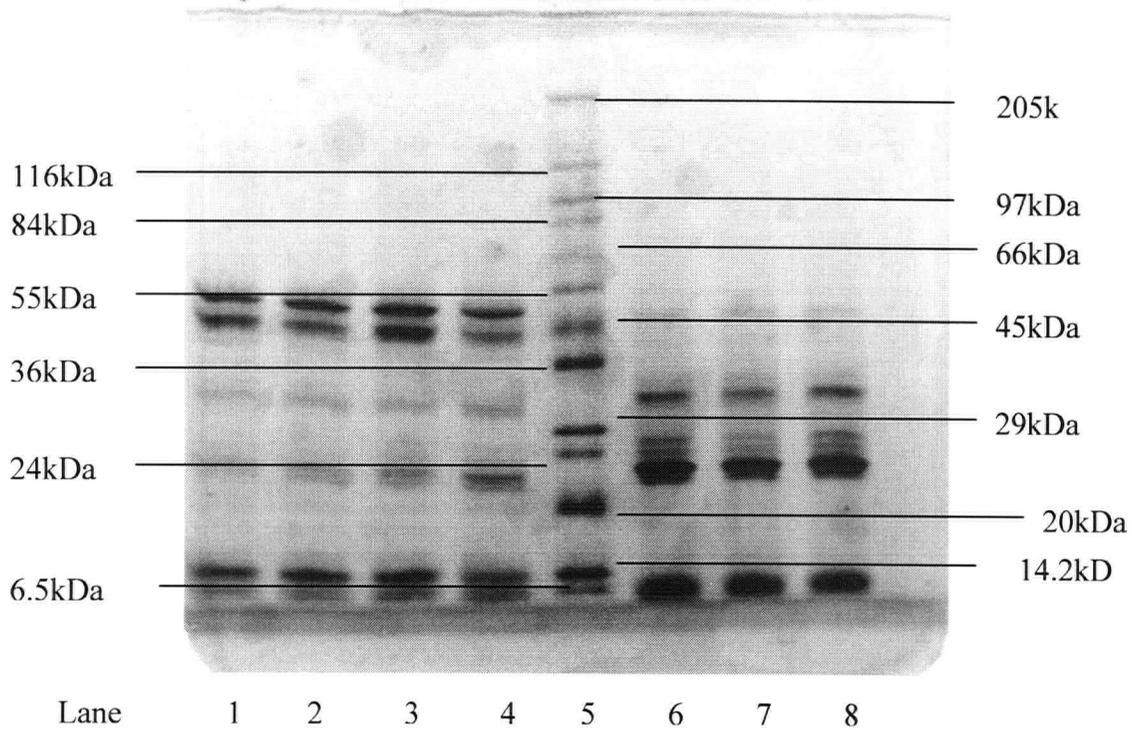
Figure 3-2d. SDS-PAGE of whole protein extracts from flaxseed Flanders on 10-15 gradient PhastGel®.

Lane 1: Flanders at Portage La Prairie without ME
Lane 2: Flanders at Rosebank without ME
Lane 3: Flanders at Morden without ME
Lane 4: CDC Normandy at Morden without ME
Lane 5: Sigma Marker Wide Range (MW range 6.5 - 205 kDa)
Lane 6: Flanders at Portage La Prairie with ME
Lane 7: Flanders at Rosebank with ME
Lane 8: Flanders at Morden with ME
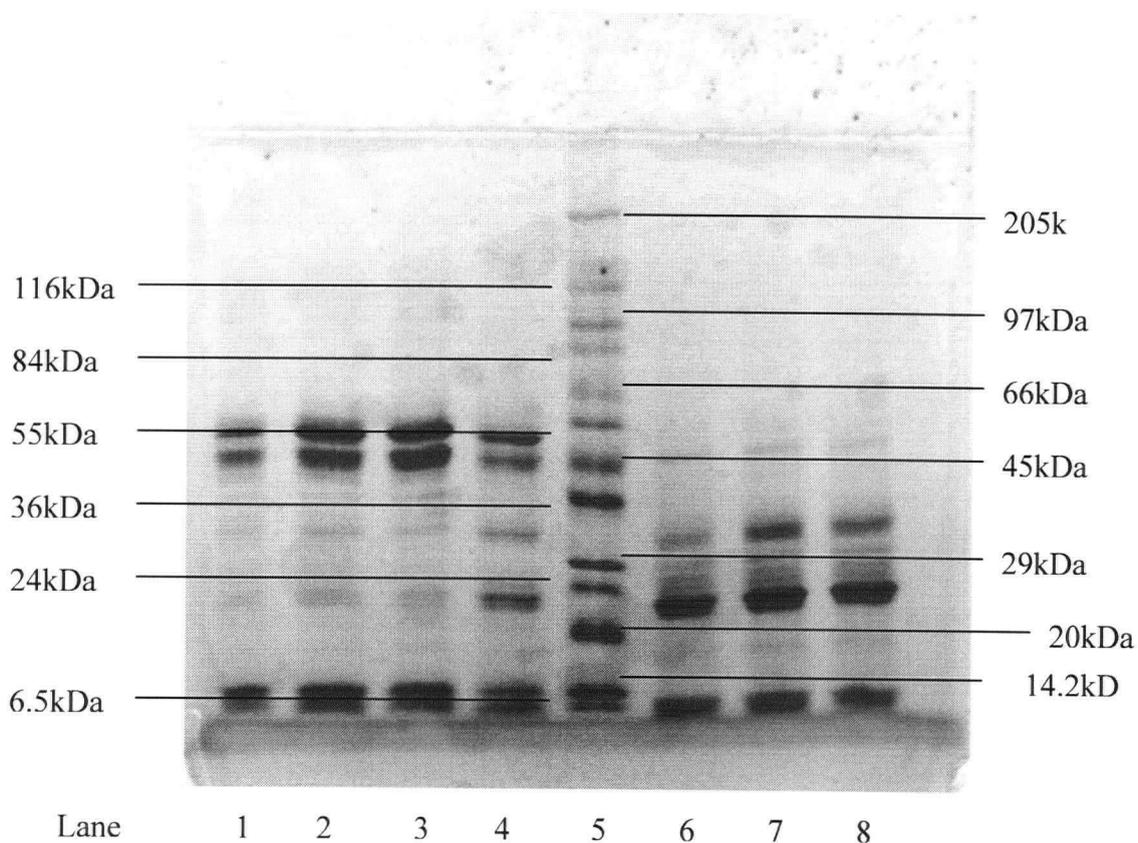
Figure 3-2e. SDS-PAGE of whole protein extracts from flaxseed NorLin on 10-15 gradient PhastGel®.

Lane 1: NorLin at Portage La Prairie without ME
Lane 2: NorLin at Rosebank without ME
Lane 3: NorLin at Morden without ME
Lane 4: CDC Normandy at Morden without ME
Lane 5: Sigma Marker Wide Range (MW range 6.5 - 205 kDa)
Lane 6: NorLin at Portage La Prairie with ME
Lane 7: NorLin at Rosebank with ME
Lane 8: NorLin at Morden with ME
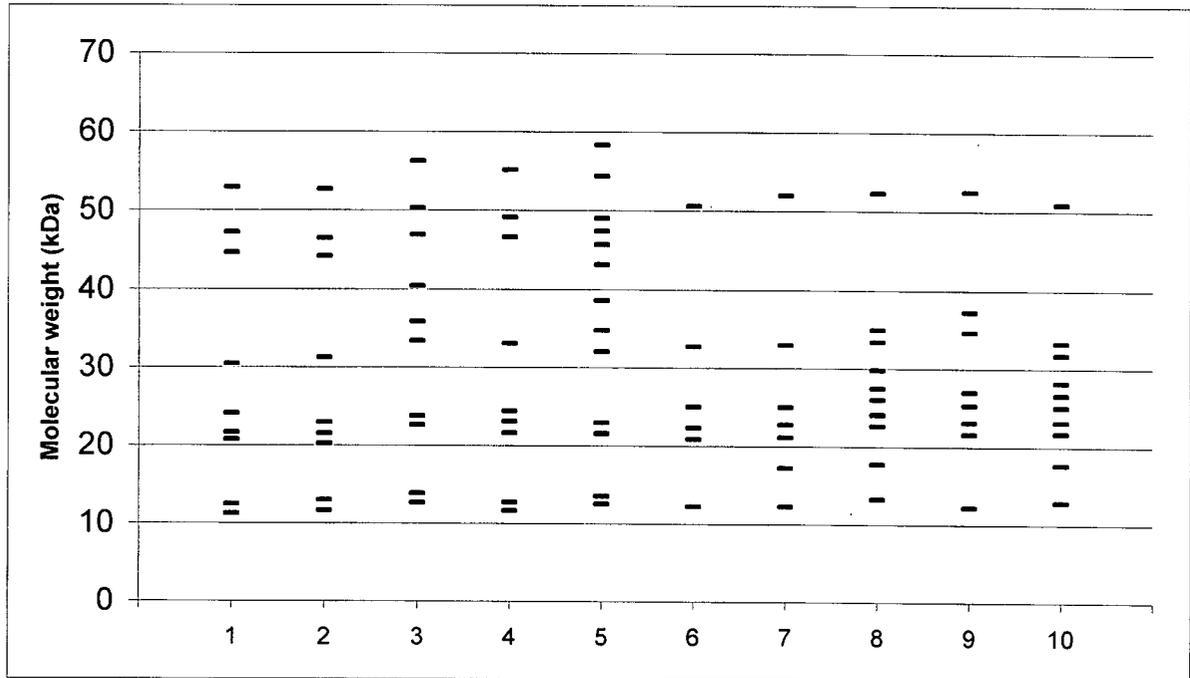
Figure 3-3. Comparison of the SDS-PAGE profiles of five flaxseed cultivars under non-reducing and reducing conditions. The data points of the molecular weights for each cultivar are the average values of that cultivar at three different locations. The position of the data points were created based on Figure 3-2 and Table 3-11.

X-axis numbers from 1 to 5: under non-reducing conditions
1:    AC Linora
2:    AC McDuff
3:    CDC Normandy
4:    Flanders
5:    NorLin

X-axis numbers from 6 to 10: under reducing conditions
6:    AC Linora
7:    AC McDuff
8:    CDC Normandy
9:    Flanders
10:    NorLin

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CHAPTER 4 CHARACTERIZATION OF Cd-BINDING COMPONENTS AND PROTECTIVE EFFECTS OF PROTEIN EXTRACTS OF FLAXSEED[1]

4.1 INTRODUCTION

Flaxseed often accumulates high amounts of Cd that exceed the dietary critical value of 0.3 ppm (Becher et al., 1997; Cieslinski et al., 1996; Moraghan 1993; Marquard et al., 1990). Cd, a widespread environmental pollutant, is a carcinogen that accumulates in the kidney cortex and is a cause of end-stage renal disease (Il'yasova & Schwartz, 2005). It is toxic to human, laboratory animals and cell cultures, and currently considered to be a human carcinogen by the International Agency for Research on Cancer of the World Health Organization (http://www.iarc.fr/).

The mechanism of Cd toxicity is not well known, but researchers have shown that Cd toxicity is related to its high capacity for binding to an intracellular ligand in the membrane (Mesonero et al., 1996). Cd affects many cellular metabolic processes including energy production, membrane transport, protein synthesis and DNA repair (Szuster-Ciesielska et al., 2000; Hartwig et al, 2002), which normally leads to cell injury or death (Santone et al., 1982). Romero-Puertas et al. (2002) reported that Cd (50 μM CdCl$_2$) induces an increase in the level of oxidized proteins in pea plants, probably mediated by H$_2$O$_2$ (Lin et al., 2002; Joseph et al, 2001; Ye et al, 1999), which affects important leaf antioxidative enzymes, and perhaps the photosynthetic carbon assimilation; these appear to be associated with the oxidative stress and the induction of senescence produced by Cd toxicity. Later on, Remero-Puertas et al. (2004) demonstrated that 50 μM

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[1] A part of this chapter has been published: Lei, B., Li-Chan, E. C., Oomah, B. D., and Mazza, G. 2003. Distribution of cadmium-binding components in flax (Linum usitatissimum L.) seed. Journal of Agricultural & Food Chemistry. 51(3):814-821.

CdCl$_2$ stress could increase H$_2$O$_2$ concentration 6 times higher in pea leave cells. In roots of wheat, Ranieri et al. (2005) also found that the increased PCs with excess Cd caused considerable amounts of H$_2$O$_2$ generated. Cultured cells exposed to high levels of Cd (Mattie & Freedman, 2001) and hydrogen peroxide (H$_2$O$_2$) (Halliwell et al., 2000) exhibit cytotoxicity.

Cd accumulation in plants such as flaxseed can lead to human exposure to this carcinogenic metal (Nawrot et al., 2006). Therefore, many studies have been focusing on understanding the mechanisms of Cd accumulation in plants, in order to create strategies to minimize the Cd content of plants (Cobbett, 2000; Klaassen et al., 1999; Rauser, 1995; 1999). The most recognized mechanisms for Cd storage and detoxification in plants are through metal chelation by specialized proteins referred to as metallothioneins (MTs) and phytochelatins (PCs) (Briat & Lebrun, 1999), but there is a paucity of information on the Cd-binding components in flaxseed.

MTs are a group of small molecular weight and cysteine-rich proteins or peptides, which in their reduced state, provide thiols for metal chelation. MTs have been found to be broadly distributed among animals, eukaryotic microorganisms, certain prokaryotes, and plants (Rauser 1999). Numerous research studies regarding MTs in animal and bacteria have been reported since the first discovery of a Cd-MT from horse kidney in 1957 (Margoshes & Vallee, 1957). However, there are very few reports of MTs isolated from plant materials.

PCs are a group of non-translational and cysteine-rich peptides, consisting of repeating units (2-11) of γ-glutamate-cysteine with a glycine, alanine, serine or other non-aromatic amino acid at the carboxy terminus (Rauser, 1990). PCs appear to be ubiquitous

in the plant kingdom (Grill, 1989) and are believed to function as detoxifying agents for Cd by virtue of their ability to bind Cd, thereby preventing it from reacting with the sulphydryl groups of vital enzymes and proteins. Apart from the typical MTs and PCs as detoxifying agents in plants, many other forms of metal chelators such as the metal-binding proteins lacking Cys or containing aromatic amino acids, and amino acids and other organic acids have been identified to play a role in Cd detoxification in plants (Rauser 1999).

The characterization of metal-binding proteins or components in plants typically includes procedures for extraction and purification, and for identification. The purification methods usually employed include solvent extraction followed by fractionation through ion exchange and size exclusion chromatographic columns. The Cd-binding peak may be monitored by the detection of eluted fraction with UV absorbance at 254 and 280 nm, direct Cd measurement by atomic absorption spectroscopy (Rauser, 1990), and/or inductively coupled plasma – mass spectrometry (ICP-MS) (Garcia et al., 2006). The structural characteristics of the Cd-binding components in the fraction have been analyzed by methods including circular dichroism, Raman spectroscopy, HPLC/MS, MALDI-TOF MS and amino acid analysis (Garcia et al., 2006; Stillman, 1995; Pande et al., 1986).

In previous studies (Chapter 2 & 3), this research established a protocol for separating most of the Cd from the major storage proteins of flaxseed, yielding a low Cd content major protein fraction and a high Cd content minor protein fraction. The latter major Cd-binding fraction contained over 50% of the Cd from flaxseed protein extract with less than 10% of the proteins. However, the basis of the high Cd-binding capacity of

this fraction is unknown. The objective of this study was therefore to characterize some properties of the components that are responsible for Cd-binding in flaxseed through the studies of the isolated major Cd-binding fraction. In addition, a second objective of this study was to evaluate the possible protective effects of flaxseed protein extracts, the major protein fraction as well as the major Cd binding fraction, against Cd and H_2O_2 induced toxicity. This research could be used as a starting point for further detailed research on the biological functions of flaxseed protein in health and medical applications.

4.2   MATERIALS AND METHODS

4.2.1   Materials

The flaxseed cultivar NorMan was used as the starting material, as described in Section 2.2 of Chapter 2. The protein samples used for the study included the protein extracts (PE) and the purified fractions from ion exchange and size exclusion chromatographic columns prepared by the methods described in Section 2.2.2 to 2.2.5 of Chapter 2. The peak eluted at 0.1 M NaCl was the major Cd binding fraction (MCF), and the peak eluted at 0.25 M NaCl was the major storage protein fraction (MPF). For the cell culture experiments, the three samples of PE, MCF and MPF were concentrated to 1.1 mg/mL as measured by BCA protein assay (Section 2.2.6.2) by ultrafiltration with a membrane of MWCO 500 Da and adjusting pH to 7.

Cd chloride, H_2O_2 solution (30%), AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride at 10mM), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and phenazine methosulfate (PMS) were from Sigma-Aldrich Canada Ltd., ON,

Canada. CellTiter 96® AQueous MTS reagent powder was from Promega Corporation, Madison, WI. All the chemicals used for preparing Dulbecco's phosphate buffered saline (DPBS), including potassium chloride, sodium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, magnesium chloride hexahydrate, and calcium chloride dihydrate, were from Fisher Scientific Ltd., ON, Canada. The cell culture medium RPMI-1640 was from American Type Culture Collection (ATCC), Manassas, VA.

4.2.2 Analytical Methods

All analyses were performed on the pooled column fractions.

4.2.2.1 Cd and protein content analyses

The Cd and protein contents were measured by the methods in Section 2.2.6.2 and 2.2.6.3 of Chapter 2.

4.2.2.2 UV/Vis absorbance characteristics

The absorbance spectrum was measured by using an ATI Unicam UV/Vis Spectrometer (Unicam Limited, Cambridge, UK).

4.2.2.3 HPLC/MS

The samples from the G50 size exclusion chromatography of NorMan flaxseed were analyzed using HPLC/MS by Mass Spectrometry Services of the University of British Columbia (Vancouver, BC, Canada). The samples were first separated by reverse-

phase HPLC on an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Pleasanton, CA), which was equipped with a C_{18} column (Reliasil, 1x 150 mm) equilibrated with 0.05% trifluoroacetic acid, and 2% acetonitrile in water. Samples were eluted with a gradient of 0 to 60% of 0.045% trifluoroacetic acid + 80% acetonitrile in water and eluted fractions were further analyzed by a PE-Sciex API III triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Toronto, ON), which had an ion-spray ionization source. In the single quadrupole mode the quadrupole mass analyzer was scanned over an m/z range of 400 to 2,000 Da with a step size of 0.5 Da and a dwell time of 1 ms per step. The ion source voltage was set at 5 kV and the orifice energy was 50 V. The molecular weight of each peak was reconstructed by Bio-Multiview software version 1.3.1 (Perkin-Elmer Sciex Instruments, Toronto, Ontario), using the "BioSpec Reconstruct" tool for the hypermass estimation. For components with low MW and few charges, the "Purity Hypermass" tool was used instead.

4.2.2.4 MALDI-TOF MS

MALDI-TOF MS (matrix-assisted laser desorption/ionisation time of flight mass spectrometry) analyses were carried out by the Nucleic Acid Protein Service Unit of the University of British Columbia (Vancouver, BC, Canada). All MALDI-TOF MS mass spectra were acquired on a Voyager-DE STR MALDI-TOF Workstation (Applied Biosystems, Foster City, CA) equipped with a nitrogen laser with an emission wavelength of 337 nm. Mass spectra were collected in the linear mode at an accelerating voltage of 25 KV and a delay of 1500 ns. The grid voltage and guide wire voltage were 94% and 0.15%, respectively, and low mass gate was set to 500 to 5,000 Da according to

the different protein fractions. Each mass spectrum collected represented the sum of the data from 100 laser shots.

4.2.2.5 Circular dichroism

The circular dichroism spectra were collected on a J-720 spectropolarimeter (JASCO, Tokyo, Japan) purged with nitrogen. Far-ultraviolet (190-300 nm) measurements were performed with a 0.5 nm bandwidth by using a U-type quartz cell of path length 1 mm. CD spectra were recorded with five accumulations, each at a scan speed of 50 nm/minute and a response time of 2 seconds.

4.2.2.6 Solubility characteristics

The solubility under different pH and temperature conditions of the major Cd-binding fraction and the major storage protein fraction eluted from ion exchange chromatography at 0.1 and 0.25 M NaCl, respectively, were compared. Conditions used were based on the methods and conditions most commonly used for solubility tests as reviewed by Vojdani (1996). The samples were dialyzed in dialysis tubing (MWCO 100) against 20 mM NaCl in deionized and distilled water. After dialysis, the samples were concentrated by ultrafiltration (MWCO 500) and protein concentration was adjusted to 1mg/mL by adding 20 mM NaCl solution. The protein content was measured by the BCA protein assay (Section 2.2.6.2).

Protein samples (5 mL aliquots) were adjusted to different pH by addition of 0.001 to 0.1N HCl or NaOH, and samples were centrifuged after 30 minutes incubation using a small benchtop Eppendorf-type microcentrifuge at its maximum speed (13,000

rpm) for 30 minutes. The supernatants were measured for protein content by the BCA protein assay. Based on the results of the solubility experiment under different pH conditions, the protein samples at the pH of least and highest solubility were used for testing the solubility as a function of temperature. Protein samples (5 mL aliquots) were heated at different temperature for 30 minutes in a water bath (≤ 100 °C) or in an autoclave (>100 °C). The inside-tube temperature was monitored with a data logger (Data Taker, Field Logger, DT 100F, Data Electronics (Aust.) Pty, Ltd, Boronia, Australia) through a 1 mm diameter needle thermocouple, which was previously calibrated with an American Society for Testing and Materials (ASTM) mercury-in-glass thermometer. The data logger collected temperatures every 20 seconds. Protein content was measured by the BCA protein assay after centrifugation (microcentrifuge, 13,000 rpm). Solubility was expressed as percent protein solubility (%PS):

$$\% \ PS = (\text{amount of soluble protein} / \text{total amount of protein}) \times 100$$

4.2.2.7 Total SH and total SH+SS contents

Total SH and total SH + SS contents were determined by using Ellman's reagent (5,5-dithiobis(2-nitrobenzoic acid), DTNB) (Ellman, 1959) and 2-nitro-5-thiosulfobenzoate (NTSB) (Thannhauser et al., 1993; Damodaran, 1985), respectively, in duplicate or triplicate with modifications of the methods as described by Chung (2001).

4.2.2.8 SDS-PAGE and IEF

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with PhastGel® high density gel, according to the PhastSystem™ Separation Technique (File No.112—SDS-PAGE of low molecular weight protein using PhastGel® high density; Amersham Pharmacia Biotech Inc., Quebec, PQ). The low range molecular weight markers (LMW 14kD - 97kD, Amersham Pharmacia Biotech Inc., Quebec, Canada) were used. Isoelectric point of the major Cd-binding fraction was determined by isoelectric focusing (IEF) following the procedure outlined by the PhastSystem™ Separation Technique (File No. 100—IEF and electrophoretic titration curve analysis). PhastGel®s with pH range from 3 –9 were used. Approximately 2 µg of sample was loaded in each lane. Samples were applied in the middle position of the gel as described in the PhastSystem™ user manual. The Sigma IEF-Mix 3.6 – 9.3, I-3018 (Sigma, St. Louis, MO) was used as marker. All PhastGels® were Coomassie stained according to the PhastSystem™ Development Technique.

4.2.2.9 Amino acid analysis

Amino acid analysis was performed by the Advanced Protein Technology Centre of The Hospital for Sick Children (Toronto, ON, Canada) by using a Waters Alliance 2690 Separation Module. The three fractions of HMW peak, Peak A and Peak B were collected after the G50 column chromatography; Peak B was dialyzed against deionized and distilled water using a dialysis membrane with molecular weight cutoff of 100 Da. The dialyzed samples were hydrolyzed manually and derivatized with PITC, and chromatographed on the Pico-Tag RP-HPLC column (3.9 mm x 15 cm) for detection of

PTC residues at 254 nm. Cysteine and cystine contents were analyzed as cysteic acid after performic acid derivatization. The data were reported as percentage of residues.

4.2.2.10 Cell culture

Human acute monocytic leukemia THP-1 cell line (classified as Biosafety Level 1) was obtained from ATCC. Cells were maintained in the RPMI-1640 medium supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U) and streptomycin (100 µg/mL) in cell culture flasks. The cells were maintained at a concentration of not more than 1×10^6 cells/mL. Fresh cell medium was added to the culture every 3 days and total cell medium replacement was done every week. All the cell cultures were incubated at 37 °C in a 5% CO_2 humidified incubator. Viable cell counts were observed by trypan blue (0.04%) exclusion dye assay using a hemacytometer under the microscope (Mishell & Shiigi, 1980), where viable cells with an intact membrane are able to exclude the dye while the dead cells without an intact membrane take up the coloring agent.

4.2.2.11 Cell count by cell proliferation assay

The cells were harvested after 2 days of culture, and were resuspended in fresh RPMI-1640 medium without addition of FBS. After incubation for one hour, 0.9 mL of cell culture was added to each well in the 24-well plates. Various concentrations of $CdCl_2$, H_2O_2, and/or protein samples were added separately to each well. DPBS was used to make the final volume to 1 mL in each well. When protein samples were to be tested, the samples were added first to the cell culture and incubated for 2 hours, before addition of $CdCl_2$ or H_2O_2.

After 4 hours of incubation, cell numbers were measured according to the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI). The reaction mixture consisted of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS). The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes in metabolically active cells. The quantity of formazan product, as measured directly in the 96-well assay plates by the absorbance at 492 nm, is directly proportional to the number of living cells in the culture. The negative control was the cell culture without addition of $CdCl_2$ and H_2O_2. The positive control, representing no viable cells, was measured by the cell culture with addition of the highest concentration of $CdCl_2$ (1mM) or H_2O_2 (0.1%), in which all cells were microscopically observed to be dead. Cell viability after different treatments was expressed as % relative absorbance using the following calculation (Promega Corporation, 2005):

Relative absorbance (%) = (mean absorbance of treated cells – mean absorbance of positive control)/(mean absorbance of negative control- mean absorbance of positive control) x 100   (Equation 1)

4.2.2.12 Statistical analysis

All experiments were conducted in triplicate, and the data were analyzed by ANOVA using MiniTab Statistical Software (Release 13.30).

4.3 RESULTS

4.3.1 UV/Vis Absorbance Characteristics

The column elution profiles showed three major peaks eluted at 0.1, 0.25, and 0.45 M NaCl from ion exchange chromatography, and three fractions of HMW peak, Peak A and Peak B eluted from size exclusion chromatography. These eluted fractions showed distinct UV absorbance characteristics comparing the ratio of the absorbance at 254 nm (A_{254}) and 280 nm (A_{280}). As shown in Figure 4-1, the A_{254} of the 0.1 M NaCl peak fraction was almost the same as A_{280}, but A_{254} was much smaller than A_{280} for the 0.25 M NaCl peak fraction, while the high salt 0.45 M NaCl peak fraction had A_{254} greater than A_{280}. For size exclusion chromatography (Figure 4-2), the HMW peak had absorbance of 254 nm higher than that of 280 nm, but the absorbance of Peak A at A_{280} was higher than A_{254}, while the absorbance of Peak B at A_{254} was almost the same as A_{280}.

UV/visible absorbance spectra of each fraction from ion exchange and size exclusion chromatographies were scanned from 190 to 500 nm, and the wavelength of maximum absorbance (λ_{max}) was noted. The 0.25 M NaCl fraction (Figure 4-3) had a peak with λ_{max} of 281 nm, which is typical of proteins containing aromatic residues, particularly tryptophan and tyrosine. The λ_{max} values for the 0.1 M NaCl and unbound fractions were 276 and 272 nm, respectively, while that of the high salt 0.45 M NaCl fraction was located at a lower wavelength of 267 nm. The 0.1 M NaCl fraction also had a smaller, broad peak between 343 to 373 nm (Figure 4-3). For the G50 column fractions (Figure 4-4), the λ_{max} for the HMW peak was near 280 nm. Peak A and Peak B both had

λ_{max} at around 275 nm, but only the major Cd-binding fraction of Peak B had an extra peak between 343 to 373 nm, similar to the 0.1 M NaCl fraction (Figure 4-4).

4.3.2 HPLC/MS

The three fractions from G50 size exclusion chromatography, namely HMW peak, Peak A and Peak B, were further analyzed by HPLC/MS. There were over 10 distinguishable peaks eluted from HPLC loaded with the HMW peak. Among them, 5 major peaks were selected for more detailed analysis of the mass spectral data. The HPLC elution profile of HMW peak and its corresponding peak analyses by MS are shown in Figure 4-5a to 4-5e. Each figure consists of three panels - the upper one is the HPLC chromatogram of TIC (total ion current) versus elution time in minutes; the middle one is the MS spectrum of the analysis result for the shaded peak in the HPLC elution profile; the lower panel is the result for molecular weight reconstruction processed by Bio-Multiview software. The strongest signals as shown in the lower panel (Figure 4-5) after molecular weight reconstruction were 11,012.0 Da for the HPLC peak eluted at 23.99 minutes (Figure 4-5a), 18,652.0 Da at 27.68 minutes (Figure 4-5b), 18,469.0 Da at 33.02 minutes (Figure 4-5c), 12,181.0 Da at 36.76 minutes (Figure 4-5d), and 23,322.0 Da at 53.51 minutes (Figure 4-5e). The highest peak in the HPLC profile was eluted at 27.68 minutes, therefore, the major component of HMW peak had molecular weight of 18,652.0 Da, while HMW peak also contained components with molecular weight at around 11 to 12, and 23 kDa. The intensity values of the mass spectral data also indicate that the component with molecular weight at 18,652.0 Da was the major one in the HMW peak (Figure 4-5b).

Three major peaks were eluted from HPLC column loaded with the Peak A sample (Figure 4-6a to 4-6c). Based on the results from Section 2.3.1.3 in Chapter 2, Peak A was eluted during size exclusion chromatography at a position corresponding to ~14 kDa. The mass spectral analysis was done by scanning the major peaks from 1,500 to 20,000 Da. It was found that the most prominent components were 2,943.0, 4,897.0, 8,160.0, 15,927.0, and 17,542.0 Da for peak eluted at 37.67 minutes (Figure 4-6a), and 2,219.0 and 10,297 Da for peak eluted at 39.49 minutes (Figure 4-6b), and 2,055.0 Da for peak eluted at 51.19 minutes (Figure 4-6c). By comparison of the signal intensity, the major components in Peak A have molecular weights of 2,943.0 and 17,542.0 Da.

Three major peaks were eluted from HPLC loaded with the Peak B sample. The major peak was eluted very early at 3.42 minutes, and two small peaks were eluted after 20 minutes (Figure 4-7a to 4-7c). Based on the fractionation range (1,500 to 30,000 Da) of the G50 column used for size exclusion chromatography, the expected molecular weight for Peak B is less than 1,500. The m/z of the base peaks in the mass spectra from HPLC of Peak B were 431.3 eluted at 3.23 minutes (Figure 4-7a), 667.3 at 21.85 minutes (Figure 4-7b), and 667.5 Da at 22.98 minutes (Figure 4-7c), corresponding to three components at MW of 1721.1, 1332.6, and 666.4 Da, respectively, after molecular weight reconstruction. The component of 1332.6 Da eluted at 21.85 minutes should be the dimer of the component 666.4 Da eluted at 22.98 minutes. Comparing the intensity and percentage, it could be concluded that the major components in Peak B were 1,721.1 and 666.4 Da.

4.3.3 MALDI-TOF MS

Five protein samples were analyzed by matrix-assisted laser desorption /ionization-time of flight mass spectrometry (MALDI-TOF MS), including the major protein and major Cd binding fractions eluted at 0.25 and 0.1 M NaCl, respectively from ion exchange chromatography, and the three peaks of HMW peak, peak A and peak B from size exclusion chromatography.

The mass spectrum of the 0.25 M NaCl fraction (Figure 4-8) showed a predominant signal at 40,519.14 Da, and other significant signals at 11,597.63, 17,071.44, 20,179,11, 42,018.54, and 51,169.33 Da over the acquisition mass range of 10 to 300 kDa. The mass spectrum of the major Cd-binding fraction eluted at 0.1 M NaCl had two predominant ion signals at 4,782.04 and 14,145.93 Da (or m/z) (Figure 4-9). Figure 4-10 shows the mass spectrum for peak A. The predominant ion signal was at 4,790.20 Da. The mass spectrum for the HMW showed three predominant peaks at 2,739.51, 2,965.23, and 3,399.75 Da (Figure 4-11). The major ion signals for peak B were at 649.66, 655.66, and 860.52 Da (Figure 4-12).

4.3.4 Circular Dichroism

Figure 4-13 shows a typical circular dichroism spectrum of the 0.45 M NaCl fraction, with a strong maximum at 267 nm and a small minimum at 235 nm. This is characteristic of Cd-thiolate coordination (Rupp & Weser, 1978). According to Rauser (1990), Cd-thiolate complexes show a positive cotton band at 275-280 nm and a negative one at 253-257 nm, with shifts in the maximum and minimum for different Cd-thiolate complexes. For typical proteins, there is no maximum above 240 nm.

4.3.5 Solubility Characteristics

The protein solubility results are shown in Figure 4-14 and 4-15. The data points were based on two individual experiments, and duplicate data deviation was less than 15% from the mean value. The pH region of least solubility (Figure 4-14) for the 0.25 M NaCl fraction from ion exchange chromatography was from pH 4 to 6, while the 0.1 M NaCl fraction was least soluble around pH 5. At the pH of least solubility, the 0.25 M NaCl fraction had about 30% soluble protein, while the 0.1 M NaCl fraction had about 60% soluble protein.

Figure 4-15 shows the results for solubility dependence on temperature at two pH conditions: pH 5 for least solubility and pH 8 for maximum solubility. The proteins in the 0.1 M NaCl fraction were more heat stable than proteins in the 0.25 M NaCl fraction. At pH 5, the solubility of 0.1 M NaCl fraction decreased from 55to around 50% with increasing temperature from 4 to 120 ^{o}C; in comparison, at the same pH, the solubility of 0.25 M NaCl decreased from 30 to around 15% over the same temperature range. At pH 8, the solubility of 0.1 M NaCl fraction was about 95% at 4 ^{o}C and decreased to 82% at 120 ^{o}C; at the same pH and temperature range, the solubility of 0.25 M NaCl fraction was decreased from 90 to 36%.

4.3.6 Total SH and Total SH + SS Contents

The total SH and total SH + SS contents are shown in Table 4-1. Because of limited sample, the total SH content of some fractions was not determined. The estimated contents of SH and SH + SS (μmol/g protein) for Peak A and Peak B should be considered as approximate values only, due to the low sample concentration and possible

inaccuracy of applying the BCA protein assay for these fractions with an unusual amino acid composition. Nevertheless, considering that the average occurrence of cysteine in protein is 1.9 % (Nelson & Cox, 2000), corresponding to a calculated total SH+SS content of 157 μmol/g protein, the values of 1104 μmol/g protein for the 0.1 M NaCl fraction and 272 μmol/g protein for Peak B are very high.

4.3.7 SDS-PAGE and IEF

The SDS-PAGE and IEF profiles of the 0.1 M NaCl fraction were obtained in order to further understand the characteristics of this major Cd-binding fraction. Figure 4-16 shows the results for non-reducing SDS-PAGE using high density (homogeneous 20%) gel, which revealed four bands in the 0.1 M NaCl fraction with estimated molecular weight of 24, 31, 56, and 71 kDa, and a couple of minor bands at > 97 Ka. The 31 kDa band was the predominant one.

Figure 4-17 shows three bands in native IEF (Figure 4-17 A), located between pI values of 4.6 to 5.9. The denaturing IEF showed more bands than native IEF, with five bands between pI values of 4.6 to 5.9 in the profiles of both reduced and non-reduced conditions (Figure 4-17 B), but the reducing IEF showed two other bands near 6.6 and 7.2, and the non-reduced denaturing IEF showed a band at around 6.6. These results agree with the solubility test at different pH, which showed that the least solubility was observed at around pH 5.

4.3.8 Amino Acid Analysis

The results of amino acid analysis for the three fractions from G50 chromatography, namely the HMW peak, Peak A and Peak B, are shown in Table 4-2. The most abundant amino acids in the three samples were Glx (Glu + Gln) and Gly. In fact, the three amino acids Asx +Glx+Gly comprised 64% of the total amino acids in Peak B. The cysteine content in the three samples was 1.7, 7.1 and 5.0% for HMW peak, Peak A and Peak B, respectively.

In addition to the usual amino acids, several unknown or unidentified peaks in the chromatographic profiles during amino acid analysis were distinctive features of the Peak A and Peak B fractions (Figure 4-18). Both Peak A and Peak B had an unknown peak at the position of 6.3 minutes, referred to as unknown peak 2 in Table 4-2, which constituted 8.7and 82.3% of the total peak area in the amino acid chromatogram of Peak A and Peak B, respectively. Peak B also had two other unknown peaks (unknown peaks 1 and 3 in Table 4-2) at 5.9 and 8.3 minutes, comprising 3.1 and 7.9%, respectively, of the total peak area. The unknown peaks were not included in the calculation of the percentage of the amino acid residues, but they were in fact major constituents, particularly of the Peak B fraction. In Peak B, considering the high levels of the three unknown peaks (93.3% of total peak area), especially the unknown peak at 6.3 minutes (82.3%), the composition of amino acids shown in Table 4-2 does not represent the actual total composition of Peak B.

4.3.9 Preliminary Antioxidant Trials of Flaxseed Protein Fractions

For preliminary trials, protein extract samples from defatted powders of flaxseed cultivar NorMan (NorMan), and cultivar Flanders grown in Morden (FM) and Portage la Prairie (FP) were used. Based on preliminary experiments, the cell culture concentration used was 5×10^5 cells per mL and the incubation time was four hours (Appendix 1). Three stresses were tested as follows: H_2O_2 (at 0.06%); hydroxyl radical from Fenton reaction (OH• from $[Fe^{2+}]$ at 25 mM + H_2O_2 at 0.03%; and peroxyl radical ROO• from AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride at 10 mM. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), the water soluble form of vitamin E, was also used as a calibrator for antioxidant activity. The results are shown in Figure 4-20. It was found that the protein extract from FM provided the THP-1 cells with the strongest protection effect against OH• and H_2O_2 stresses, but had no effect on AAPH stress. The NorMan and FP protein extracts showed very little protection effect.

This experiment showed that flaxseed protein extract might have antioxidant activity or protective effect against hydroxyl radical and direct oxidative stress from H_2O_2. However, it had to be further tested and no conclusion could be drawn. There were two problems in the experiment. First, the samples used for antioxidant activity test were quite old. The FM and FP liquid samples collected from chromatographic column had been stored already in the freezer (-18 °C) for almost 6 months, while the NorMan sample had been in the freezer already over two years. Second, the sample concentration was too high, both for Trolox and for protein extracts.

New protein extract sample was prepared from defatted powder of flaxseed NorMan. Ion exchange and size exclusion chromatography were conducted, and fractions

were collected and used to evaluate antioxidant activity in the THP-1 cell cultures subjected to H_2O_2 (0.06%) oxidative stress. The experimental results are summarized in Table 4-4. Because of limited sample, further processing of fractions to achieve equivalent concentrations (1.4-1.5 mg/mL as the original extracts) was not done. Nevertheless, the three protein extracts were in a similar concentration range, and it was found that FM and NorMan provided very strong protective effect against H_2O_2 stress, while FP did not. Contrary to the result of the previous experiment, NorMan sample had the strongest protective effect; this suggested that the antioxidant activity of the protein extract was also "time dependent", i.e. the antioxidant activity of the samples may be degraded after long-term frozen storage.

Samples of NorMan 0.1 M NaCl (major Cd binding) fraction and its three fractions from size exclusion column all showed various degrees of protective effect against H_2O_2. The NorMan 0.25 M NaCl fraction showed no obvious protective effect compared with the H_2O_2 treatment. The viable cells were expressed as % relative absorbance, assuming 0% relative absorbance for H2O2 treatment and 100% for Trolox treatment. The protein extract from NorMan showed 95% relative absorbance, while the HMW peak after size exclusion chromatography, at only 0.005 mg/mL protein concentration, showed almost 50% relative absorbance. Upon comparing the protective effect based on unit protein (enhancement efficiency index), it was more clearly demonstrated that the HMW peak had the strongest protective effect, followed by Peak B and 0.1 M NaCl, peak A, and then the NorMan protein extract (Table 4-4). However, this speculation could only be used as a reference for further study, because the protein concentration as determined by BCA protein assay may not accurately reflect the actual

concentration of compounds responsible for this protective effect in each sample of HMW peak, Peak A and Peak B, as discussed in Section 2.4 of Chapter 2.

4.3.10 Growth Promoting Effect of Protein Extracts

According to the results in Section 4.3.9, protein extract of NorMan flaxseed conferred protective effects against oxidative stress; however, the concentration used for the test was high. In order to find the appropriate concentration for the toxicity experiment, a series of different concentrations of protein extracts from flaxseed NorMan were tested. Unexpectedly, it was found that the flaxseed protein extract could promote THP-1 cell growth. Figure 4-21 shows the dose dependent effect of protein extract on THP-1 cell viability. The growth promoting effect of flaxseed protein extract was more pronounced when present in the range of 0.07 to 0.28 mg/mL than at protein concentrations higher than 0.28 mg/mL. In order to reveal if the protein extract has specific protective effect against Cd and H_2O_2 toxicity, a lower protein concentration with minimal growth promoting effect should be used. A concentration of 0.11 mg/mL was selected, which gave a 20% growth promotion effect. Because of limited sample, the major Cd fraction and major protein fraction were not tested for this effect.

4.3.11 Effects of Flaxseed Protein Samples on Cd and H_2O_2 Induced Cytotoxicity

Preliminary experiments were conducted to test the effects of different concentrations of H_2O_2, H_2O_2 with Fe^{2+} (the Fenton reaction) and Cd on THP-1 cell death (Appendices 2, 3 and 4). Based on the results from these preliminary experiments, three flaxseed samples were then tested for protective effects against cytotoxicity induced by

300 μM Cd and 0.06% H_2O_2. The three samples, at a final concentration of 110 μg/mL in the cell culture, were protein extract (PE) from NorMan, major Cd-binding fraction (MCF) eluted from ion exchange chromatography at 0.1 M NaCl, and major protein fraction (MPF) eluted at 0.25 M NaCl. Similar results were found for both Cd and H_2O_2 cytotoxicity experiments (Figures 4-22 and 4-23). It was noted that MCF conferred the highest protective effects for THP-1 cells against both Cd and H_2O_2 toxicity, resulting in increases of relative absorbance of 48 and 89%, respectively. It is also clear that MCF is a stronger protective agent against H_2O_2 toxicity than Cd toxicity.

The treatments with PE also showed significant protective effects against Cd and H_2O_2 toxicity as indicated by the bar charts in Figures 4-22 and 4-23. However, considering the promoting effect of PE on the cell culture (Figure 4-21), additional specific protective effects of PE against Cd toxicity may be relatively small, since there was only a 14% increase in relative absorbance compared with the treatment of cell culture plus Cd. On the other hand, greater protective effect of PE on cells against H_2O_2 toxicity is observed with a 44% increase in the relative absorbance, which is greater than the 20% promoting effect of PE. The treatments with MPF had no protective effects against cytotoxicity according to statistical analysis (Figures 4-22 and 4-23).

Cd at a concentration of 300 μM caused 72% cell death in the cell culture compared with control, but the addition of 110 μg/mL of protein extract diminished the cell death to 58%, while the addition of major Cd-binding fraction eluted by 0.1 M NaCl from ion exchange chromatography lowered the cell death to 24%. The inclusion of 0.06% H_2O_2 caused 96% cell death in the culture; the addition of 110 μg/mL of protein

extract lowered the cell death to 52%, while the addition of major Cd-binding fraction dramatically lowered the cell death to only 7%.

4.4   DISCUSSION

4.4.1   Structural Characteristics of Flaxseed Protein Fractions

The results of this study suggest the presence of multiple Cd-binding factors in the protein fractions of flaxseed, as the Cd was present in the unbound fraction, as well as in each of the salt eluted fractions from ion exchange chromatography. One preliminary indicator of Cd-binding protein or peptide was the higher absorbance at 254 nm than at 280 nm for protein fraction (Li-Chan et al., 2002).   After fractionation of flaxseed protein extracts by ion exchange chromatography (Figure 4-1), the 0.25 M NaCl fraction had $A_{280} > A_{254}$, which is typical for proteins, while the 0.45 M NaCl fraction had $A_{254} > A_{280}$, which is characteristic of metal-binding proteins. The 0.1 M NaCl fraction had $A_{254} \cong A_{280}$. The high salt fraction (0.45 M NaCl) may contain some specific Cd-binding factor(s) as indicated by the higher A_{254} than A_{280}. This fraction showed a distinctive ultraviolet absorbance spectrum (Figure 4-3 B). The peaks were very broad and the wavelength of maximum intensity was at 267 nm for the 0.45 M fraction, and 258 nm for the 0.50 and 1.0 M fractions. It has been reported that Cd complexes have a shoulder between 250 and 275 nm typical of metal thiol coordination (Rauser, 1990). The absorption spectra of the 0.45 and 0.50 M fractions were similar to those of PC complexes from *Schizosaccharomyces pombe* (Plocke & Kagi, 1992).

Figure 4-13 shows a typical circular dichroism spectrum of the 0.45 M NaCl fraction, with a strong maximum at 267 nm and a small minimum at 235 nm. This is

characteristic of Cd-thiolate coordination (Rupp & Weser, 1978). According to Rauser (1990), Cd-thiolate complexes show a positive cotton band at 275-280 nm and a negative one at 253-257 nm, with shifts in the maximum and minimum for different Cd-thiolate complexes. For typical proteins, there is no maximum above 240 nm. This result was in agreement with the studies from a Cd-binding MT (Pande et al., 1986), where the Cd-containing MT was compared with apo-MT in circular dichroism spectrum; it was found that the apo-MT had a negative shoulder at 222.5 nm and no positive bands, but the Cd-MT displayed positive and negative ellipticity bands between 210 and 300nm, originating from metal-ligand charge-transfer transition. A similar conclusion was also made by Li-Chan et al., 2002, who suggested that the high salt fractions of 0.45 and 0.50 M NaCl were probably metal-binding PCs; however, these fractions accounted for recovery of only a minor percentage of the Cd found in the original flaxseed.

Considering that over 50% of Cd was eluted in the 0.1 M NaCl fraction with about 10% of the proteins (Chapters 2 & 3), the 0.1 M NaCl fraction should contain the major Cd-binding components in flaxseed. Therefore, most of the current studies focused on this fraction. The studies of the UV/visible absorbance spectra from ion exchange chromatography (Figure 4-3) and size exclusion chromatography (Figure 4-4) revealed that the λ_{max} values for the 0.1 M NaCl and G50 Peak B were both near 276 nm, and both of these two fractions also had an additional smaller peak between 343 to 373 nm. These unique absorbance spectra of Cd-binding fractions of 0.1 M NaCl and Peak B may be an indication that these two fractions contained phenolic compounds. Phenolic compounds exhibit two major absorption bands in the UV/visible region: a first band in the range between 320 and 380 nm and a second band in the 250 to 285 nm range (Matthaus, 2002).

However, this is not likely to be true based on the discussion on phenolics in Peak B in Section 2.4. According to amino acid analysis (Section 4.3.8), more than 90% of Peak B was composed of unknown components; these components may exhibit special characteristic absorbance.

The studies from MALDI-TOF MS spectra of the major Cd-binding fraction, eluted from ion exchange chromatography at 0.1 M NaCl, and the major protein fraction, eluted at 0.25 M NaCl, indicated that these two fractions were different. The mass spectrum of the 0.25 M NaCl fraction (Figure 4-8) showed a predominant signal at 40,519.14 Da, and other significant signals at 11,597.63, 17,071.44, 20,179,11, 42,018.54, and 51,169.33 Da over the acquisition mass range of 10 to 300 kDa. According to previous research (Chung et al., 2005), the molecular weight of the major storage protein in the 0.25 M NaCl fraction was determined by size exclusion chromatography to be 365 kDa; reducing and non-reducing SDS-PAGE revealed 3 predominant bands at 20, 23 and 31 kDa and 2 predominant bands at 40 and 48 kDa, respectively, as well as several minor bands.

The mass spectrum of the major Cd-binding fraction eluted at 0.1 M NaCl from ion exchange chromatography had two predominant ion signals at 4,782.04 and 14,145.93 Da (or m/z) (Figure 4-9), and a small peak observed at 28,391.13 Da, which could be a dimer of the 14,145.93 Da component (28,391.13/14,145.93 = 2.0), and the latter could be in turn a trimer of the 4,782.04 Da component (14145.93/4782.04 = 3.0). These results agree with G50 size exclusion chromatography of the 0.1 M NaCl fraction, which revealed that the major protein peak eluted around the 14 kDa position (Section 2.3.2.2). Leopold et al. (1999) reported a high molecular weight Cd-PC complex

estimated to be 13 kDa by ICP-MS analysis. According to the review of Prasad (1995), two major groups of Cd-binding complexes have been isolated from different higher plants/cultures *in vitro*: they are 8 to 14 kDa complexes similar to those of metallothioneins in some aspects and the low molecular weight PCs, $(\gamma\text{-Glu-Cys})_n\text{Gly}$, with n usually 2 to 5 and sometimes up to 11 (Cobbett, 2000).

The major Cd-binding fraction eluted with 0.1 M NaCl was further separated into three peaks (HMW peak, Peak A and Peak B). The HPLC/MS analysis indicated that the major component in HMW peak had MW at 18,652.0 Da (Figure 4-5b), while the base peak of the major component in Peak A eluted at 37.67 minutes was 2,943.0 Da as shown in Figure 4-6a, and the second major component in Peak A was 17,542.0 Da, which could be the hexamer of the component of 2,943.0 Da (17,542.0/2,943.0 = 6.0). Peak B had major components at 1,721.1 and 666.4 Da (Figure 4-7a & 4-7c). These results were consistent with the observations from G50 size exclusion chromatography, which had a fractionation range between 30,000 and 1,500 Da (Figure 2-6), in which the HMW peak was eluted at the void volume V_o and Peak A was eluted before V_t.

MALDI-TOF MS has a higher mass range than other mass spectrometry techniques and, unlike HPLC/MS, produces mostly singly charged ions (Shoji et al., 2006). One feature of this technique is its ability to detect biomolecules in complex mixtures in the presence of larger molar excesses of salts and buffers. Another feature of MALDI-TOF MS is that it exhibits good detection limits providing the identification of low femtomole quantities of proteins (Garcia et al., 2006; Yang & Chien, 2000). The predominant component in peak A was at 4,790.20 Da (Figure 4-10), while the mass spectrum for the HMW showed three predominant peaks at 2,739.51, 2,965.23, and

3,399.75 Da (Figure 4-11), and the major components for peak B were at 649.66, 655.66, and 860.52 Da (Figure 4-12).

Comparing the results from the two mass spectrometry techniques, MALDI-TOF MS indicated that the major component in Peak A was 4,790.2 Da (Figure 4-10), while HPLC/MS revealed that the major component in Peak A was 2,943.0 Da with a hexamer at 1,7542.0 Da shown in Figure 4-6a. It was also noticed that another peak at 14,706.0 Da could be a pentamer of the 2,943.0 Da component (14706.0/2943.0 = 5.0), and this pentamer could also be the trimer of the 4,897.0 Da component (14,706.0/4,897.0 = 3.0) shown in Figure 4-6a. This implied that the dominant component in Peak A could probably be some protein with MW around 14 kDa before electrospray ionization, as demonstrated by the result from G50 size exclusion chromatography of Peak A (Section 2.3.1.3 of Chapter 2)

Similar analysis revealed complementary results from HPLC/MS and MALDI-TOF MS for HMW peak and Peak B. For the HMW peak, HPLC/MS gave one peak at 18,652 Da (Figure 4-5b), while MALDI-TOF MS yielded three major peaks at 2,739.51, 2,965.23, and 3,399.75 Da (Figure 4-11), which could be components for 18,652 Da (2,739.51 + 2,965.23+ 3,399.75 = 18,209). For Peak B, the HPLC/MS gave two major components at 1,721.1 Da (Figure 4-7a) and 666.4 Da (Figure 4-7c), and the MALDI-TOF MS gave 646.66, 655.66, and 860.52 Da (Figure 4-12), while the latter component could be the subunit of the 1,721.1 Da (860.52 x 2 = 1,721.04).

From the above analyses of HPLC/MS, MALDI-TOF MS, UV/Vis and circular dichroism spectra, and the result from size exclusion chromatography, it was understood that the three fractions eluted by 0.1, 0.25 and 0.45 M NaCl from the ion exchange

chromatographic column were different in composition. The MW of the major proteins in the 0.1 and 0.25 M NaCl fractions were 14 and 40 kDa, respectively, while the high salt (0.45 M NaCl) eluted peak was a non-protein Cd binding fraction. The MW of the major components of the G-50 separated HMW peak and Peak A were 19 and 14 kDa, respectively, while the major Cd-binding factors in Peak B were unidentified components with molecular weight at 0.6 to 0.9 kDa.

4.4.2 Elucidation of the Characteristics of Major Cd-Binding Components

After extensive comparison and research, phytic acid (660.04 Da), SDG (secoisolariciresinol diglycoside, 686.71 Da), and other organic acids such as α-linolenic acid (278.43 Da), and citric acid (192.12 Da) were all excluded as possible candidates that could be tentatively assigned to the major components in Peak B as identified by MALDI-TOF MS, because of apparent mismatch in molecular mass (Table 4-3). Phytic acid was also excluded as a component in the major Cd-binding fraction based on Raman spectral data from Dr. Eunice Li-Chan's research lab (data not shown here). In the spectrum of MALDI-TOF MS (Figure 4-12), the peak at 649.66 Da could, however, be assigned as Cd binding PC Cd-(γ-Glu-Cys)$_2$Gly, or Cd-PC$_2$, which has calculated molecular weight of 649.96 (mass error < 0.05%). Another peak at 537.96 m/z could be the apo-PC: (γ-Glu-Cys)$_2$Gly (Figure 4-12), which has a calculated molecular weight of 539.55 (mass error < 0.3%) (Table 4-3). The Cd induced peptide in maize with a peak at 538 Da by mass spectrometry (Meuwly et al., 1995) was also interpreted as (γ-Glu-Cys)$_2$Gly. It should be stressed that Cd is by far the most potent inducer of PC synthesis. Cd^{2+} has been found to be 6 times stronger as a PC inducer than Cu^{2+} in *R. serpentina* cell

culture, and 40 times stronger than Cu^{2+} in *S. pombe* (Kotrba et al., 1999). Potentiometric titrations indicate that PC_2 forms very stable complexes with Cd^{2+} ion over a wide pH range (Dorcak & Krezel, 2003).

The peaks at 655.66 and 860.52 Da have not been assigned and could be polypeptides with unusual amino acids or other unknown compounds, since amino acid analysis revealed three unidentified peaks eluting after Tyr and Met (Table 4-2 & Figure 4-18). In addition to the usual amino acids, several unknown or unidentified peaks in the chromatographic profiles during amino acid analysis were distinctive features of the Peak A and Peak B fractions (Figure 4-18). Both Peak A and Peak B had an unknown peak at the position of 6.3 minutes, referred to as unknown peak 2 in Table 4-2, which constituted 8.7 and 82.3% of the total peak area in the amino acid chromatogram of Peak A and Peak B, respectively. Peak B also had two other unknown peaks (unknown peaks 1 and 3 in Table 4-2) at 5.9 and 8.3 minutes, comprising 3.1 and 7.9% respectively of the total peak area. The unknown peaks were not included in the calculation of the percentage of the amino acid residues, but they were in fact major constituents, particularly of the Peak B fraction. In Peak B, considering the high levels of the three unknown peaks (93.3% of total peak area), especially the unknown peak at 6.3 minutes (82.3%), the composition of amino acids shown in Table 4-2 does not represent the actual total composition of Peak B. In view of the complexity of Cd-binding components reported to exist in different organisms (Rauser, 1999; Stone & Overnell, 1985), Peak B may contain some unusual amino acids or other organic acids that can effectively bind Cd. The characterization of these unknown peaks will be the subject of continuing study.

PCs have been observed to be aggregated to different complexes in solution (Prasad, 1995; Cobbett, 2000), such as the 8 to 14 kDa complexes. A pure preparation of Cd-PC$_2$ (MW 649.96) that was loaded to a size exclusion chromatographic column was reported to elute at a position corresponding to molecular weight of 3.4±0.5 kDa (Vacchina et al., 1999). Grill et al (1985, 1987) demonstrated that the two Cd-binding PCs with apparent molecular weight of 4 and 1.8 kDa, consisted of (γ-Glu-Cys)$_2$-Gly and (γ-Glu-Cys)$_3$-Gly. Based on these reports, it was speculated that the predominant peak at 4,790.20 Da of Peak A (Figure 4-10) could be an aggregate of (γ-Glu-Cys)$_2$-Gly, which is the major component in peak B (Figure 4-12); it was also speculated that the 14 kDa component in 0.1 M NaCl fraction (Figure 4-9) could be the further aggregate of the component from peak A. Mass spectra of the 0.1 M NaCl fraction and peak A showed a mass difference of 8 between 4,782.04 Da for the 0.1 M NaCl fraction and 4,790.2 Da for peak A, which could be the result of protonation at different pH. The 0.1 M NaCl fraction had a pH of 8.6 and peak A had a pH of 7; more H$^+$ would be lost at basic pH. A fully protonated PC$_2$ molecule undergoes two reversible proton dissociation steps in fairly well separated pH ranges. The protons of the three carboxyl groups dissociate in the acidic pH range while the protons of ammonium and two sulphhydryl groups dissociate in the basic pH region (Dorcak & Krezel, 2003). It was therefore postulated that the 4.7 kDa components in peak A and 0.1 M NaCl fraction were composed of 8 (γ-Glu-Cys)$_2$-Gly units and 4 Cd atoms, with a calculated molecular weight for this Cd$_4$-PC$_8$ combination of 4,766 Da. Figure 4-19 shows the possible three-dimensional structure by a computerized model.

If the 14 kDa peptide was indeed the aggregate of Cd-PC$_2$, the Cys content should be higher than 15% (Rauser 1990) and the UV spectra of Peak A and Peak B should be similar. However, the results of amino acid analysis and UV absorbance characteristics did not support the above speculation. In fact, amino acid analysis (Table 4-2) indicated that the Cys content was 7.1% for the highest Cys fraction of Peak A. Furthermore the UV absorbance characteristics of the 0.1 M NaCl and Peak A fractions were quite similar, but those of HMW peak, Peak A and Peak B were quite different from each other (Figure 4-1 & 4-2). These results suggest that while the major component of 14 kDa in the 0.1 M NaCl fraction could be eluted into Peak A, the Cd-binding factors in Peak A and B should be different. The Cd-binding component of the 14 kDa peptide in the 0.1 M NaCl fraction therefore does not appear to be a MT or a PC, but a novel Cd-binding protein eluting in Peak A after size exclusion chromatography, which along with the Cd-PC$_2$ and other unknown components in Peak B, bound over 50% of the Cd in flaxseed.

Small metal binding peptides of 2 to 4 kDa behave like entities of MW 10 to 13.8 kDa during size exclusion chromatography (Rauser, 1990). This could explain our results from MS analysis, where 2.9 and 4.8 kDa peptides were determined in Peak A, while in the size exclusion chromatography, the apparent molecular weight was 14 kDa. Studies using marine algae showed that a 14 kDa polypeptide was eluted from G75 size exclusion chromatographic column loaded with Cd treated cell extract, and this was the predominant Cd-binding protein that did not exist in the control cells (Garcia & Salazar, 2003). Three Cd-binding protein bands were observed in the gills of Cd-exposed mussels corresponding to molecular weights of 14, 11, and 9.5 kDa (Geret & Cosson, 2002; Pavicic et al., 1991; Roesijadi et al., 1989). Cd in oyster and rat organs was principally

bound to proteins of 9.2 and 13.8 kDa (Casterline & Yip, 1975). A Cd binding protein was purified from *Hansenula anomala*, the MW was approximately 33 kDa, which was composed of two subunits having a MW of 18 and 14 kDa, respectively (Yu et al., 1990).

Because the 0.1 M NaCl peak was the major Cd-binding fraction, the identified 14 kDa component is probably the major protein component responsible for binding the 21% of the eluted Cd from size exclusion chromatography of NorMan flaxseed (Section 2.3.1.3). MT or PC are usually Cys-rich protein or peptides. The total SH and total SH + SS content of the 0.1 M NaCl fraction was measured along with other fractions to make comparison (Table 4-1). The 0.1 M NaCl fraction did contain much higher total SH + SS content, but the further separated fractions of HMW peak, Peak A and Peak B did not show corresponding high values. For a more sensitive and accurate analysis of the Cys content, each fraction of the three peaks was subjected to amino acid analysis (Table 4-2 and Figure 4-18). The Cys content was 1.7, 7.1 and 5.0% for HMW peak, Peak A and Peak B, respectively. The most abundant amino acids in the three samples were Glx (Glu + Gln) and Gly, and these samples also contained aromatic amino acids.

The results from this thesis study on Cd-binding components in flaxseed are similar to other reported studies on Cd-binding components. Jemal et al. (1998) studied Cd-binding components in pepper, which showed that the pepper plants respond to Cd stress by the synthesis of PCs, desglycyl PCs, and two 10 kDa proteins, which were the major Cd-binding components in the highest Cd-binding fraction eluted from a G50 size exclusion chromatographic column. The amino acid analysis of these two 10 kDa proteins showed that the Cys content were 2.6 and 3%, and the most abundant amino acids in these two peaks were Glu + Gly, and they also contained aromatic amino acids.

In another study, a 14 kDa protein was isolated from rainbow trout and characterized as a non-MT Cd-binding protein containing aromatic amino acids and 3.5% Cys (Mullins et al., 1999). Study using Sephadex G-25 and SDS-PAGE found two Cd binding proteins with MW of 21 and 9.2 kDa, and amino acid analysis revealed that these proteins contained aromatic amino acids, and the most aboundant amino acid were Asx (Asp + Asn) and Glx (Glu + Gln), but only contained 1.9% cysteine (Yoshida, 1986).

The Cd-binding components in different organisms are very complex, and the ligands for Cd are found in various forms including proteins, peptides, amino acids and other organic acids (Rauser, 1999). Many of these components show distant relationship or no relation to the typical horse metallothioneins or PCs, and their cysteine contents vary widely. The cysteine contents in Peak A and Peak B in this study were much higher than the average value of 1.9% of cysteine in proteins (Nelson & Cox, 2000), but still very much lower than the average value of 30% reported for animal metallothioneins (Stone & Overnell, 1985). According to Rauser (1990), Cys, Glu and Gly account for 45-97 and 19-92% of the amino acids found in Cd-binding and Cu-binding PC complexes, respectively, with Cys ranges of 8 to 43 and 3 to 39%, respectively. Stone and Overnell (1985) reviewed a wide range of Cd-binding proteins from various organisms including animals, plants and microorganisms, and concluded that Cd can bind to proteins through thiol and non-thiol groups, and that the Cys contents in Cd-binding proteins varied from 0 to 34%. A glycoprotein characterized from mushroom *Agaricus macrosporus* contained no Cys, while Glu and Asp together accounted for 35% of the amino acids (Meisch et al., 1983). The Cd-binding protein from whelk was reported to contain aromatic amino acids and only 4 to 4.9% Cys (Dohi et al., 1983).

The result from HPLC/MS and MALDI-TOF MS indicated that the 0.1 M NaCl fraction contained two major components with molecular weights at 4.8 and 14.1 kDa, with the latter probably being a trimer of the former. The SDS-PAGE of the 0.1 M NaCl fraction had a very intense band estimated to be 31 kDa (Figure 4-16). Considering this result was obtained under non-reducing conditions, and that the 0.1 M NaCl fraction contained high SH+SS content, this 31 kDa component could be the combination of the 4.8 and 14.1 kDa components linked by disulfide bands. The existence of the disulfide bands was also indicated by the results from IEF of the 0.1 M NaCl fraction (Figure 4-17), where more bands were found in denatured condition than in native condition.

One of the distinctive characteristics of the Cd-binding proteins that has been reported is their thermal stability (D'Auria et al., 2001); it has been reported that the metal binding protein can withstand heating at $100^{\circ}C$ for a couple of minutes (Kubo et al., 2000; Kayano et al., 1990). The stability is attributed to the metal-SH link (Bragigand et al., 2003). The thermal stability can be measured by many different methods such as NMR spectroscopy or circular dichroism (D'Auria et al., 2001). However, a practical approach often used in food science to evaluate the thermal stability of a protein is to measure the solubility of protein at different temperatures. The major Cd-binding protein in the 0.1 M NaCl fraction in this study was more heat stable over a wide range of temperature and also was more stable in alkaline condition than proteins in the 0.25 M NaCl fraction (Section 4.3.5).

4.4.3 Protective Effects of Flaxseed Proteins

Recently, the preventive and therapeutic potential of flaxseed has received increased scientific attention from evidence of beneficial biological effects in reducing the risk of degenerative diseases. The result has been a proliferation of literature on flaxseed with emphasis on human nutrition and disease prevention (Oomah & Mazza, 2000; Oomah & Mazza, 1998). However, most of the research studies are focused on health benefits and disease preventive effects of flaxseed oil, polysaccharide and phenolic components. There are only a few research studies that have been directly related to flaxseed proteins, as reviewed by Oomah and Mazza (2000), and most of these experiments were investigating nutritional aspects. To our knowledge, there have been no reported research studies on the possible effects of flaxseed or flaxseed proteins on cell growth or toxicity.

In this study, THP-1 cells were selected to study the potential protective effects of flaxseed protein extracts and fractions on cytotoxicity induced by Cd or H_2O_2. Cells of the mononuclear phagocytic system (such as THP-1 cell line) are strategically located at portals of entry in humans and therefore may be particularly at risk for Cd exposure though contaminated air, food, and drinking water (Funkhouser et al., 1994).

Flaxseed protein extract showed strong growth promoting effect on THP-1 cells (Figure 4-21). This result is very important in that the protein extract may be used as an additive to the medium for the growth of cultured cells for research purpose. THP-1 cells are human acute monocytic leukemia cells, which can be used for studies on induction of differentiation; the cells were reported to produce lysozyme and to be phagocytic (Tsuchiya et al., 1980; 1982). The present research is the first report that a protein extract

can promote THP-1 cell growth, although growth promoting effects of externally added proteins on other cell lines have been reported. MT has been reported to stimulate the growth and survival of cultured neurons, and accordingly the use of MT as an active ingredient in neuronal therapy provides a novel method of treatment for a range of neuronally based disease states (West et al., 2005). Dialyzed serum albumin had a considerable growth-promoting effect on cultivated hamster cells (Nilausen, 1978). Mincheva and co-workers (1990) found that high concentration of crude colostral milk proteins had an inhibitory effect on human T lymphocyte cell growth while low concentration enhanced T cells growth. The studies from Saito et al. (1996) found apo-LF, Cu-LF and Zn-LF inhibited the growth of *E. coli* and *S. aureus*, but apo-LF, Cu-LF and Fe-LF promoted the growth of *Bifidobacterium* strains. Franek and Katinger (2002) used synthetic peptides in hydridoma cell cultures, and they observed an apparent growth-promoting effect. In their batch cultures, the viable cell density increased to 184% relative to control when 0.25% tetraglycine was introduced into the medium. Zou et al. (1991) reported dose dependent growth-promoting activity of egg white in promoting the proliferation of two kidney fibroblast cell lines (CV-1 and BHK-21) and a rat epithelial cell line (IEC-18); the optimal concentration was 10% (v/v) of egg white in the cell culture medium, and the cell growth increased to 120 - 140% compared to the control. Yoo et al (1997) found that bovine lactoferricin (Lfcin-B) induced apoptosis in THP-1 cells in dose- and time-dependent manner. In comparison, the research presented here showed that the addition of 0.056% (0.56 mg/mL) flaxseed protein extract increased the cell growth to 187% relative to the control, and the growth promotion was observed to be dependent on protein concentration to the tested level of 1.4 mg/mL.

The specific molecular mechanisms, by which Cd produce its cytotoxic effects, are not well understood. Most studies in this area have focused on identifying the mechanisms by which Cd kills cells. Results of those studies have shown that Cd^{2+} can disrupt a variety of intracellular processes that could lead to the metabolic derangement and death of cells (Prozialeck et al., 1993). The same studies also observed that ionic Cd (Cd^{2+}) selectively damages the junctions between LLC-PK1 cells. Exposure to micromole concentrations of $CdCl_2$ for 1-4 hours causes the cells to separate from each other without killing them. Liu et al. (1996) demonstrated by primary cell culture that cytotoxicity of $CdCl_2$ occurred at and above 129 μM following 4 h of exposure. Even though different cell lines were used, the experimental results of this paper are in agreement with others. Exposure to Cd at relatively high and low levels has been reported to cause necrosis and apoptosis, respectively, which suggests that the mode of cell death by Cd is dependent upon its exposure level (Satoh et al., 2003). It has also been reported that Cd markedly induced apoptosis in the rat tests at a dose of 5 μmol Cd/kg body weight while 10 and 20 μmol Cd/kg body weight caused more necrosis than apoptosis (Zhou et al., 1999). Lopez et al. (2003) demonstrated in neuron cell culture that Cd induces apoptosis at very low concentration (100 nM) and necrosis at the higher concentrations (10-100 μM).

According to the present research, there was no significant difference between the % relative absorbances (representing cell viability) of the treatments with Cd less than 40 μM and the control, but the treatment with Cd at 150 μM decreased the relative absorbance by 50% (Appendix 4). The cytotoxic actions of Cd were investigated using freshly isolated hepatocytes by Stacey et al (1980), who observed a dose- and time-

dependent increase in lipid peroxidation in isolated rat hepatocytes incubated for up to 75 minutes in 50 – 400 μM CdCl$_2$. Santone et al. (1982) also found a dose dependent effect of CdCl$_2$ toxicity on hepatocytes. They noted that in the cultures after 1 hour exposure to 50-400 μM of CdCl$_2$, little evidence of toxicity was observed as evaluated by total cellular protein content and cell viability. In their studies, cell viability was 64% at 400 μM Cd, while in the present study, only 16% relative absorbance was observed at 400 μM Cd. The THP-1 cells used in this study therefore appeared to be more sensitive to Cd toxicity than cultured hepatocytes. One of the reasons may be because the medium used for incubation of THP-1 cells with Cd contained no FBS. A previous study (Borenfreund & Puerner, 1986) indicated Cd incubated with cultures in medium containing 1% FBS was 3-4 times more toxic than in medium with 10% FBS. Nevertheless, the major reason for the observed difference between Cd-induced cytotoxicity in THP-1 cells and cultured hepatocytes could be the cancerous nature of the former cells. Research from Szuster-Ciesielska et al. (2000) and other previous studies (Koizumi & Sone, 1991; Mishima et al., 1995, Waalkes et al, 1996; Waalkes & Diwan, 1999) have shown that tumor cells, i.e. liver, lung tumor cells and HeLa cells, are hypersensitive to Cd. This heightened sensitivity may be due to poor expression of metallothionein genes, since it is well known that metallothioneins sequester Cd and thereby mitigate its toxicity.

The MCF had significant protective effect against Cd toxicity. This protein fraction is a high Cd-binding fraction from flaxseed. A 14 kDa protein along with some unknown components with MW of 0.6 to 0.9 kDa are the major components in MCF responsible for high Cd binding ability. Metallothioneins and phytochelations sequester Cd intracellularly and thereby act as a detoxifying agent. A previous study has shown that

174

primary cultures of rat renal epithelial cells were more sensitive to Cd-MT toxicity than to Cd^{2+} (Cherian, 1985). However, other researchers have reported that Cd is very toxic to cultured cells while Cd-MT is not, at least during the first 24-hour incubation period (Prozialeck et al., 1993). The prolonged incubation time needed for Cd-MT to cause toxicity is because the MT has to be taken up by cells, and Cd bound to the MT can be released intracellularly through the actions of lysomal enzymes (Prozialeck et al., 1993). In the present study, MCF protected cells from Cd toxicity, even though MCF itself carried more Cd than other fractions. One of the possible explanations is that MCF sequestered Cd in the culture to protect cells from absorbing Cd and thus preventing cells from Cd toxicity. Cambar et al. (1998) found that very high dilution of Cd solution can induce MT formation in the cells and therefore render the cultured cells insensitive to Cd toxicity. This can be another explanation to the protective effect of MCF, if the added MCF was taken up by the cultured cells.

High levels of H_2O_2 are cytotoxic to a wide range of animal, plant and bacterial cells, although LD_{50} values and the mode of cell death depend on multiple parameters, including cell type, its iron content, duration of exposure to H_2O_2, the H_2O_2 concentration, and the cell culture media (Halliwell et al., 2000). H_2O_2 can inflict cellular injury at multiple sites either directly or through the generation of more reactive intermediates. It is also capable of inactivating critical enzymes directly by oxidation of thiol groups at the active site of the enzyme (Salahudeen et al., 1991). Recently, Matthaus (2002) reported that the extract from defatted residue of flaxseed showed strong antioxidant activity measured by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method, β-carotene bleaching method and electron spin resonance, but the

active component was not identified. The present study indicates that MCF also protected against cytotoxicity induced by H_2O_2; the possible mechanism for this protective effect may be attributed to the thiol rich nature of this MCF fraction (Lei et al., 2003). The toxic effect of H_2O_2 could be reduced after the oxidation of sulphydryl (-SH) groups contained within a cysteine of the protein structure. Similar to Cd toxicity, the protective effect of flaxseed protein against H_2O_2 toxicity should be further studied in order to clarify the real mechanism under this effect.

4.5   CONCLUSIONS

This study provided evidence to support previous speculations on the multiplicity of Cd-binding factors in flaxseed (Lei et al., 2003; Li-Chan et al., 2002), The comparative studies employing the techniques from spectroscopic, mass, and amino acid analyses demonstrated that a 14 kDa protein and the Cd-PC_2 along with some unknown amino acids or organic acids with MW in the range of 0.6 to 0.9 kDa could be the major Cd binding factors in flaxseed responsible for binding over 50% of the Cd in the protein extract, or over 40% of the Cd in the original seeds. Considering that over 70% of Cd was found in Peak B, and also considering that the unknown peaks observed in amino acid analysis accounted for over 90% of the fraction of Peak B, the most important Cd-binding components in flaxseed could be the unknown amino acids or organic acids eluted in Peak B by size exclusion chromatography of the 0.1 M NaCl fraction from ion exchange chromatography. Since Peak A contained about 20% of Cd, the 14 kDa protein in Peak A could also be considered as one of the important Cd-binding factors in flaxseed. Studies from the UV/Vis and circular dichroism spectra also indicated that the

fraction eluted at 0.45 M NaCl contained Cd-thiolate coordination, but this high salt eluted fraction only contributes a minor role in terms of total amount of bound Cd.

This study also demonstrated that the proteins extracted (PE) from flaxseed defatted powder exhibited a dose-dependent promotion of the THP-1 cell growth. Furthermore, the major Cd binding fraction (MCF) isolated by ion exchange chromatography of PE was shown to protect THP-1 cells from the toxicity induced by Cd and the oxidative stress of H_2O_2.

4.6   ACKNOWLEDGMENTS

4.7   TABLES AND FIGURES

Table 4-1. SH and SS contents (μmol/g protein)[a] of the fractions from ion exchange and G50 size exclusion chromatography of NorMan flaxseed

Samples	Total SH	Total SH + SS
Protein extract	9.23	82.8
Protein extract[b]	NA[c]	124
0.25 M NaCl fraction[b]	NA	64.8
0.1 M NaCl fraction	60.5	1104[d]
HMW peak	NA	15.6
Peak A	0.9	38.8
Peak B	66.3	272

[a] The values shown are the average of triplicate measurements.

[b] data from Chung, 2001.

[c] NA: not analyzed.

[d] average of the data from two separate experiments (1037 and 1170 μmol/g protein), each determined in triplicate.

Table 4-2. Amino acid composition (g/100 g total amino acids) of the fractions from G50 size exclusion chromatography of NorMan flaxseed

	HMW peak	Peak A	Peak B
Asx (Asp + Asn)	9.5	5.8	16.3
Glx (Glu + Gln)	14.2	22.4	30.7
Ser	6.1	5.3	5.2
Gly	12.4	16.3	17.0
His	1.6	1.6	0.7
Arg	5.6	7.6	2.3
Thr	5.4	3.7	2.2
Ala	9.8	5.2	1.9
Pro	5.1	2.8	3.9
Tyr	1.9	1.6	2.1
Unknown Peak 1	-	-	$(3.1)^b$
Unknown Peak 2	-	$(8.7)^b$	$(82.3)^b$
Val	6.7	4.1	2.4
Met	1.9	1.4	0.5
Unknown Peak 3	-	-	$(7.9)^b$
Cys[a]	1.7	7.1	5.0
Ile	3.6	2.8	3.6
Leu	6.2	5.4	3.1
Phe	2.4	2.0	1.4
Lys	5.9	4.9	1.7

[a] Determined as cysteic acid after performic acid oxidation.
[b] The data for the three unknown peaks are the area of each peak expressed as a percentage of the total peak area of all the amino acids plus the three unknown peaks.

179

Table 4-3. Molar masses of the possible candidate components of Peak B

Chemical entity	Molar mass			
	X	n = 2	n = 3	n = 4
	--	482.50	714.74	946.98
	Gly	539.55	771.79	1004.03
$(\gamma\text{-Glu-Cys})_n X$	β-Ala	553.57	785.81	1018.05
	γ-Glu	611.61	843.85	1076.09
	Gln	610.61	842.85	1075.09
	Ser	569.57	801.81	1034.50
Atom	Molar mass			
Cd	112.41			
Cu	63.55			
Zn	65.41			
Ca	40.08			

Table 4-4. Effects of flaxseed protein extracts and chromatographic fractions on hydrogen peroxide induced toxicity in THP-1 cells[a]

Sample	mg/mL[b]	Relative absorbance %[c]	Cell numbers x 10^6	Enhancement efficiency index[d]
Trolox (negative control)	2.5	100	2.2	
FM extract	1.5	52	1.3	35
FP extract	1.4	-8	0.1	-6
NorMan extract	1.4	95	2.1	68
NorMan 0.25M[e]	0.11	2	0.3	22
NorMan 0.1M[e]	0.04	35	1.0	872
NorMan HMWpk[f]	0.005	46	1.2	9231
NorMan peak A[f]	0.15	29	0.8	196
NorMan peak B[f]	0.02	27	0.8	1332
H_2O_2 (positive control)	0.06%	0	0.3	

[a] The oxidative stress from H_2O_2 (0.06%) was tested using THP-1 cell culture with addition of different flaxseed protein fractions. The cell count was measured by the method described in Section 4.2.2.11.

[b] mg/mL was the final concentration in the cell culture

[c] Relative absorbance % = (absorbance of treatment – absorbance of H_2O_2 treatment) / (absorbance of Trolox treatment – absorbance of H_2O_2 treatment) x 100

[d] Enhancement efficiency index: was the protection effect based on unit protein (relative absorbance/protein in mg/mL).

[e] NorMan 0.25M & NorMan 0.1M: the 0.25M and 0.1M NaCl eluted fractions from ion exchange chromatography loaded with protein extract from flaxseed cultivar NorMan.

[f] MorMan peak HMWpk, A, B: the peaks eluted from size exclusion chromatography loaded with 0.1M NaCl fraction from ion exchange chromatography.
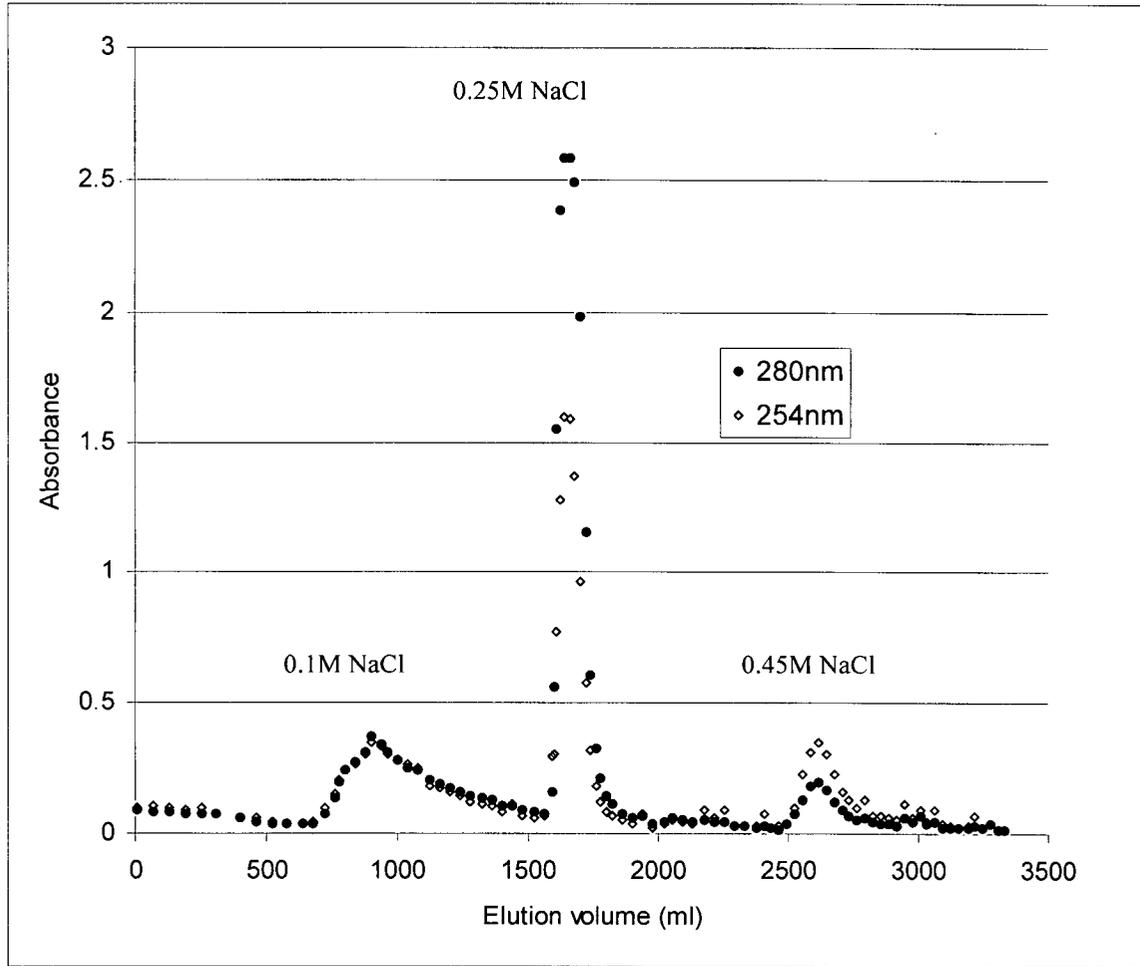
**Figure 4-1. DEAE sephacel ion exchange chromatography of protein extract from NorMan flaxseed.** Fractions were eluted by stepwise gradient with 0.1 M, 0.25 M, and 0.45 M NaCl and monitored by absorbance at 254 nm and 280 nm.
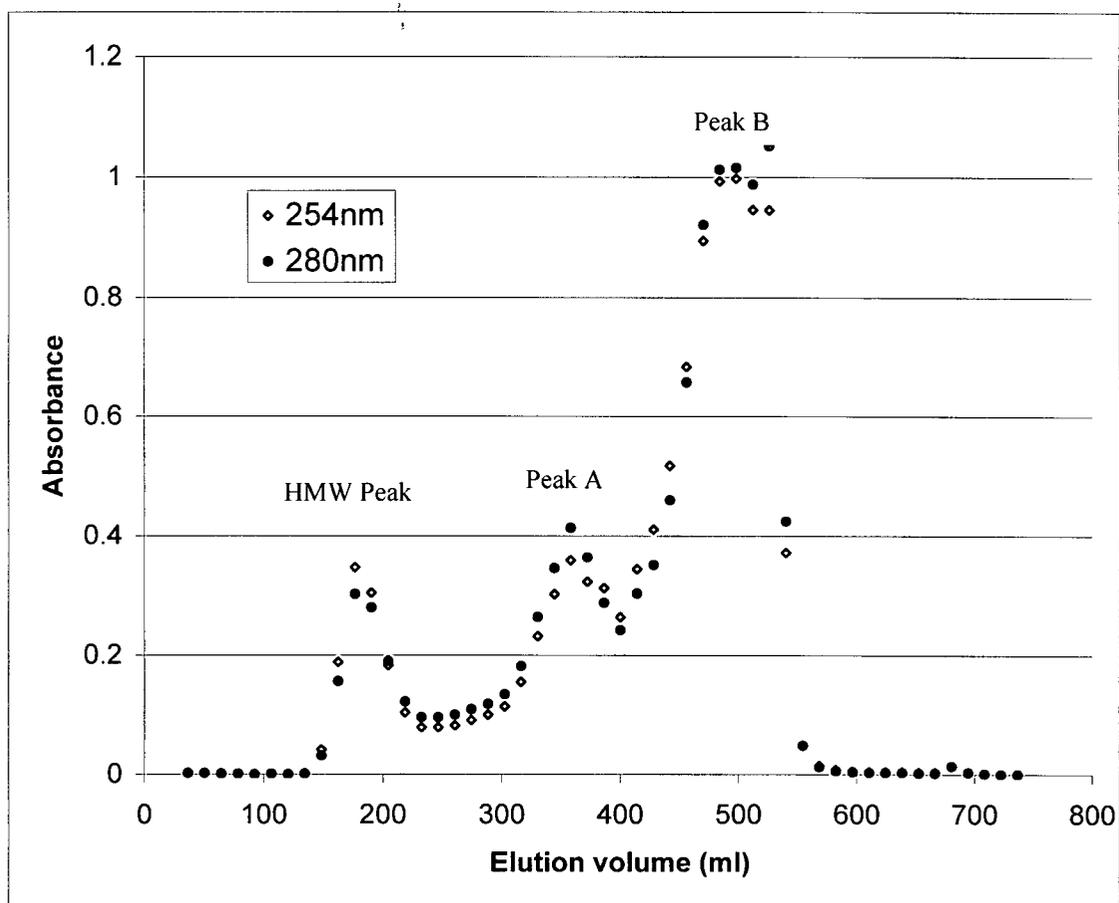
Figure 4-2. Size exclusion chromatography of the 0.1M NaCl fraction from Figure 4-1. Fractions were eluted in deionized and distilled water and monitored by absorbance at 254 nm and 280 nm.
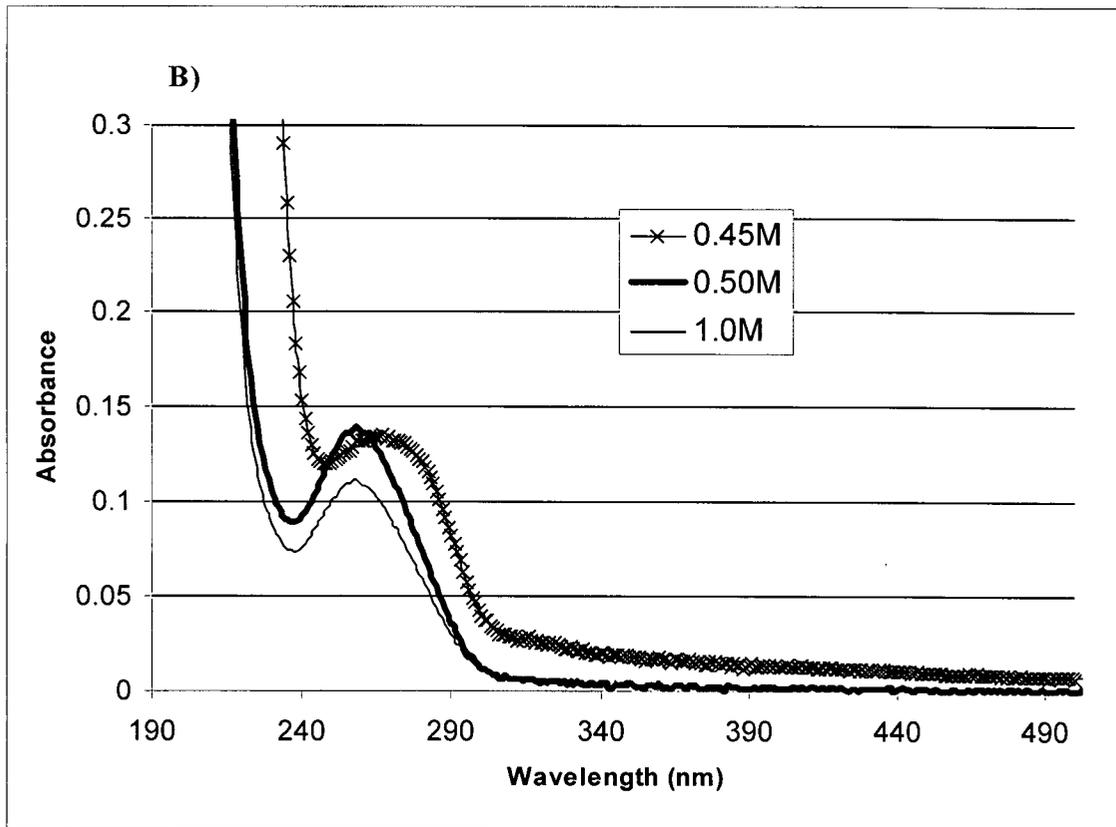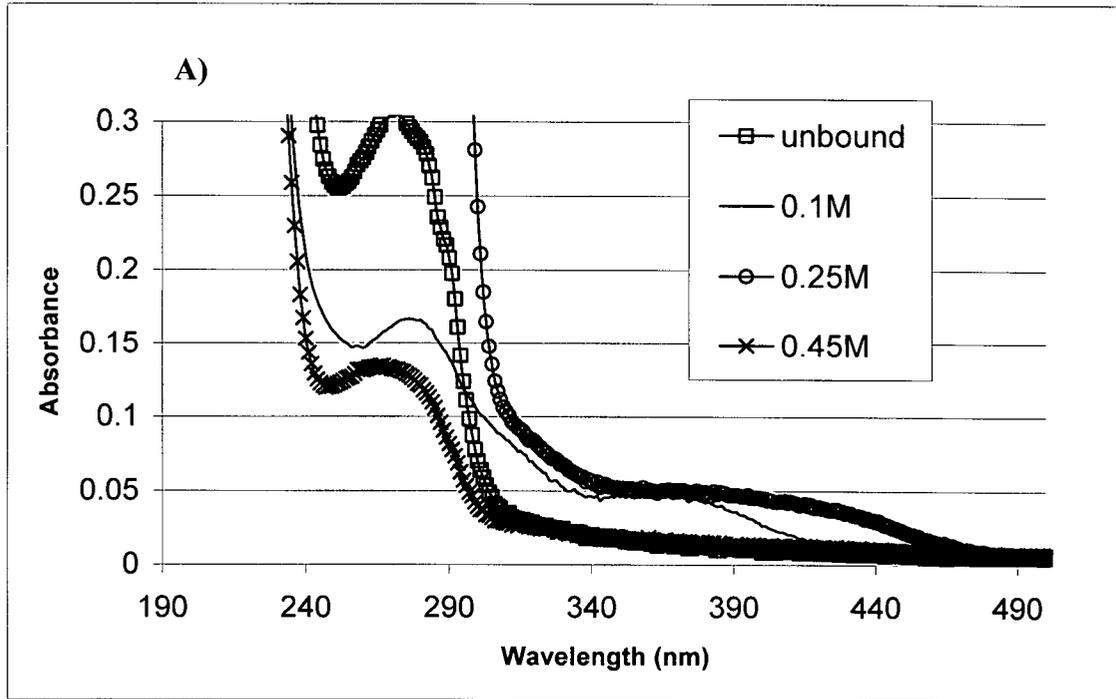
Figure 4-3. Typical UV/Vis absorbance spectra of each fraction from ion exchange chromatography of NorMan flaxseed. A) shows fractions of unbound, 0.1 M NaCl, 0.25 M NaCl, and 0.45 M NaCl; B) shows fractions of 0.45 M NaCl, 0.50 M NaCl, and 1.0 M NaCl.
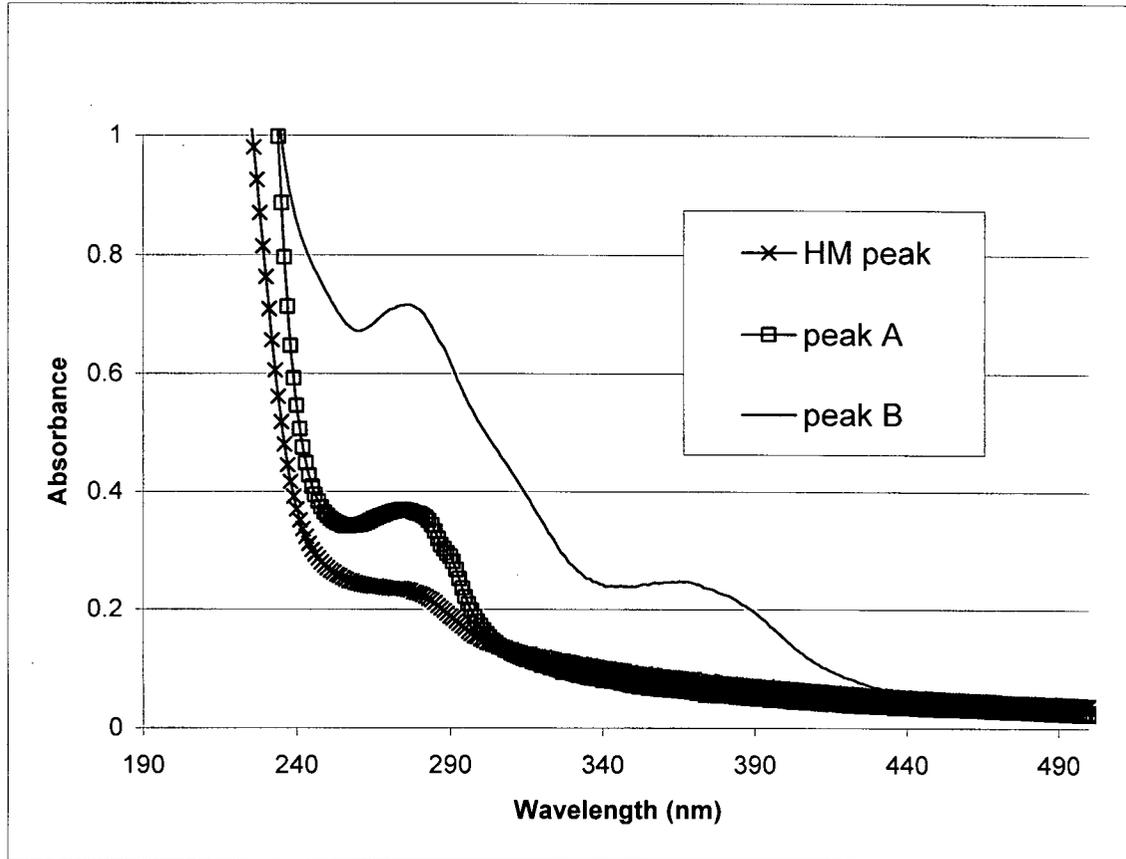
Figure 4-4. Typical UV/Vis absorbance spectra of fractions from size exclusion chromatography of NorMan flaxseed. The protein concentrations as determined by BCA method were 128, 375, and 148 μg/mL, respectively, for HMW peak, Peak A, and Peak B.
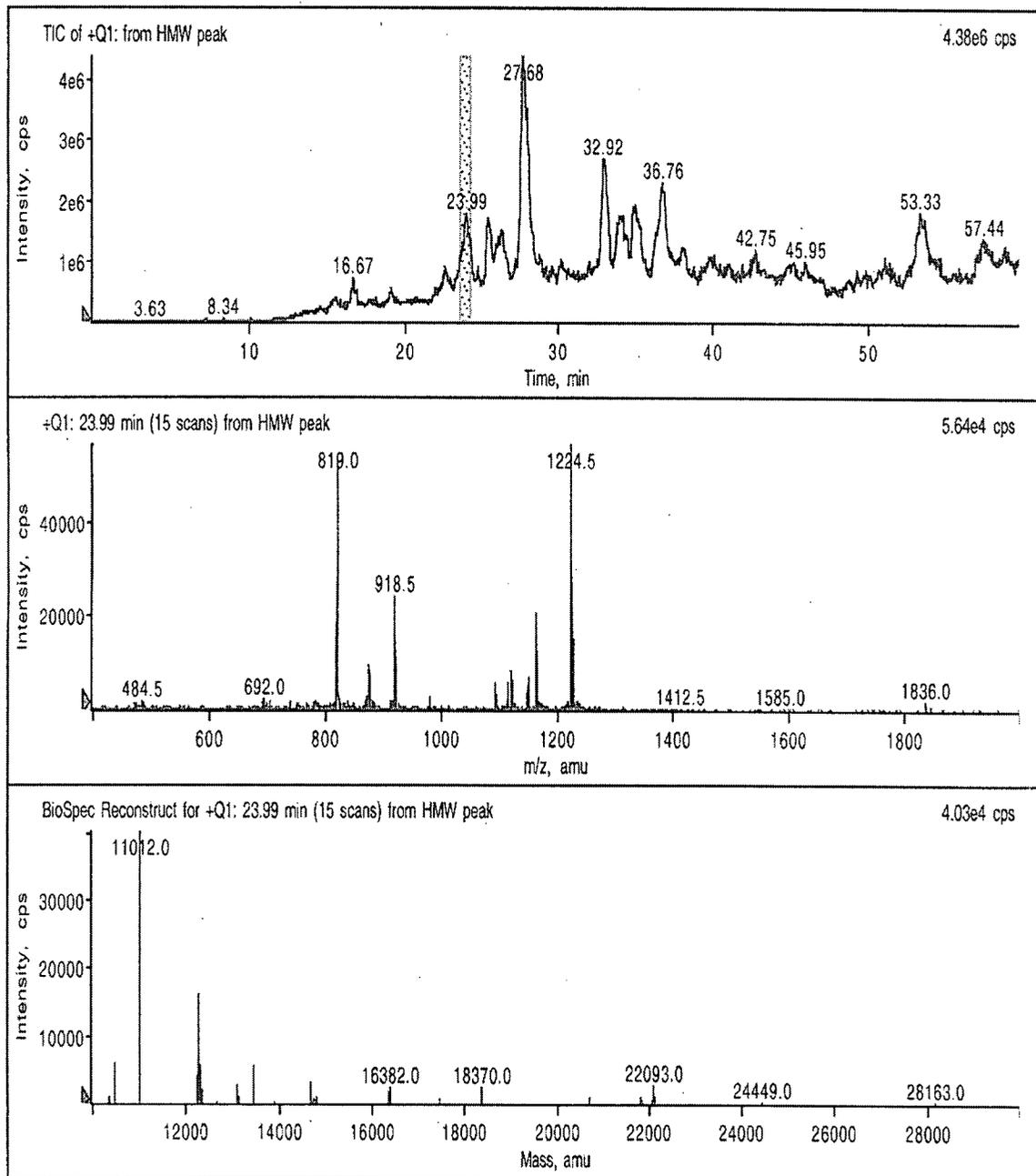
Figure 4-5a. HPLC/MS analysis result of HMW peak showing molecular weight reconstruction of the peak at 23.99 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the spectrum for molecular weight reconstruction. HMW peak was the first peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.

186
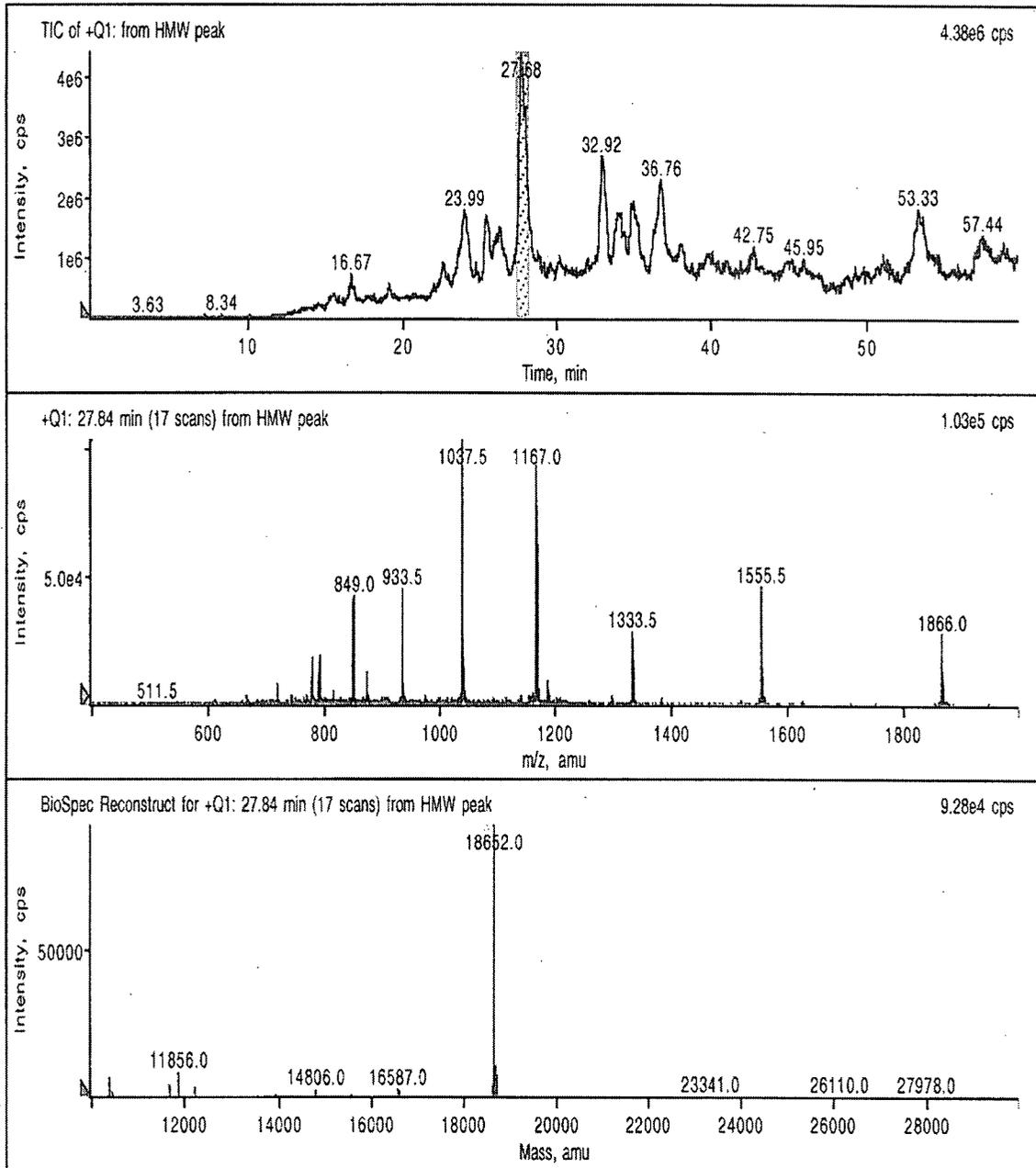
Figure 4-5b. HPLC/MS analysis result of HMW peak showing molecular weight reconstruction of the peak at 27.68 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the spectrum for molecular weight reconstruction. HMW peak was the first peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.

187
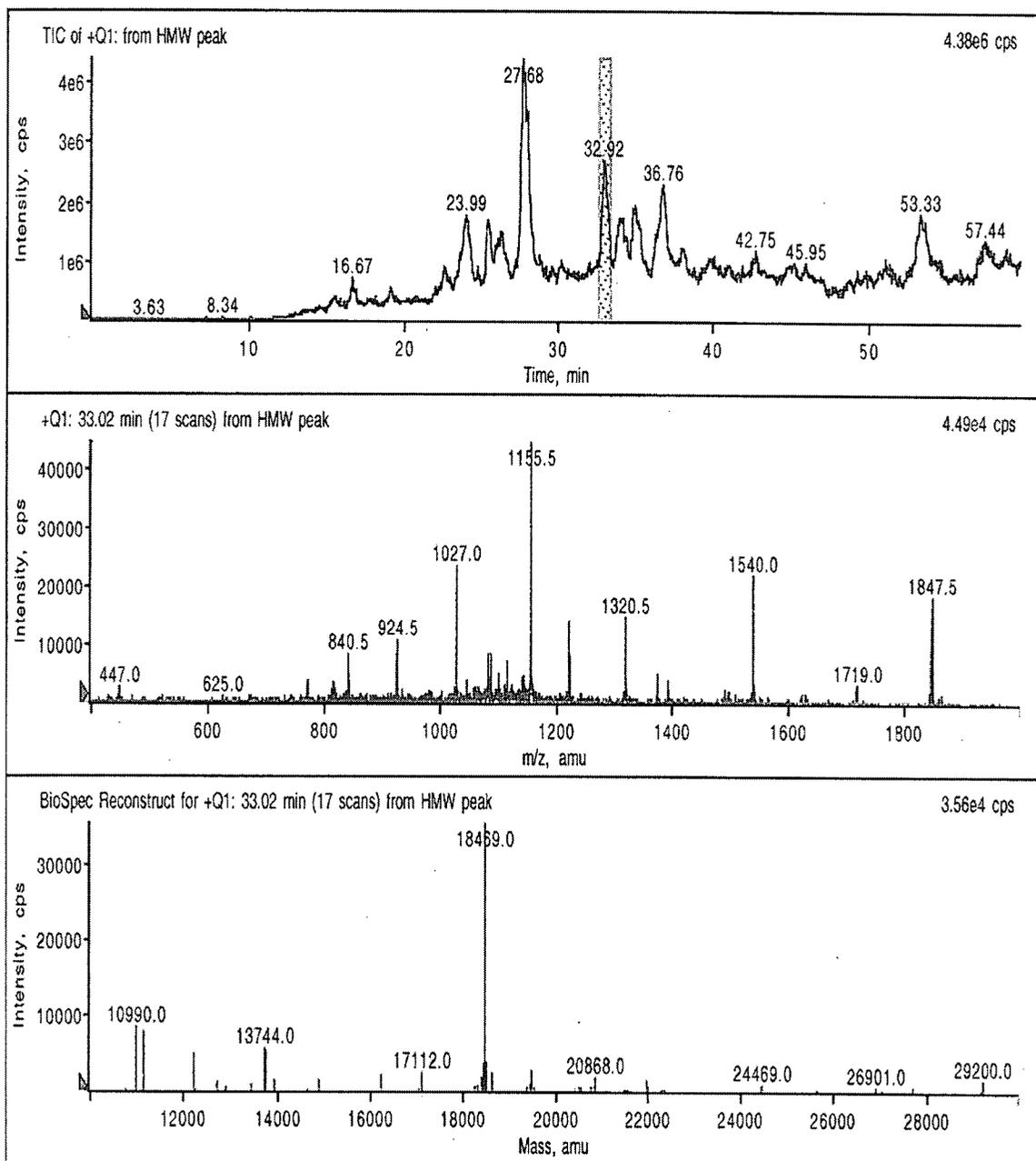
Figure 4-5c. HPLC/MS analysis result of HMW peak showing molecular weight reconstruction of the peak at 32.92 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the spectrum for molecular weight reconstruction. HMW peak was the first peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.
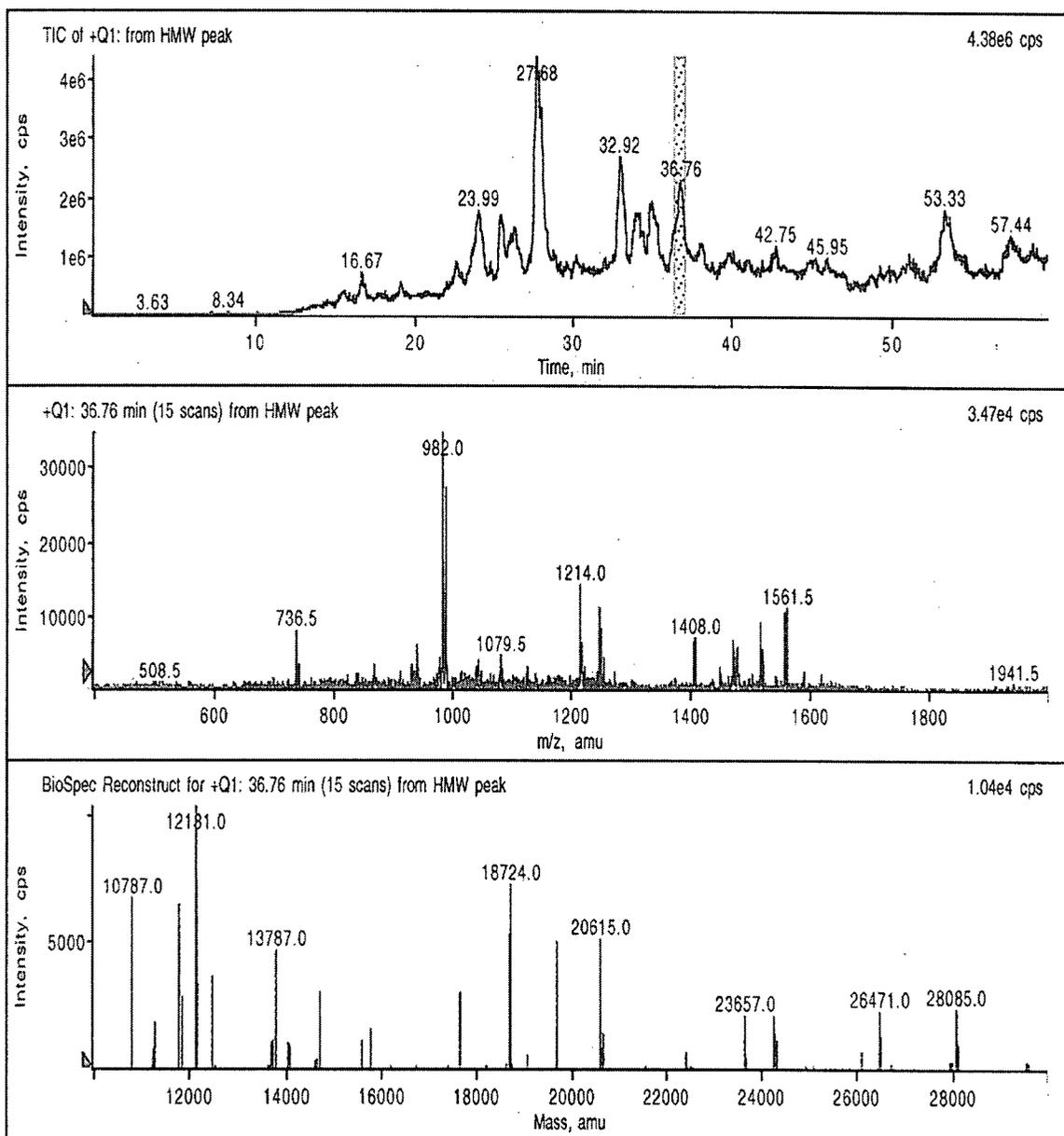
Figure 4-5d. HPLC/MS analysis result of HMW peak showing molecular weight reconstruction of the peak at 36.76 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the spectrum for molecular weight reconstruction. HMW peak was the first peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.
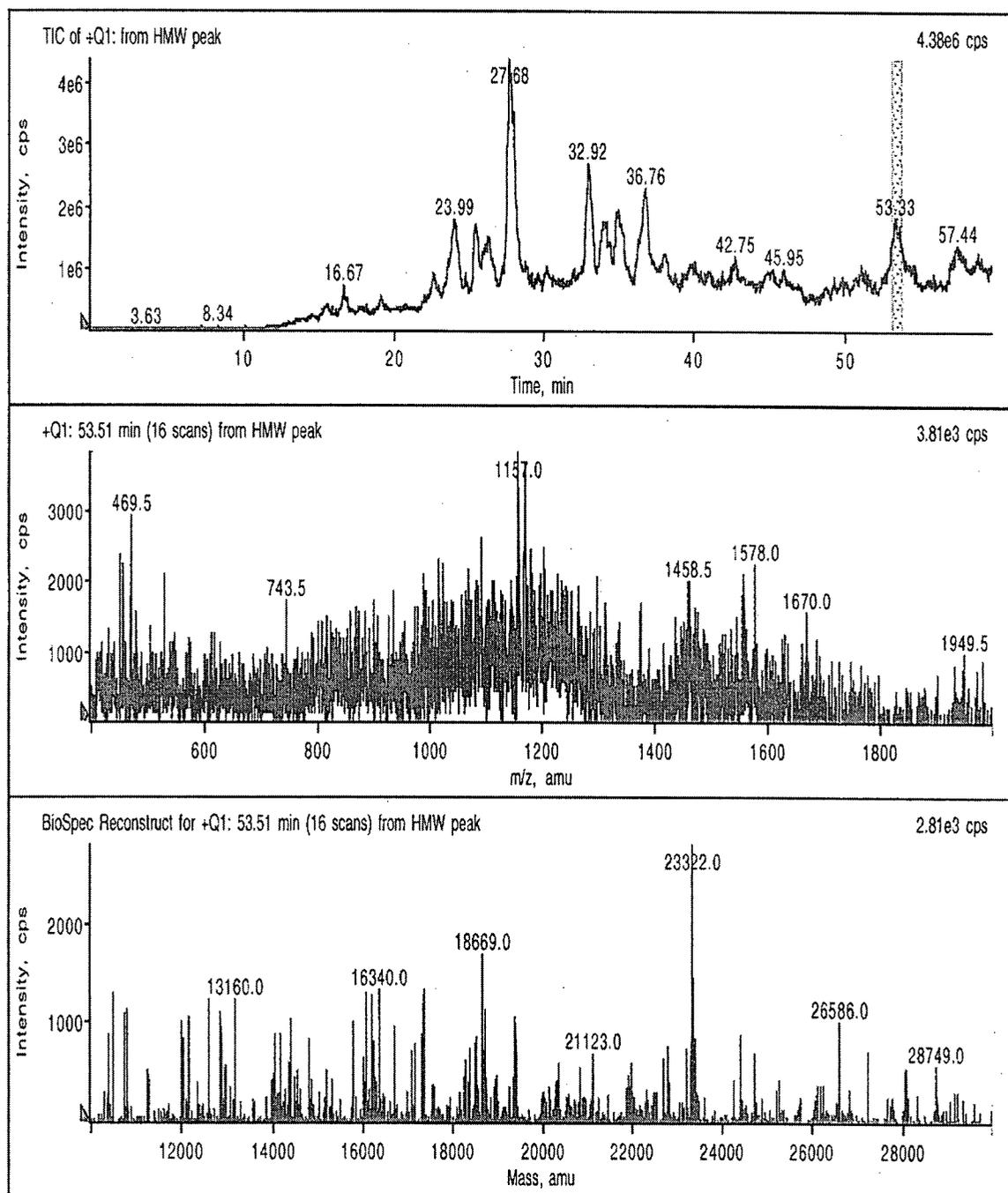
Figure 4-5e. HPLC/MS analysis result of HMW peak showing molecular weight reconstruction of the peak at 53.33 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the spectrum for molecular weight reconstruction. HMW peak was the first peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.

190
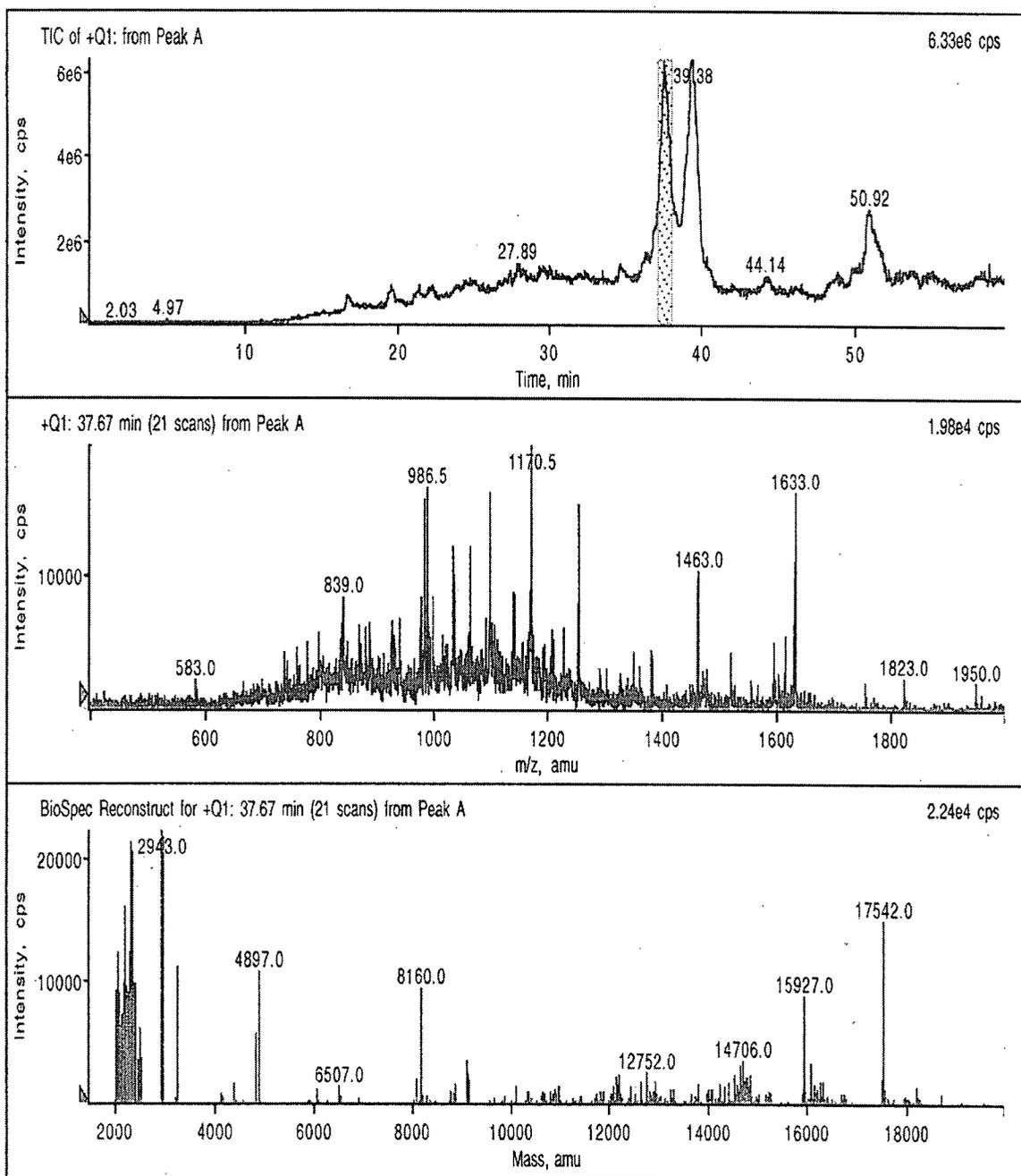
Figure 4-6a. HPLC/MS analysis result of Peak A showing molecular weight reconstruction of the peak at 37.67 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the spectrum for molecular weight reconstruction. Peak A was the second peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.
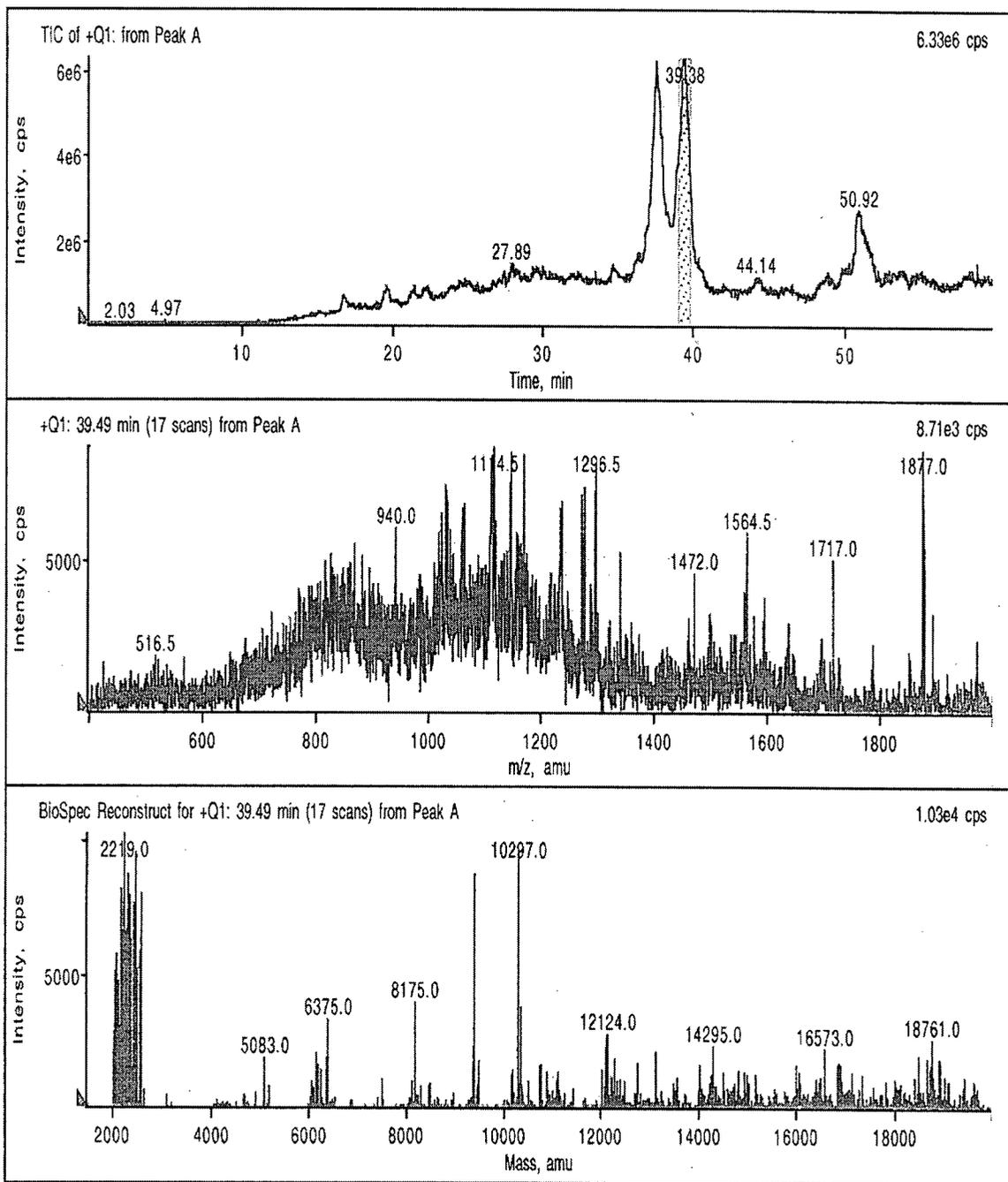
Figure 4-6b. HPLC/MS analysis result of Peak A showing molecular weight reconstruction of the peak at 39.38 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the spectrum for molecular weight reconstruction. Peak A was the second peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.
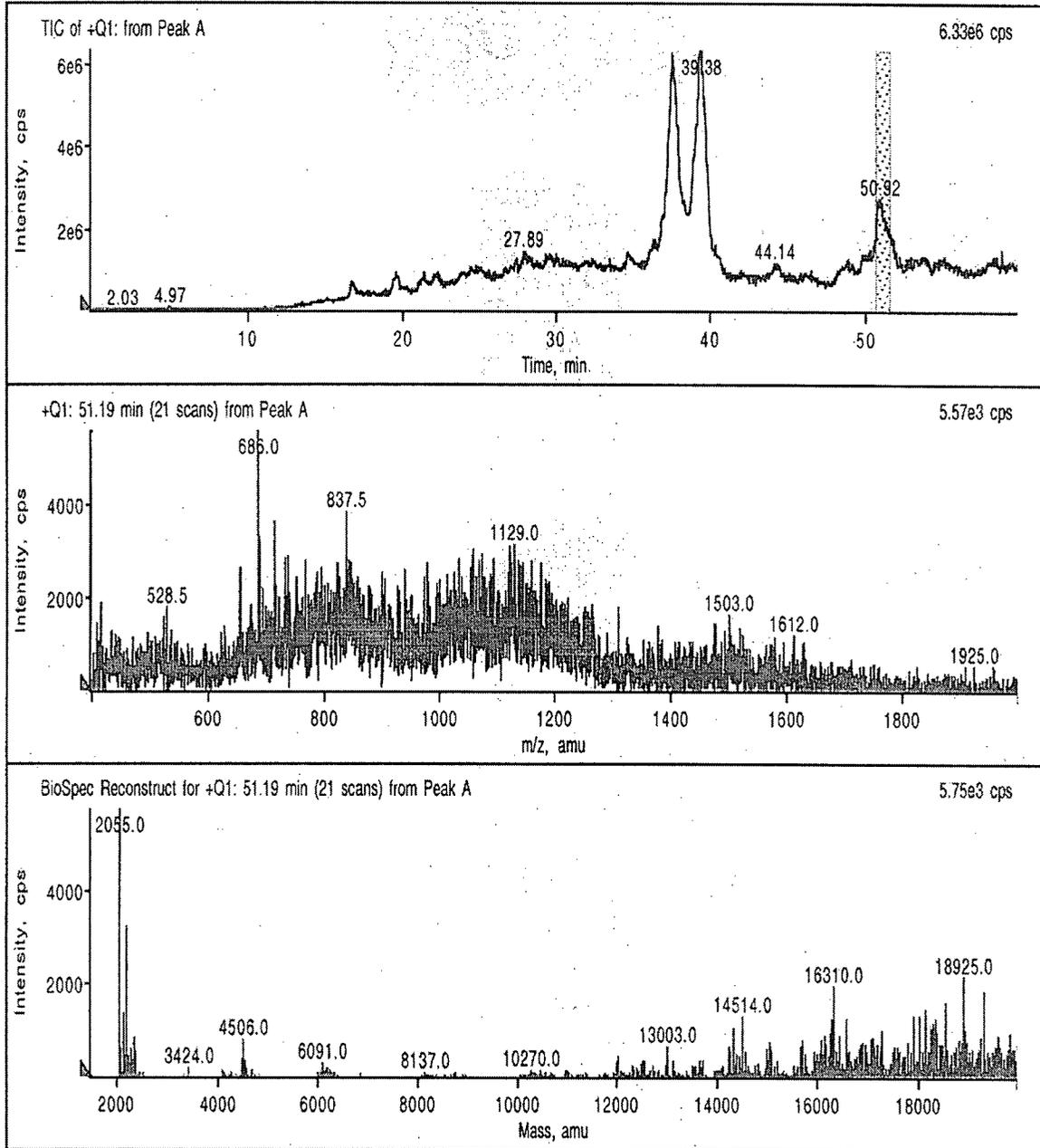
Figure 4-6c. HPLC/MS analysis result of Peak A showing molecular weight reconstruction of the peak at 50.92 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the spectrum for molecular weight reconstruction. Peak A was the second peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.
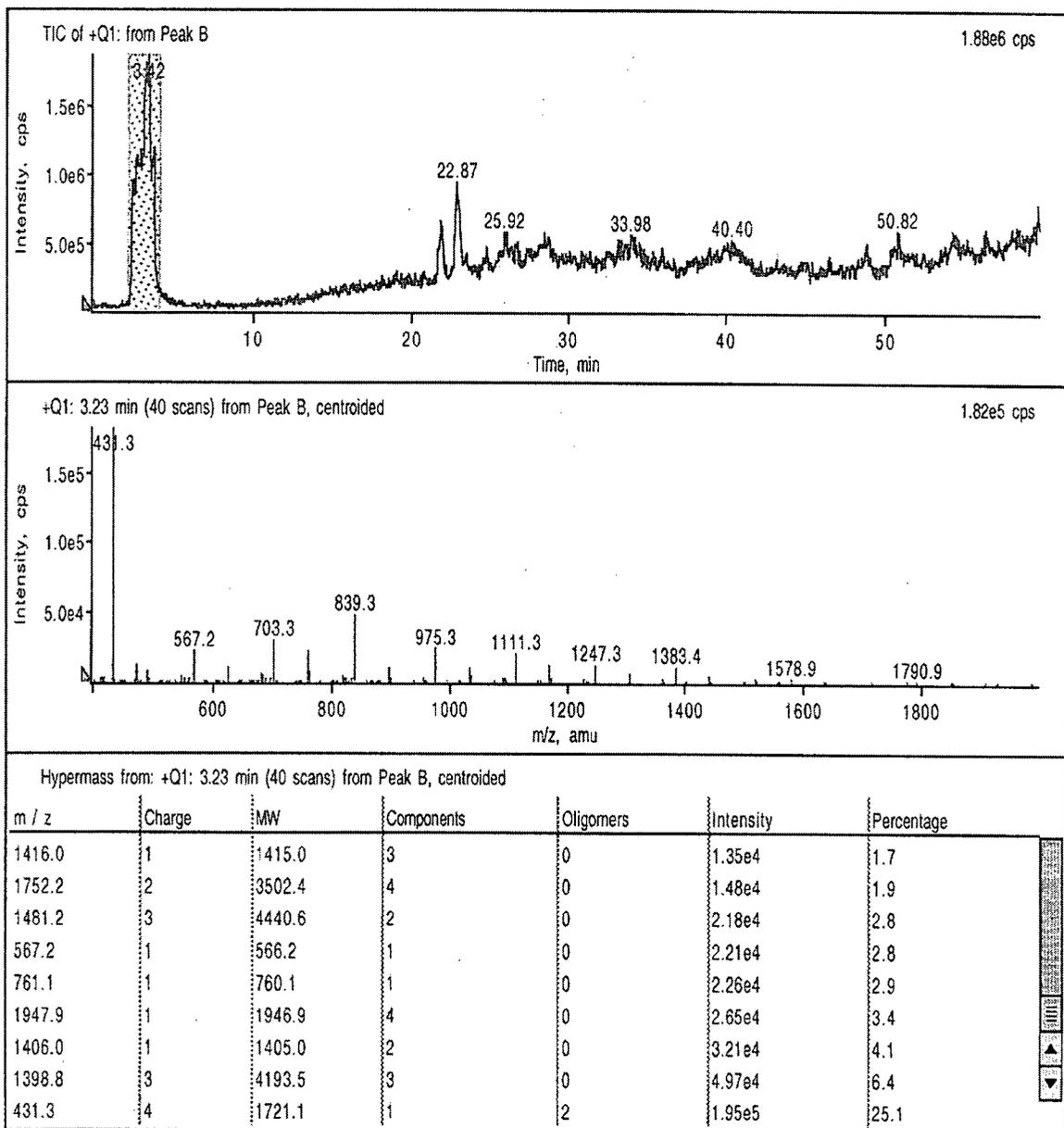
Figure 4-7a. HPLC/MS analysis result of Peak B showing molecular weight reconstruction of the peak at 3.42 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the table for molecular weight reconstruction calculation by the purity function of the Multiview software. Peak B was the third peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.

TIC of +Q1; from Peak B   1.88e6 cps

3.42
22.87
25.92   33.98   40.40   50.82

+Q1: 21.85 min (15 scans) from Peak B, centroided   5.99e4 cps

667.9
605.4
464.9 521.1   804.2   1073.7 1165.1   1381.2   1520.1

Hypermass from: +Q1: 21.85 min (15 scans) from Peak B, centroided

m / z	Charge	MW	Components	Oligomers	Intensity	Percentage
1360.1	1	1379.1	3	0	4.13e3	1.4
1269.8	2	2537.5	4	0	4.20e3	1.5
623.6	1	622.6	1	0	4.33e3	1.5
1074.0	2	2145.9	3	0	4.47e3	1.6
1400.1	1	1399.1	3	0	4.87e3	1.7
973.3	2	1944.6	3	0	6.20e3	2.2
1393.9	1	1392.9	3	0	6.80e3	2.4
605.4	1	604.4	1	0	1.07e4	3.7
1075.9	3	3224.8	3	0	1.20e4	4.2
667.3	2	1332.6	2	2	6.33e4	22.1
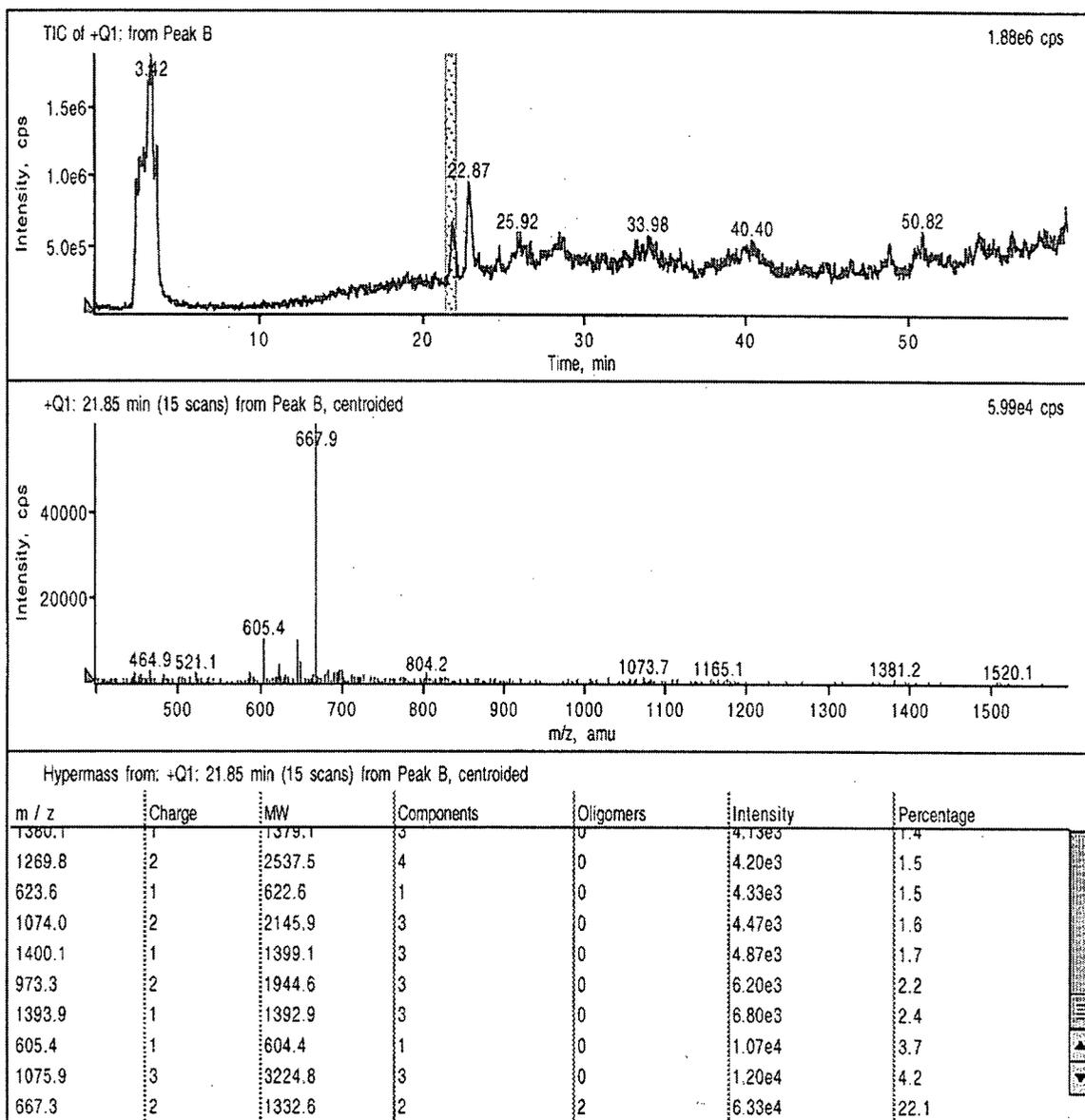
Figure 4-7b. HPLC/MS analysis result of Peak B showing molecular weight reconstruction of the peak at 21.85 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the table for molecular weight reconstruction calculation by the purity function of the Multiview software. Peak B was the third peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.

195

TIC of +Q1: from Peak B   1.88e6 cps

3.#2

1.5e6

1.0e6

5.0e5

22.#7

25.92   33.98   40.40   50.82

10   20   30   40   50

Time, min

+Q1: 22.98 min (15 scans) from Peak B, centroided   7.39e4 cps

66#.5

60000

40000

20000

454.3   953.2   1096.0   1268.5   1544.1

600   800   1000   1200   1400   1600

m/z, amu

Hypermass from: +Q1: 22.98 min (15 scans) from Peak B, centroided

| m / z	Charge	MW	Components	Oligomers	Intensity	Percentage
1398.9	1	1397.9	2	0	6.33e3	1.5
673.9	1	672.9	1	0	6.60e3	1.5
649.5	1	648.5	1	0	8.07e3	1.9
947.2	2	1892.5	2	0	9.20e3	2.1
709.6	1	708.6	1	0	1.39e4	3.2
1290.0	1	1289.0	2	0	1.67e4	3.9
605.8	1	604.8	1	0	1.84e4	4.3
623.9	2	1245.8	1	2	2.71e4	6.3
667.5	1	666.4	1	2	1.07e5	24.7
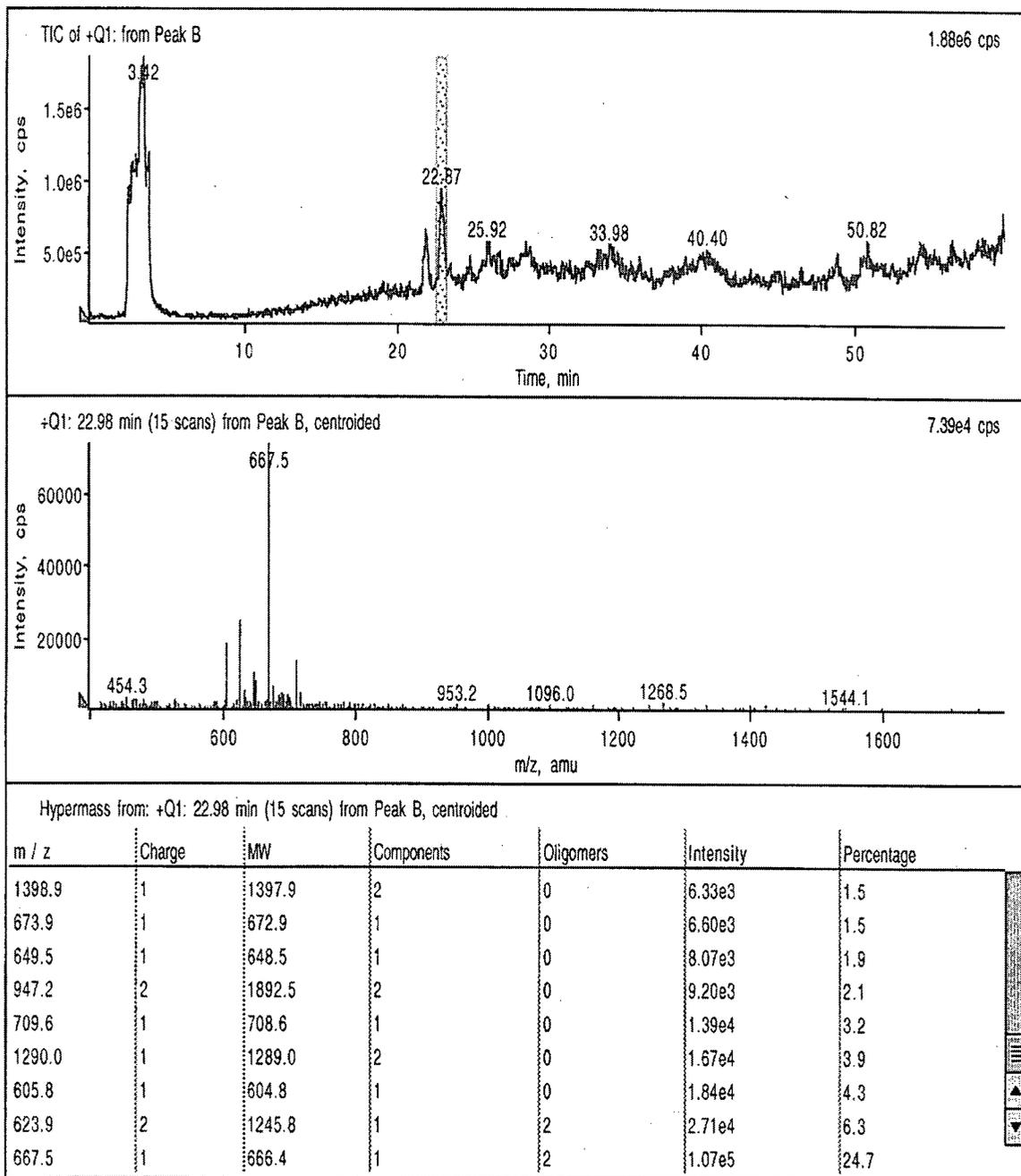
Figure 4-7c. HPLC/MS analysis result of Peak B showing molecular weight reconstruction of the peak at 22.87 min eluted from HPLC. The upper, middle and lower panels are HPLC TIC diagram, MS spectrum, and the table for molecular weight reconstruction calculation by the purity function of the Multiview software. Peak B was the third peak eluted from size exclusion chromatography loaded with 0.1 M NaCl fraction of ion exchange chromatography of Norman flaxseed.

196
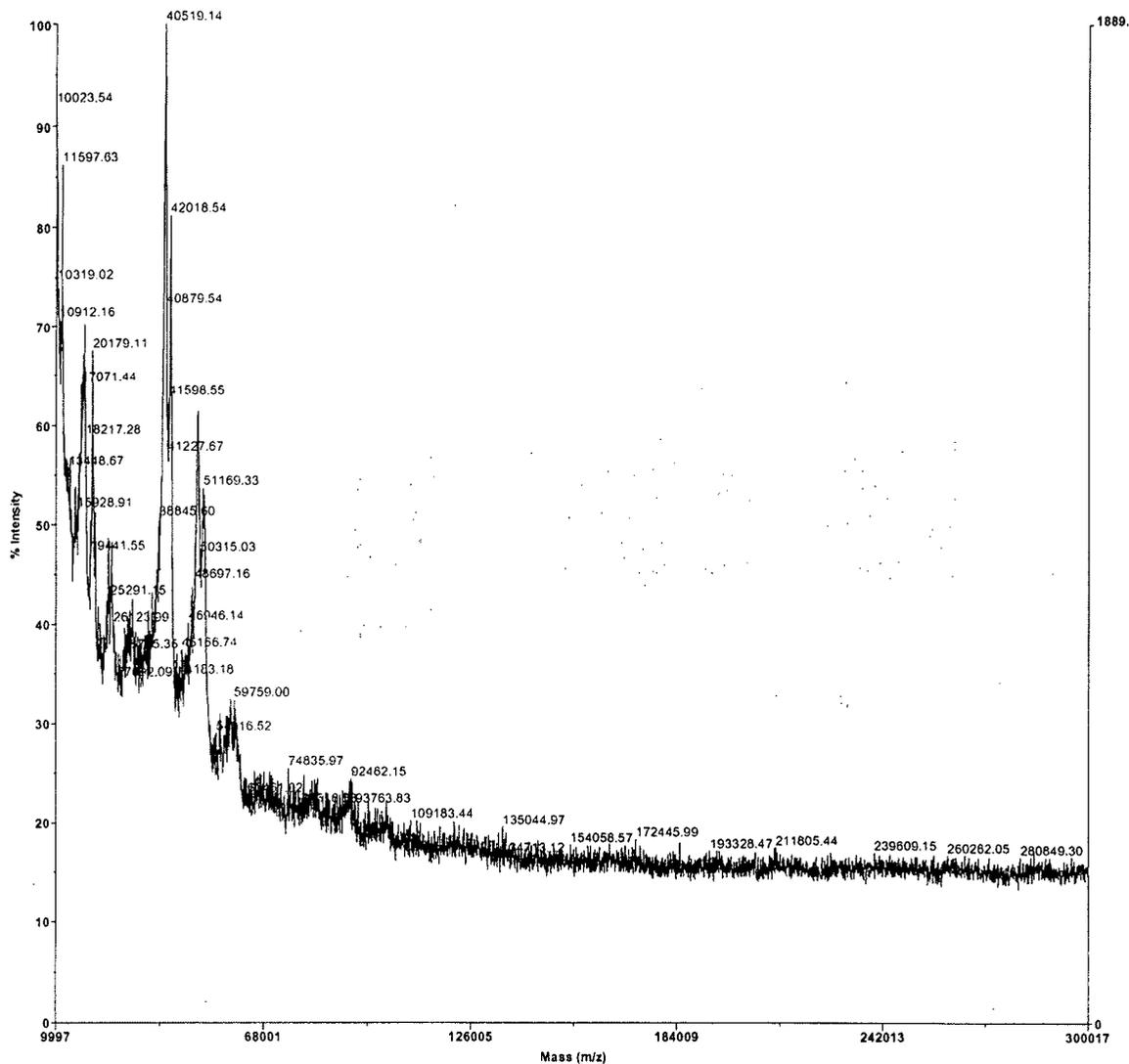
Figure 4-8. MALDI-TOF/MS spectrum of major protein fraction of NorMan flaxseed eluted at 0.25 M NaCl from ion exchange chromatography (refer to Figure 2-5).
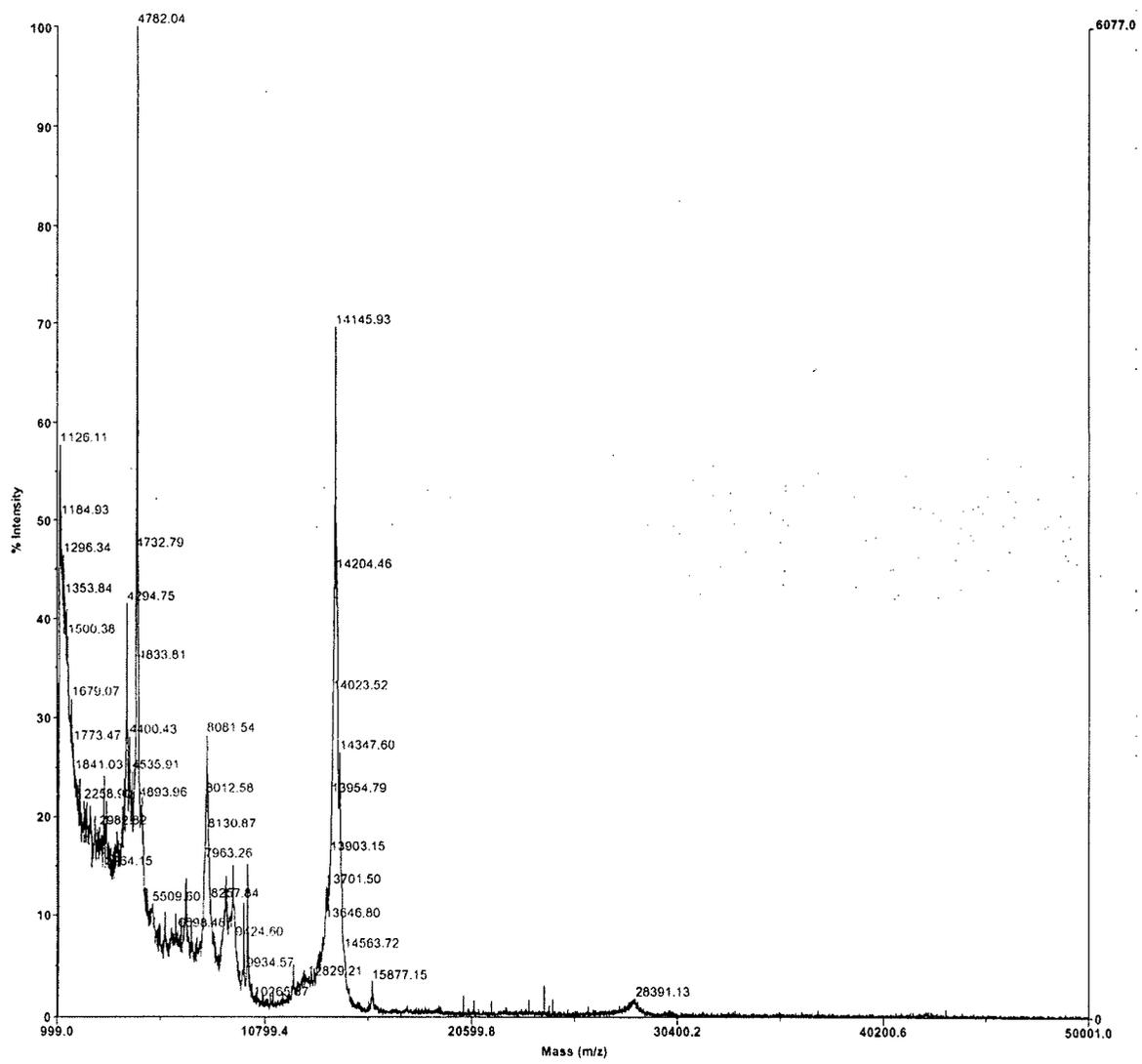
Figure 4-9. MALDI-TOF/MS spectrum of major Cd-binding fraction of NorMan flaxseed eluted at 0.10 M NaCl from ion exchange chromatography (refer to Figure 2-5).
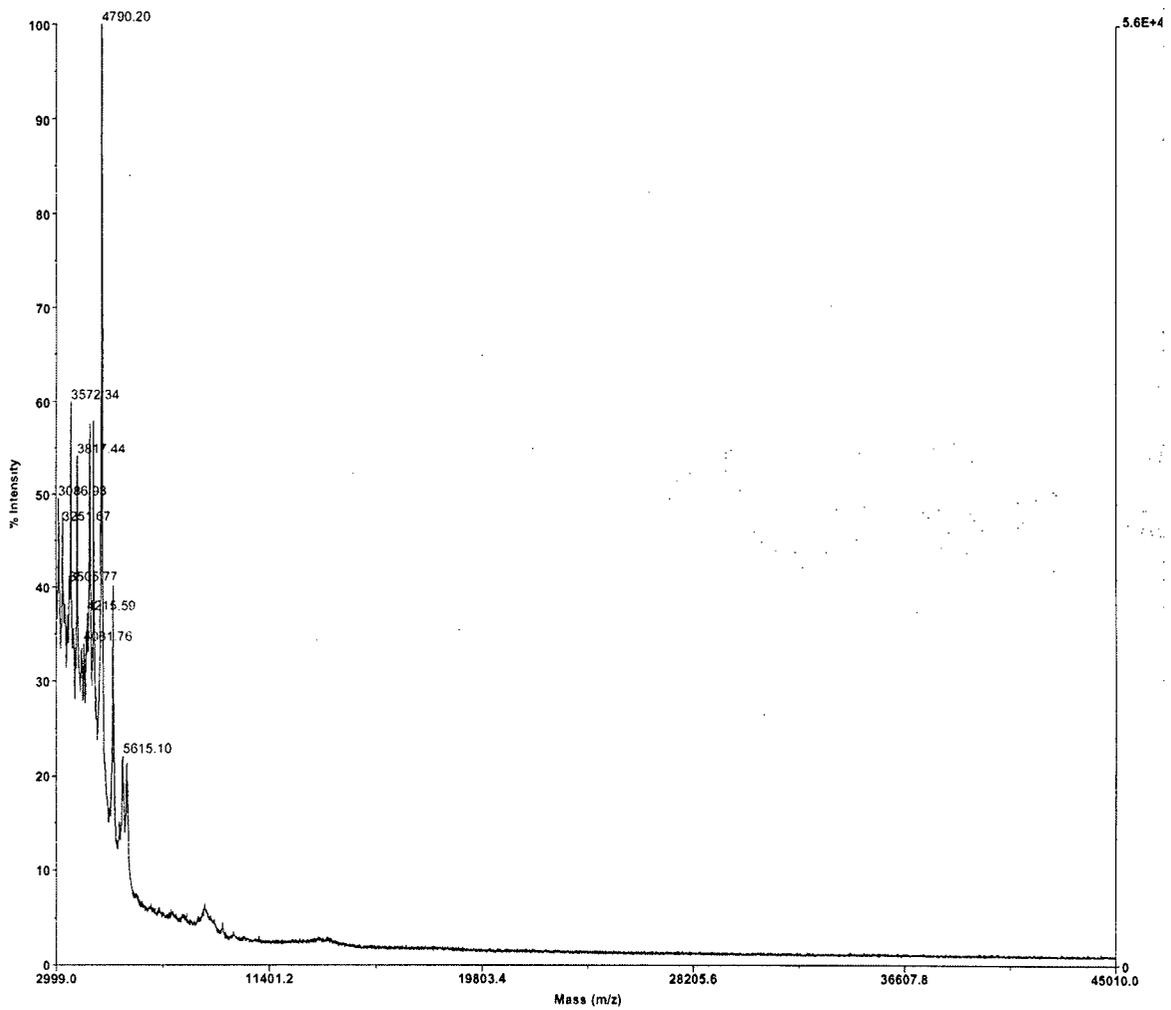
Figure 4-10. MALDI-TOF/MS spectrum of Peak A fraction of NorMan flaxseed eluted as the second peak from size exclusion chromatography loaded with the major Cd-binding fraction eluted at 0.10 M NaCl from ion exchange chromatography (refer to Figure 2-6).
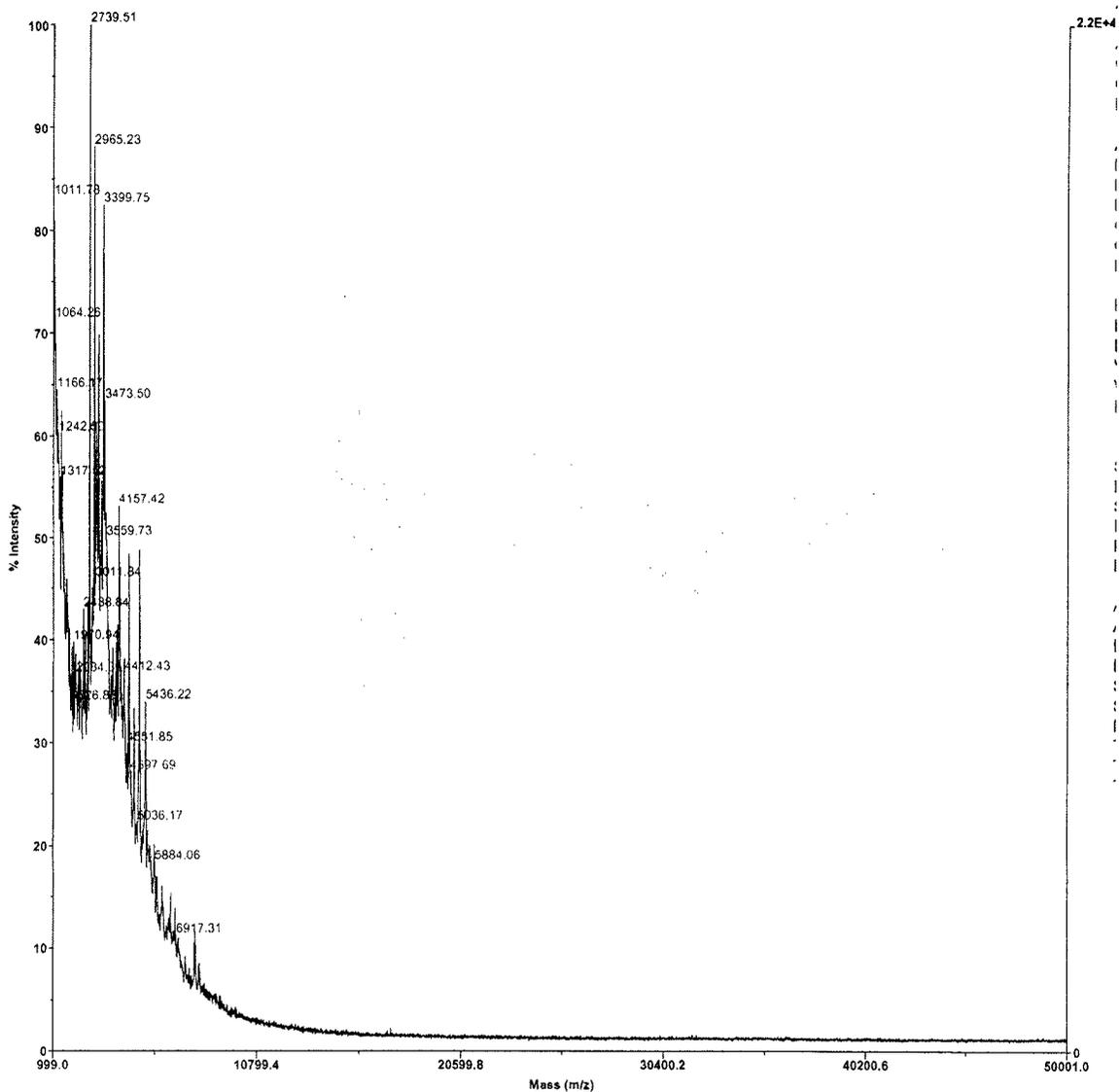
Figure 4-11. MALDI-TOF/MS spectrum of HMW peak fraction of NorMan flaxseed eluted as the first peak from size exclusion chromatography loaded with the major Cd-binding fraction eluted at 0.10 M NaCl from ion exchange chromatography (refer to Figure 2-6).

200
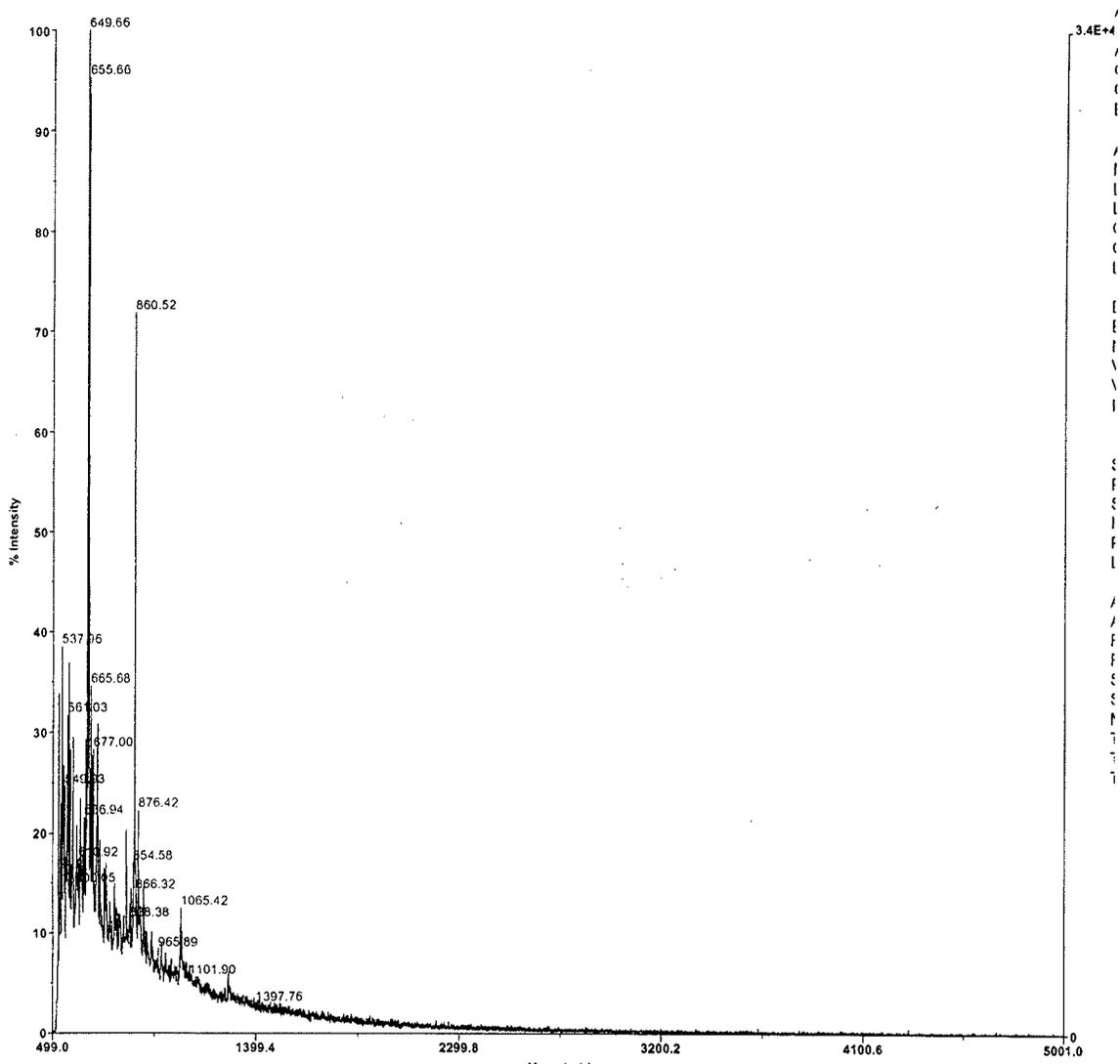
Figure 4-12. MALDI-TOF/MS spectrum of Peak B fraction of NorMan flaxseed eluted as the third peak from size exclusion chromatography loaded with the major Cd-binding fraction eluted at 0.10 M NaCl from ion exchange chromatography (refer to Figure 2-6).
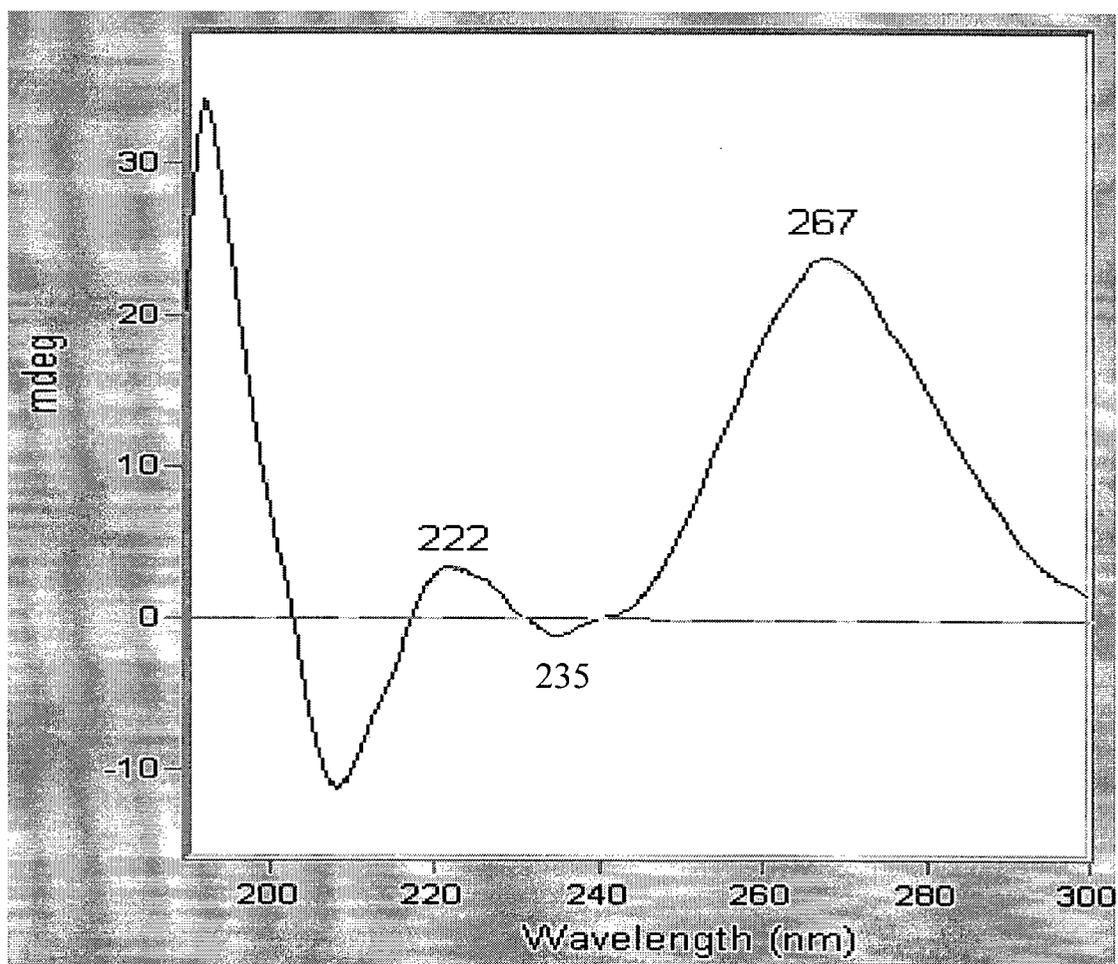
Figure 4-13. Circular dichroism (CD) spectrum for high salt 0.45 M NaCl fraction eluted from ion exchange chromatography loaded with protein extract of NorMan flaxseed.
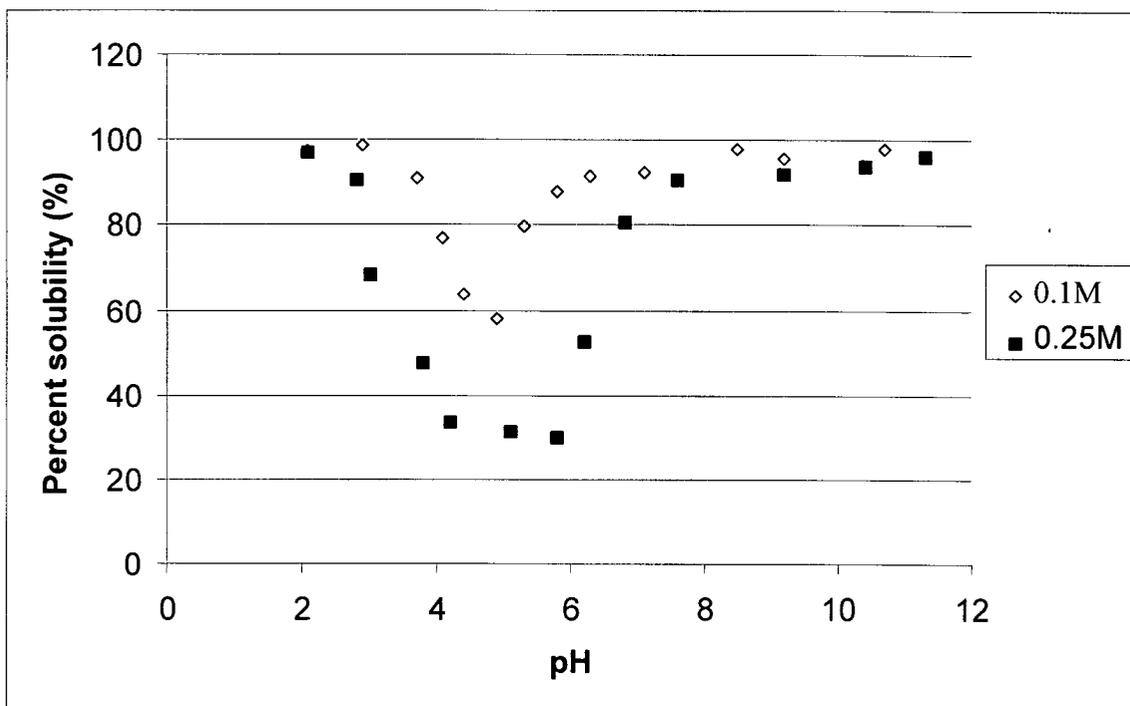
Figure 4-14. Solubilities as measured by BCA protein assay of the major Cd-binding fraction (0.1 M NaCl fraction) and major storage protein fraction (0.25 M NaCl fraction) at different pH values in 20 mM NaCl solution. Data points are based on the average values of two independent experiments. Sample was prepared from NorMan flaxseed.
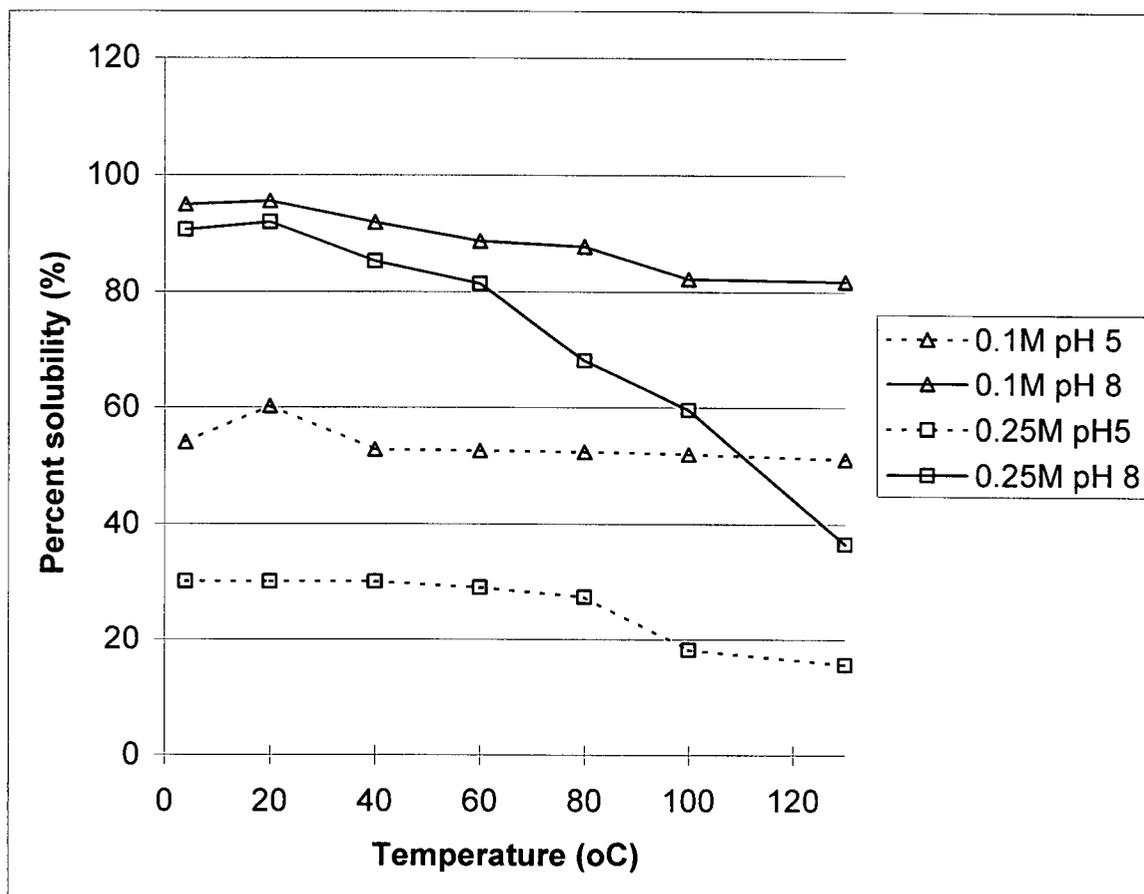
Figure 4-15. Solubilities as measured by BCA protein assay of the major Cd-binding fraction (0.1 M NaCl fraction) and major storage protein fraction (0.25 M NaCl fraction) at pH 5 or pH 8 as a function of temperature. Data points are based on the average values of two independent experiments. Sample was prepared from NorMan flaxseed.

97.0 KDa
66.0 KDa

45.0 KDa
30.0 KDa

20.1 KDa
14.4 KDa

MW Markers
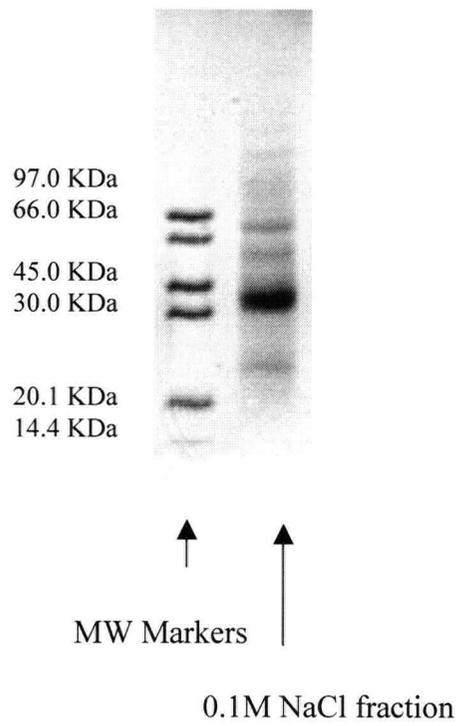
0.1M NaCl fraction

Figure 4-16. Non-reducing SDS-PAGE on PhastGel high density gel (20%) for the major Cd-binding fraction (0.1M NaCl fraction). Sample was prepared from NorMan flaxseed.
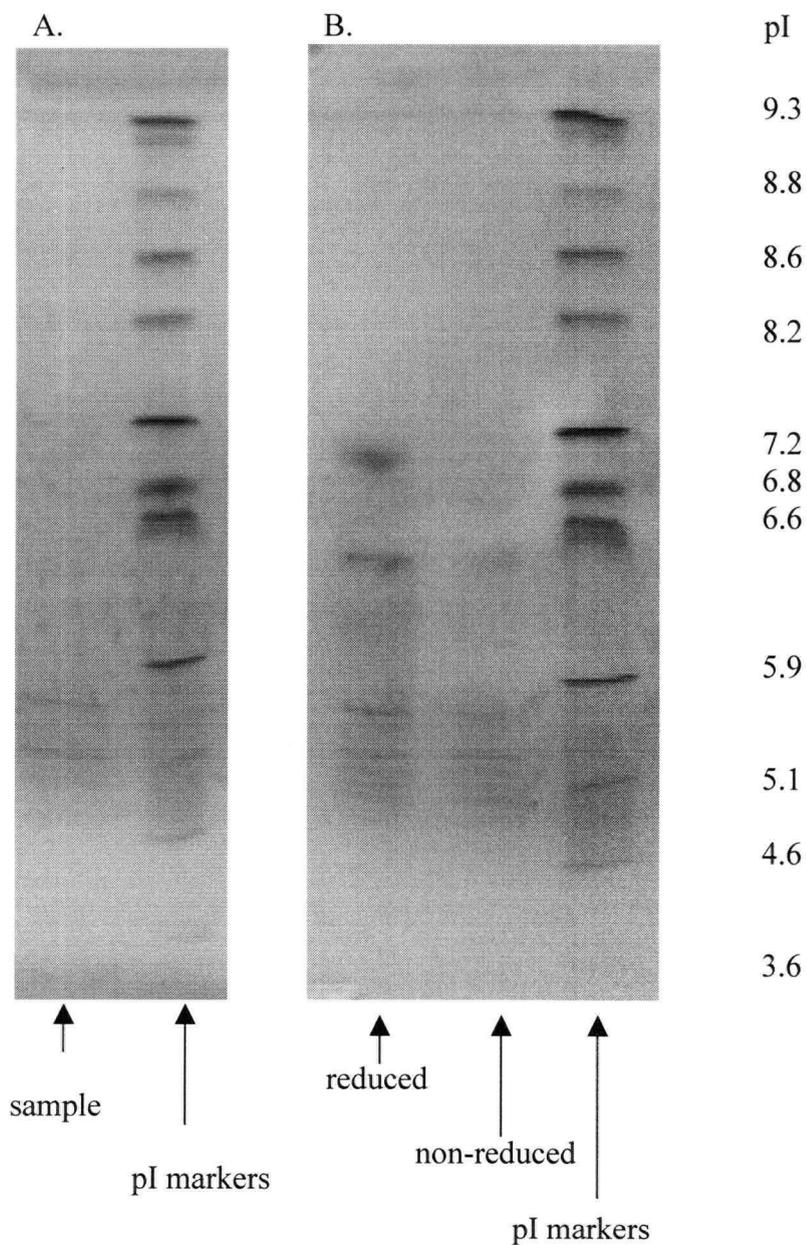
Figure 4-17. Isoelectric focusing of major Cd-binding fraction (0.1M NaCl fraction) under native (A) and denatured condition (B). In the denatured condition, sample was treated by 6 M urea + 10mM dithiothreitol (reduced) or 6 M urea only (non-reduced). Sample was prepared from NorMan flaxseed.
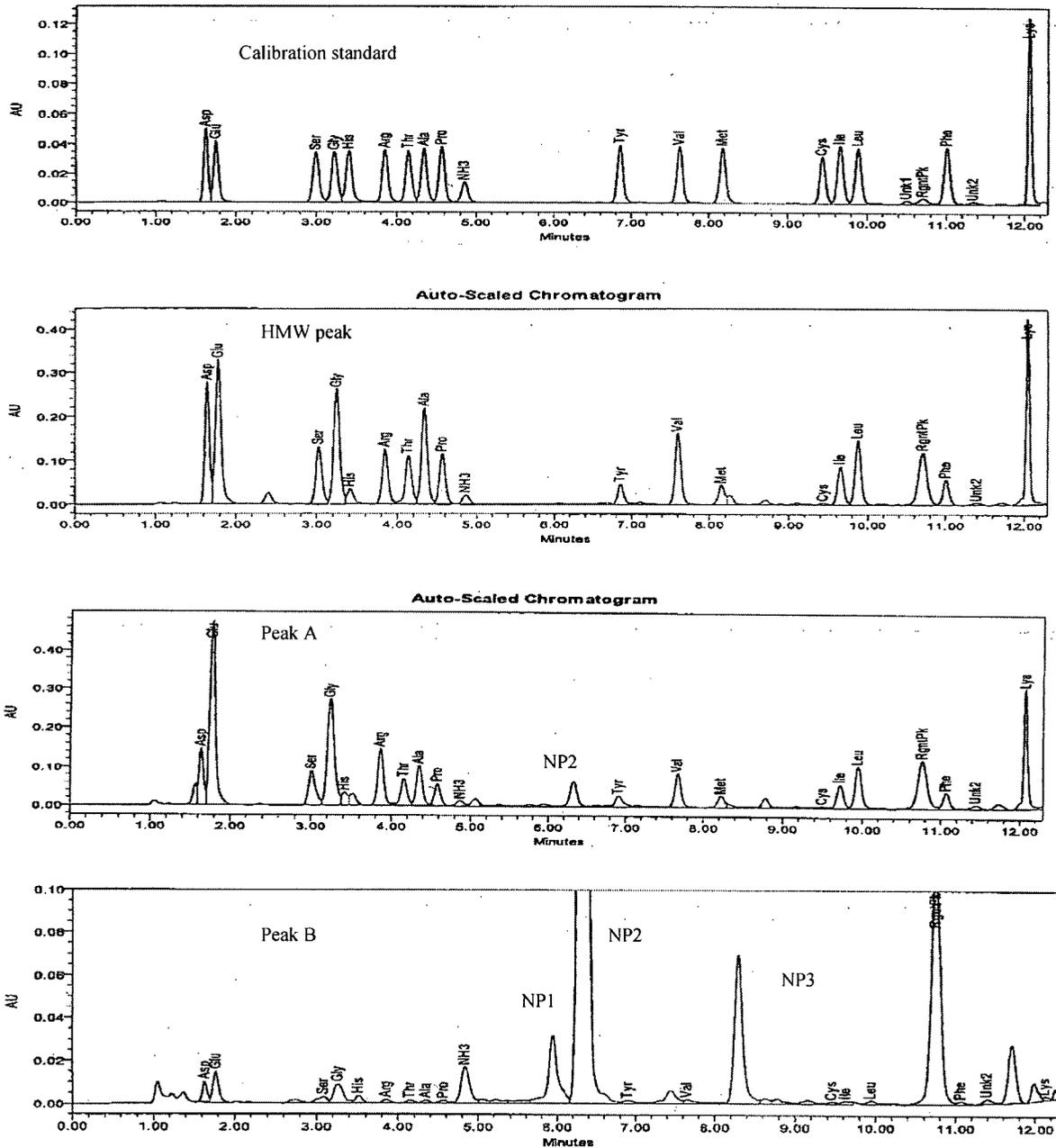
Figure 4-18. HPLC chromatograms of amino acid analysis of the three fractions of HMW peak, peak A, and peak B. The three fractions were purified by size exclusion chromatography on Sephadex G50 column. NP1, NP2 and NP3 indicate unknown peak 1, unknown peak 2, and unknown peak 3. Sample was prepared from NorMan flaxseed.
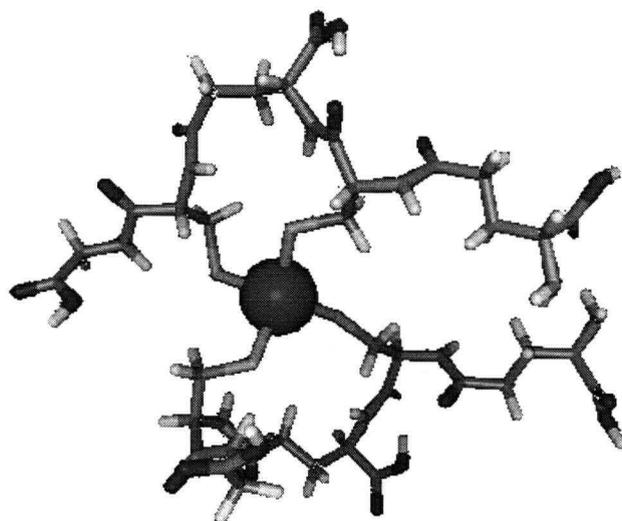
Figure 4-19. Optimized structure of the Cd-PC complex. The Cd atom is in the center, chelated with 4 sulfur atoms donated by 2 molecules of (γ-Glu-Cys)$_2$-Gly (Manunza et al., 2000).
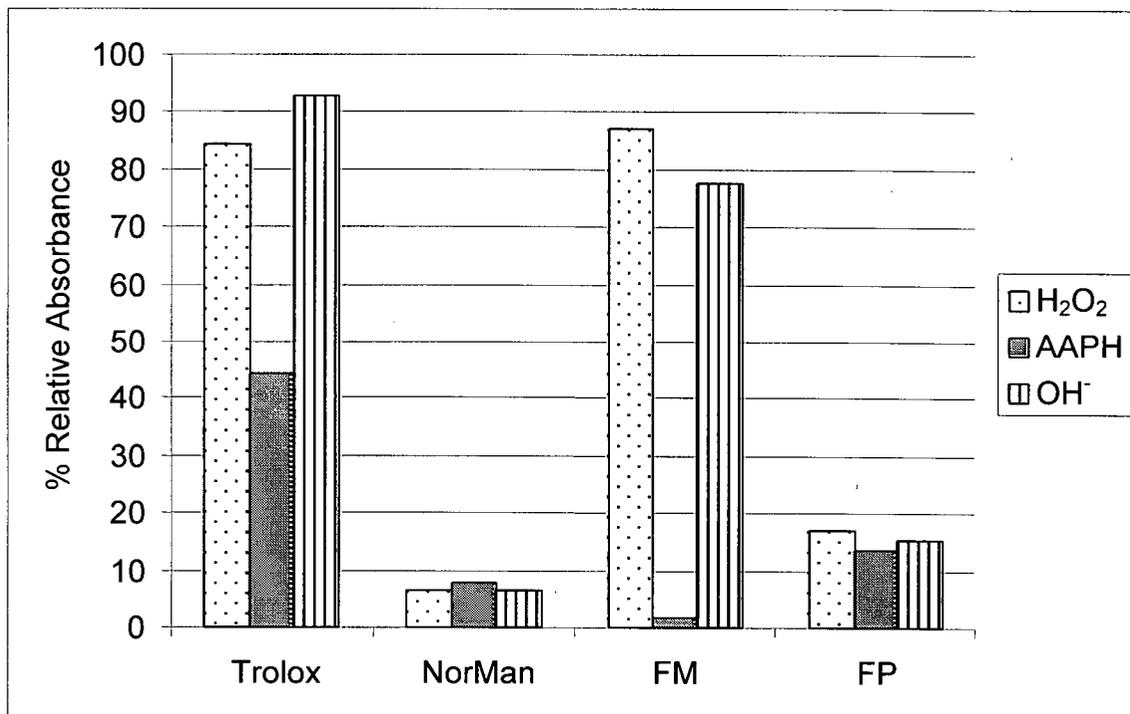
Figure 4-20. Antioxidant activity of Trolox (2.5 mg/mL) and protein extracts from flaxseed: NorMan (2.1 mg/mL), FM (2.5 mg/mL) and FP (2.5 mg/mL) against three oxidant stresses in THP-1 cells: 0.06% hydrogen peroxide, 10 mM AAPH, and hydroxyl radical from 25 mM Fe^{2+} + 0.03% hydrogen peroxide. Note that NorMan was frozen for over two years before use, and FM (Flanders grown in Morden) and FP (Flanders grown in Portage la Prairie) was frozen for over 6 months before use.
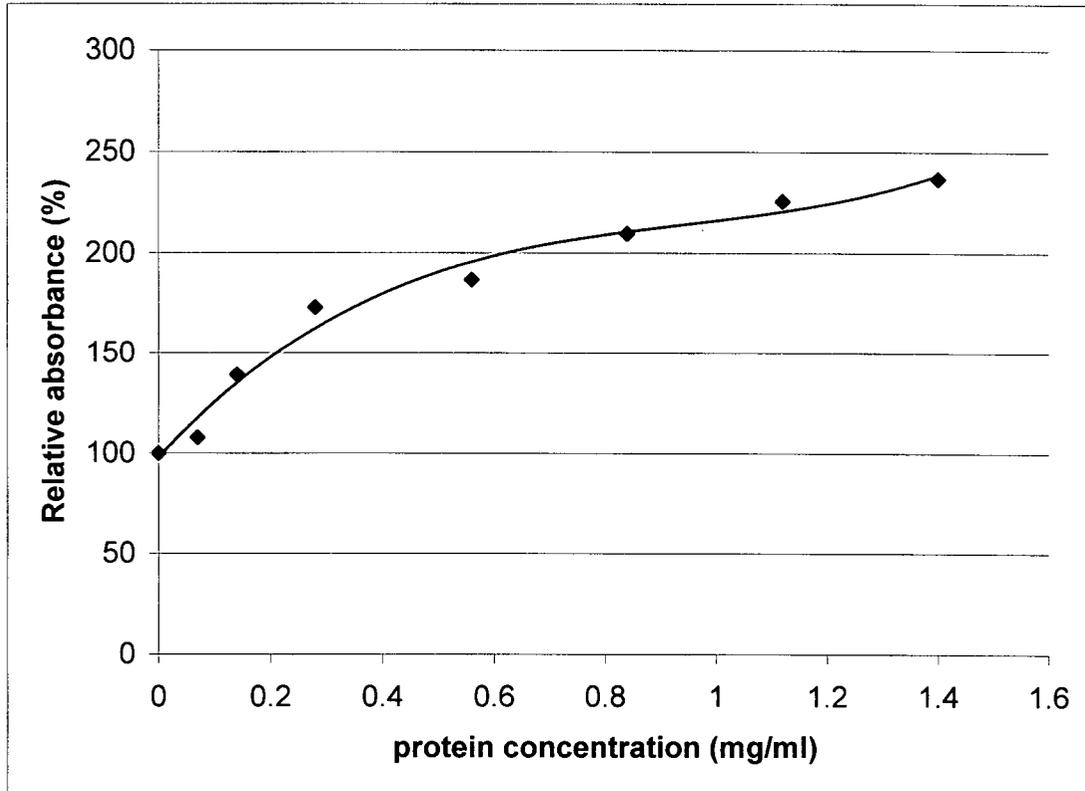
Figure 4-21. Growth promoting effect of protein extract (PE) of NorMan flaxseed on THP-1 cells. Each data point represents the mean of three determinations with S.D. value less than 5% of the mean value.
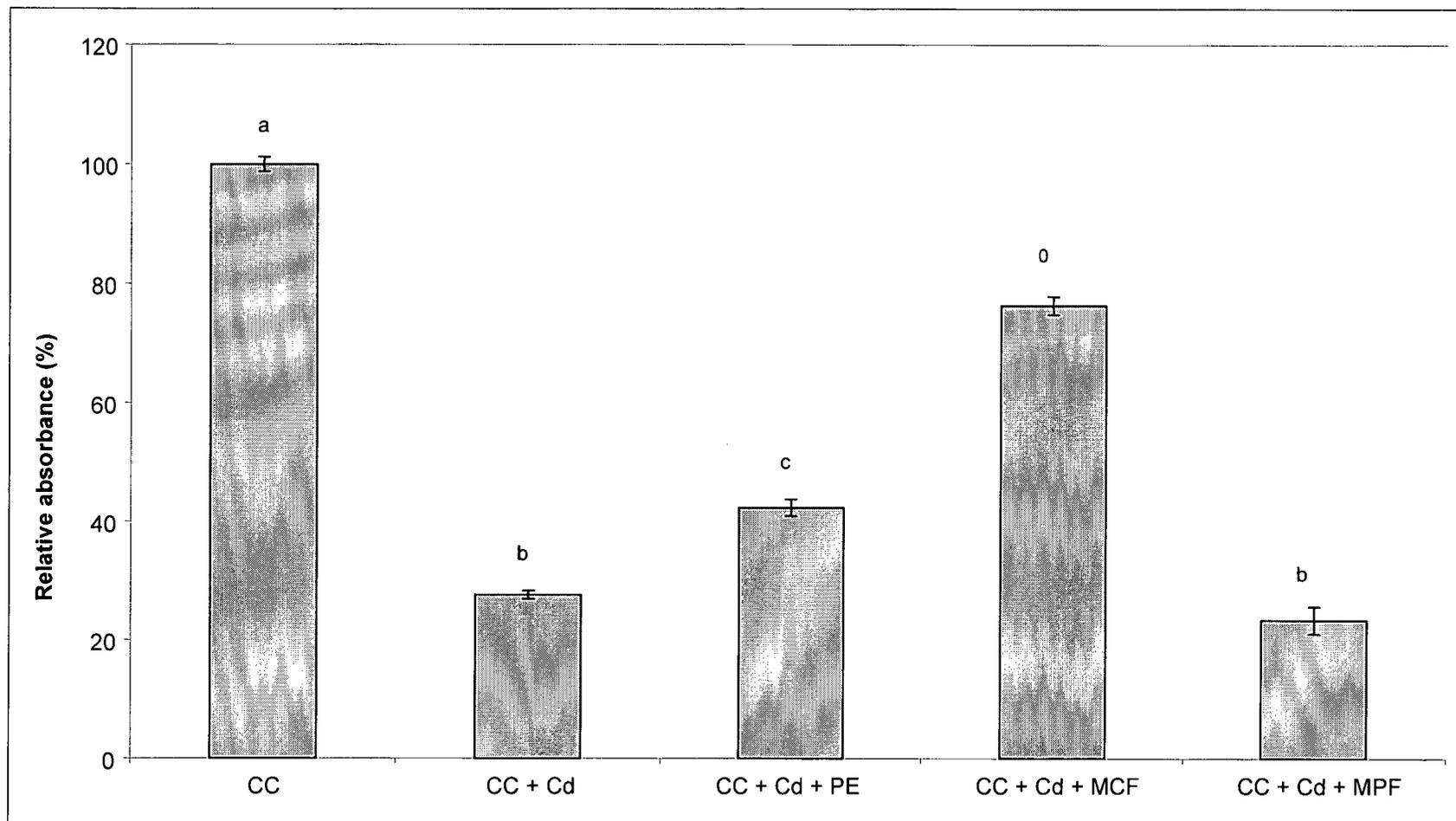
Figure 4-22. Protective effects of protein samples against cytotoxicity caused by 300 μM cadmium. CC: THP-1 cell culture; PE: 110 μg/mL protein extract; MCF: 110 μg/mL major cadmium fraction; MPF: 110 μg/mL major protein fraction. Bars with same letters are not significantly different by Fisher's pairwise comparisons at 5% level. The error bar indicates the standard deviation range for each treatment based on three determinations.
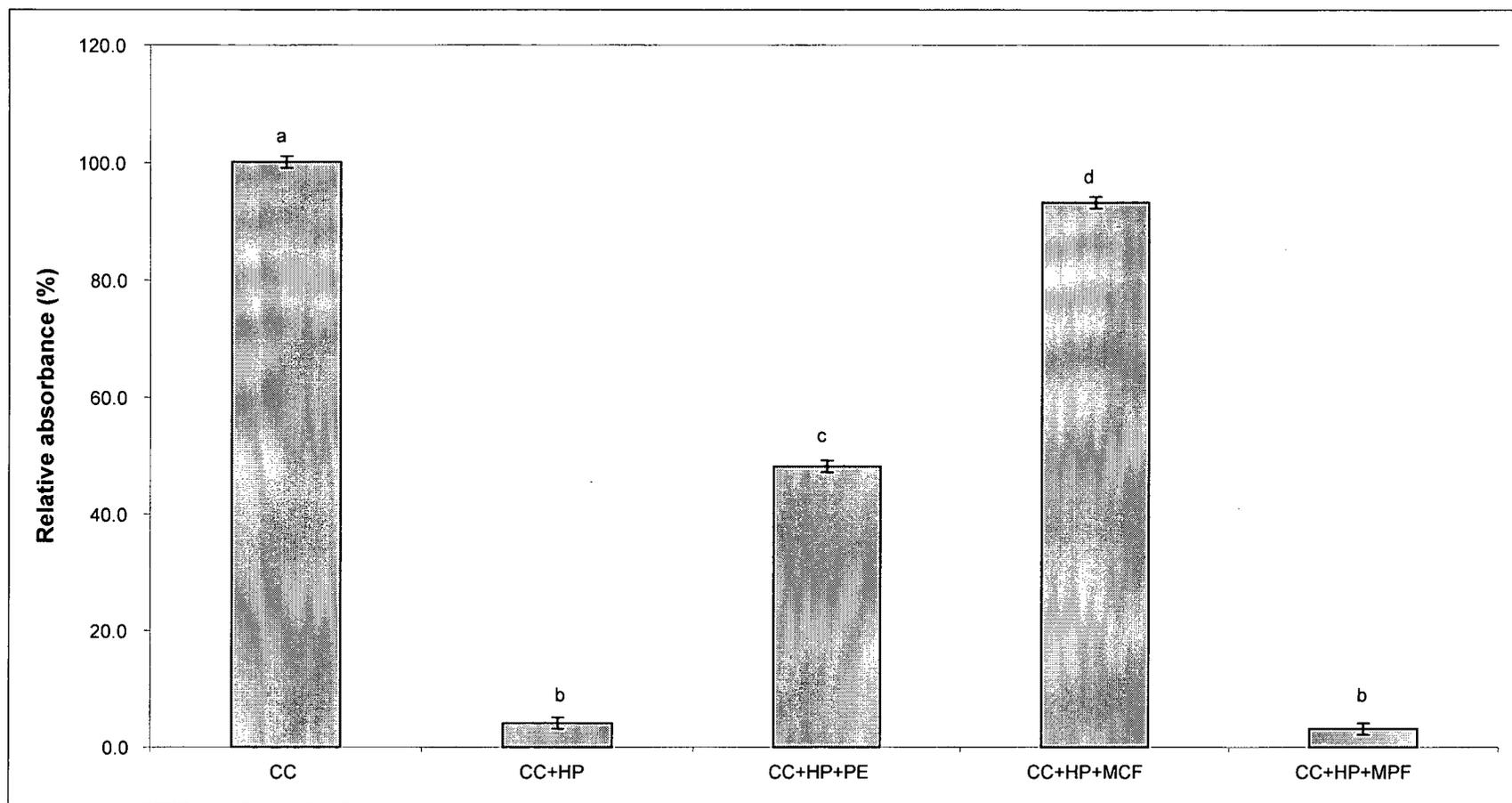
Figure 4-23. Protective effects of protein samples against cytotoxicity caused by 0.06% hydrogen peroxide. CC: THP-1 cell culture; HP: hydrogen peroxide; PE: 110 μg/mL protein extract; MCF: 110 μg/mL major cadmium fraction; MPF: 110 μg/mL major protein fraction. Bars with same letters are not significantly different by Fisher's pairwise comparisons at 5% level. The error bar indicates the standard deviation range for each treatment based on three determinations.

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214

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CHAPTER 5 SUMMARY OF MAIN FINDINGS AND RECOMMENDATIONS FOR FUTURE STUDIES

5.1 SUMMARY

5.1.1 Isolation and Purification of Cd-Binding Fractions from Flaxseed

In this study, the flaxseed cultivar NorMan with 0.53 ppm of Cd was investigated for the distribution of Cd in the seed. Over 95% of the Cd from the whole seed was in the defatted flaxseed powder, and over 82% of the Cd was recovered in the proteins extracted using 0.1 M Tris buffer with 0.1 M NaCl + 0.01M mercaptoethanol at pH 8.6, indicating that Cd in flaxseed was distributed in the protein and not the oil or hull fractions, which is consistent with findings reported by Kolodziejczyk and Fedec (1995). The protein extract was fractionated by ion exchange chromatography on a DEAE Sephacel column yielding a 0.1 M NaCl fraction with 66% of the Cd in only 7% of the total eluted protein from the column, whereas the major protein fraction (72%) that was eluted by 0.25 M NaCl contained only 25% of the eluted Cd. Further separation of the major Cd-binding fraction by size exclusion chromatography of the 0.1 M NaCl fraction on a Sephadex G-50 column resulted in three peaks, referred to as HMW peak, peak A and peak B. The HMW peak having 26% protein contained no detectable Cd. The peak A having 60% protein and peak B having 14% protein were two Cd-binding fractions containing 21 and 79% of the eluted Cd, respectively, and major Cd binding components with MW of 14 and 0.6 to 0.9 kDa, respectively.

The isolation protocol established for flaxseed cultivar NorMan was applied to another flax cultivar Flanders, producing similar results. About 50% of the Cd concentrated into the major Cd-binding fraction eluted by 0.1 M NaCl, which contained about 8% of the proteins. The major protein fraction that was again eluted at 0.25 M NaCl, contained about

60% of the eluted protein with about 14% of the eluted Cd. Further separation of the 0.1 M NaCl fraction by size exclusion chromatography again resulted in three peaks; however, the HMW peak was very small and contained less than 0.4% of the eluted protein without Cd. The cultivar Flanders grown in Morden had 1.54 ppm Cd in the seeds, and the Cd and protein distributions in the fractions from size exclusion chromatography were similar to that of NorMan. Peak A and B with 62 and 38% of protein containing 27 and 73% of eluted Cd, respectively. However, for the low Cd Flanders grown in Portage la Prairie, Peak A contained no detectable Cd, and Peak B contained 100% of the eluted Cd in 32% of the eluted protein.

The Cd level in the flaxseed cultivar NorMan major protein fraction eluted with 0.25 M NaCl from ion exchange chromatography was reduced substantially from 3.2 ppm (ng Cd/mg protein) in the protein extract to 0.9 ppm in the major protein fraction. Similarly, using flaxseed cultivar Flanders grown in Morden and Portage la Prairie, the Cd level was reduced to 1.3 and 0.4 ppm in the 0.25 M NaCl fractions, respectively, from 6.0 and 2.2 ppm, respectively, in the unfractionated protein extracts. Further studies using five flaxseed cultivars grown in three locations showed that the average Cd content in the 0.25 M NaCl fraction expressed on a protein basis was 2.55 (±0.65) ppm, representing a 40% decrease from 4.23 (±0.59) ppm Cd in the original unfractionated protein extracts. These results indicate the potential to isolate the major storage proteins with lower Cd content than the whole seed, which can then be used safely as a functional food ingredient. An industrial scale process can be potentially developed based on this laboratory-scale protocol.

5.1.2 Cultivar and Location Effects on Cd Distribution in Flaxseed

The applicability of the Cd distribution profile and the protein purification protocol for separating major Cd-binding fraction from major storage proteins are very important in processing flaxseed into a functional food ingredient with low level of the toxic metal Cd. In order to verify the relevance of the experimental results and the established protocols to other flaxseed cultivars, and also to reveal the possible relationships of the Cd distribution pattern in flaxseed as influenced by different cultivars and locations, further experiments on a larger sample size were conducted, which involved five different flaxseed cultivars grown in three different locations. The results indicated that the purification protocol employed was applicable to all the 15 samples including 5 cultivars grown in 3 locations. The major Cd-binding fraction was eluted in the 0.1 M NaCl fraction, which contained 52% of the total eluted Cd in 12% of the total eluted proteins; the major storage protein fraction was in the 0.25 M NaCl fraction, which had 39% of the total eluted Cd in 66% of the total eluted proteins. However, some exceptions occurred; for AC McDuff grown in Morden and Flanders grown in Rosebank, the 0.25 M NaCl fraction contained the highest amount of both Cd and proteins compared with other fractions. After studying and comparing the Cd content in each fraction based on unit protein (ng Cd/mg protein), it was found that the 0.1 M NaCl fraction contained the highest amount of Cd on a protein basis for all five cultivars grown in three locations. The average Cd content (ng Cd/mg protein) in the 15 samples was 0.92 (±0.63), 19.36 (±3.64), 2.55 (±0.65), and 3.53 (±2.87) ppm for the unbound, 0.1, 0.25, and 0.50 M NaCl fractions, respectively. The Cd levels were 4.6 times higher in the major Cd-binding fractions eluted by 0.1 M NaCl, and 40% less in the major protein fractions eluted by 0.25 M NaCl, compared to the Cd levels in the protein extracts (4.23 ± 0.59 ng/mg). These

experimental results further demonstrated that the protocols established using flaxseed NorMan and Flanders were successful and should be applicable as a general guideline for scaling-up a commercial process and producing flaxseed proteins with low Cd content.

The Cd accumulation in flaxseed was greatly influenced by growing location, and this effect was highly significant at a confidence level of 99%. The cultivars grown in Morden had a Cd level three to four times higher than those growing in Portage la Prairie and Rosebank. This heavy location influence from Morden on Cd level in flaxseed may mask cultivar effect to some extent. The analysis without the Morden data did reveal a slight cultivar effect on Cd level in flaxseed, with the flaxseed of cultivar Flanders grown in Rosebank containing the highest Cd in the seeds. The dependence of Cd level on flaxseed genotype had been reported earlier (Becher et al, 1997; Li et al., 1997), but our experimental results showed that location effect on seed Cd accumulation was predominant, and this conclusion confirmed previous report of Grant et al (2000) that the location had the greatest effect on Cd accumulation.

A similar location effect was observed for Cd/protein ratios in protein extract, 0.1 and 0.25 M NaCl fractions. The Cd level expressed as ng Cd/mg protein was much higher in the flaxseed grown in Morden than in Portage la Prairie and Rosebank. Since the much higher Cd level in flaxseed grown in Morden could mask the effects from cultivars, the percentage rather than absolute values of Cd distribution in each fraction was calculated for comparison. It was found that there was no significant difference in Cd distribution in each salt eluted fraction (0.1, 0.25, and 0.50 M NaCl). This indicated that the fractionation protocol by ion exchange chromatography separated major Cd binding fraction from the major protein fraction in the protein extract independent of flaxseed sources including cultivar and growing

location, and further demonstrated that this purification protocol can be used as a general guideline for flaxseed protein purification. A significant difference in % Cd distributed in the unbound fraction of flaxseed was observed among different cultivars at 99% confidence level, but no difference was noted among locations. This means that the purification protocol could be adjusted for each different cultivar to minimize or maximize Cd retention in the unbound fraction; however, in practice this would be unnecessary considering Cd in the unbound fraction was relatively low (3.4 ± 2.0%).

5.1.3 Characterization of Cd-binding Components in Flaxseed

The analyses including UV/Vis absorbance, circular dichroism, HPLC/MS and MALDI-TOF MS, pointed out that the three fractions eluted at 0.1, 0.25 and 0.45 M NaCl from ion exchange chromatography were different in composition. The 0.1 and 0.25 M fractions were protein in nature, but the 0.45 M NaCl fraction was not proteinaceous based on analyses of UV/Vis and circular dichroism. This high salt eluted fraction of 0.45 M NaCl was composed of Cd-binding factors having Cd-thiolate coordination according to circular dichroism analysis and previous research (Li-Chan et al., 2002). However, this fraction accounted for recovery of only a minor percentage of the Cd found in the flaxseed.

The MALDI-TOF MS analysis demonstrated that the predominant component in the 0.25 M NaCl major protein fraction had a molecular weight of 40.5 kDa, and other significant components at 11.6, 17.1, 20.2, 42.0, and 51.2 kDa. Previous research (Chung et al., 2005), using SDS-PAGE, indicated that there were 3 predominant bands with apparent molecular weight at 20, 23, and 31 kDa under reducing condition, and 2 predominant bands at 40 and 48 kDa on non-reducing condition. This MALDI-TOF profile of the 0.25 M NaCl

fraction was different from the 0.1 M NaCl major Cd-binding fraction. In the 0.1 M NaCl fraction, the predominant components were proteins with MW of 4.8 and 14.1 kDa, the latter was probably a trimer of the former. This result was in agreement with the result from size exclusion chromatography, which showed that the major protein peak of the 0.1 M NaCl fraction was eluted at 14 kDa position from the G50 column.

The analyses of the results from HPLC/MS and MALDI-TOF MS spectra of the HMW peak, Peak A and B, in conjunction with information on the elution behavior on G50 size exclusion chromatography, led to a conclusion that the 0.1 M NaCl fraction contained a 19 kDa component in HMW peak, 14 kDa in Peak A, and several components in the range of 0.6 to 0.9 kDa in Peak B. Studies from spectral characteristics and amino acid analysis suggested that HMW peak, Peak A and B were different in composition. The HMW peak and Peak A components were protein in nature, while Peak B was not. The 0.1 M NaCl fraction contained very high values of total SH + SS contents compared with other fractions (Table 4-1), while amino acid analysis showed 7.1 and 5.0% of Cys contents in Peaks A and B, respectively (Table 4-2). Solubility studies showed high thermal stability of the major Cd-binding fraction, which is a typical characteristic of Cd-binding proteins. Based on Cys amino acid content and the analysis of MALDI-TOF MS spectrum of Peak B, one of the chelators for Cd in the Peak B could be Cd-PC$_2$. However, because the amino acid analysis showed that over 90% of the fraction was composed of unknown peaks, the most important chelators in Peak B are unidentified unusual amino acids or organic acids with the molecular weight in the range of 0.6 to 0.9 kDa. Because over 50% Cd was eluted into the major Cd-binding fraction of the 0.1 M NaCl, in which 0-20 and 70-100% of Cd were further separated into Peaks A and B, respectively, by size exclusion chromatography, it can be concluded that

the unusual amino acids/organic acids with MW of 0.6 to 0.9 kDa and Cd-PC$_2$ as well as a 14 kDa protein were responsible for binding the majority of Cd in the protein extract and in the original seeds.

5.1.4 Protective Effects of Protein Extracts from Flaxseed against Cd and H$_2$O$_2$

Previous studies reported in this thesis have demonstrated that flaxseed proteins can be fractionated into the major Cd-binding fraction and the major protein fraction with at least 40% less Cd comparing with the original flaxseed proteins. The major protein fraction can be safely used as a functional food ingredient, and the major Cd-binding fraction can be used for investigating the mechanism of Cd retention in flaxseed. A preliminary study on flaxseed protein fractions was carried out in order to further explore these potential biological characteristics. A study reported that a 25 kDa protein from flaxseed strongly inhibited the growth of the agronomically important pathogen *Alternaria solani*, and the human pathogen *Candida albicans* (Borgmeyer, et al., 1992). Matthaus (2002) reported that the extract from defatted residue of flaxseed showed strong antioxidant activity as measured by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method, β-carotene bleaching method and electron spin resonance. In this study, proteins extracted from flaxseed defatted powder and the major cadmium binding fraction eluted at 0.1 M NaCl from ion exchange chromatography of the protein extract conferred protective effects against acute toxicity by cadmium and strong oxidative stress by hydrogen peroxide. The addition of 110 μg/ml of protein extract and major cadmium binding fraction can reduce cadmium (300 μM) toxicity to cells as observed by the reduction of relative absorbance by 14 and 44%, respectively; the addition of 110 μg/ml of protein extract and major cadmium binding fraction decreased

hydrogen peroxide (0.06%) toxicity to cells with the relative absorbance reduced by 48 and 89%, respectively. The protein extract was also found to promote THP-1 cell growth in a dose dependent manner. The protective effects of protein extract on Cd and hydrogen peroxide toxicities may be the results of combined effects of protection against toxicity and promotion of cell growth.

5.2 RECOMMENDATIONS FOR FUTURE STUDIES

Flaxseed accumulates high Cd content depending on growing location. Over 80% of Cd in flaxseed can be extracted with proteins in 0.1 M NaCl Tris buffer at pH 8.6. This protein extract can be further separated into a major storage protein from a major Cd-binding fraction. This major storage protein fraction is recommended to be used as a functional food ingredient, whereas, the major Cd-binding fraction can be used for further study of Cd retention mechanism. The studies of the Cd distribution and fractionation method were based on a commercial flaxseed cultivar NorMan, and were further confirmed by 5 other flaxseed cultivars grown in three different locations.

In this study, the flaxseed protein extract was successfully separated into a major Cd-binding fraction and a major protein fraction by ion exchange chromatography. The protocol established here was on a lab scale, it would need to be scaled up for an industrial purpose. This scaling-up experiment could be done by loading more samples to a bigger column. Alternatively, based on the knowledge obtained from this study, a commercial purification protocol producing major storage protein with low Cd content can also be developed using a different approach. A new purification protocol could be designed to bind the major Cd-binding fraction more tightly onto the column while eluting the storage proteins in the

unbound fraction, by employing cation instead of anion exchange chromatography along with the adjustment of pH and salt concentration in equilibrating buffer. This modification would provide a more effective and economical method for industrial purpose. Another way to design a new purification protocol is to use ultrafiltration or size exclusion chromatography. The major storage proteins of flaxseed are in the range of 320 kDa (Marcone et al., 1998) and 365 kDa (Chung et al., 2005), while the Cd-binding proteins are much smaller (Section 4.4).

Previous research (Li et al., 1997; Cieslinski et al., 1996; Marquard et al., 1990) confirmed that Cd accumulation was genotype dependent as well as being affected by growing location. However, studies reported in this thesis showed a strong location effect, but only very slight cultivar effect, which could be a result of the much higher level of Cd accumulated in the flaxseed grown in Morden that masked the cultivar effect. Further investigation of the cultivar effect on Cd accumulation in flaxseed without the influence of growing location should be carried out. Three to five locations with low, medium and high level of Cd in the soil, and three to five cultivars with three repeats in each location would make a better set of data for statistical analysis and reveal the cultivar and location effect at the same time.

This study showed that over 40% of Cd was bound by a 14 kDa protein and the unidentified unusual amino acids/organic acids along with Cd-PC_2. The best way to determine the sequence of the 14 kDa in Peak A would be the capillary electrophoresis (CE), which is the most universal and most frequently used electroseparation technique for qualitative and quantitative analysis of small peptides in biological samples (Perez-Rama et al., 2005; Kasicka 2003). Peak A could be further concentrated and filtered by ultrafiltration

using membranes of different molecular weight cutoffs, for example, membrane of 30,000 MWCO for removing of any bigger and aggregated proteins, and then filtered by membranes of 5,000 MWCO to remove any buffer salts and smaller molecules. The concentrated protein fraction would then be applied to CE for further separation. The individual peaks eluted from CE could be applied to MS and amino acid analysis (Garcia & Salazar, 2006; Stillman 1995).

Further detailed study of the behavior of Cd-binding components in flaxseed under different conditions could be carried out by using immunological techniques (Jones et al., 2002; Khosraviani et al., 1998; Talbot et al., 1986). The 14 kDa protein could be purified and characterized as mentioned above, then the monoclonal antibody cell line against this protein could be produced by hybridoma technology, and the monoclonal antibody produced by this cell line could be used for affinity purification process to trap the bulk of similar proteins in the protein extract from flaxseed. In addition to providing information on the quantity of this protein and its Cd-binding capacity in flaxseed, this approach could be used to study the changes of this 14 kDa protein in the flaxseed under different conditions such as cultivar differences, growing location effect and parameter changes from soil like Cd and pH. Information from this study would be valuable in creating a comprehensive strategy for managing Cd content in flaxseed cultivars. The preliminary research using this technique was reported before for studying the Cd-binding protein in rainbow trout (Mullins et al., 1999).

However, the future study should be focused on the major Cd-binding fraction of Peak B, which was responsible for over 35% of the Cd in the flaxseed protein extract. The amino acid analysis showed over 90% of the components in Peak B were unidentified. MS analysis showed the PC_2-Cd could be one of the unidentified components, but this has to be further confirmed by other experiments. Peak B could be further separated by HPLC and

then subjected to the analysis by nuclear magnetic resonance (NMR) spectroscopy (Lee et al., 1997). This would provide a detailed structural profile of the small molecules in Peak B, and the low molecular weight Cd-binding components as well as $Cd-PC_2$ could be identified. Leopold et al. (1998) introduced a method of HPLC coupled with ICP-MS to separate and detect Cd-binding protein from plant cell, the detected Cd binding fraction was further identified by ESI MS to be $Cd-PC_2$. The 0.45 M NaCl fraction could be studied further using these methods to isolate and characterize the putative compounds containing putative Cd-thiolate coordination.

Peak B and the 0.45 M NaCl fraction are expected to be composed of small organic molecules responsible for Cd binding, especially the Peak B, which contains the 0.6 to 0.9 kDa unknown components. One possible candidate for this MW range would be phenolics. Flaxseed contains high level of phenolics, but the levels of bound phenolics acids are very low (Oomah et al., 1995). It is expected that most of the phenolics in the flaxseed would be removed by the dehulling process and others would remain in the pellet after aqueous extraction using Tris buffer (Section 2.2.2), therefore, the major components in Peak B and the 0.45 M NaCl fraction would not be phenolics. However, this has to be confirmed by direct experimentation. The structural characterization with HPLC and NMR, or HPLC with MS could be the best solution.

From the preliminary studies on antioxidant activity of different protein fractions, it was found that HMW peak has the strongest effect against cytotoxicity caused by H_2O_2. However, further studies using this HMW peak fraction were unsuccessful, because of the very limited sample quantity obtained from size exclusion chromatography. It is suggested to use bigger sample size and column to obtain more proteins from HMW peak, or characterize

the major components in this fraction, and then use affinity column combined with the immunological technique to trap more proteins directly from the protein extract of flaxseed.

The current studies showed that the protein extract and the major Cd-binding fraction of 0.1 M NaCl from ion exchange chromatography had significant protective effects against the cytotoxicity by H_2O_2 and $CdCl_2$. Protein extract also had obvious promoting effect on THP-1 cell growth. The reasons for the protective effects and also the cell growth promoting effect from flaxseed proteins are not known. Future experiments should be planned to explore the relationship between flaxseed protein and cell growth, and also the underlying mechanism for this phenomenon. The future studies could be focused on the samples from HMW peak, Peaks A and B to study the growth promoting effect and protective effect against the cytotoxicity by H_2O_2 and $CdCl_2$. As well a comparison between the effects of the whole protein extract and the major Cd-binding components of the 0.1 M NaCl fraction should be conducted to pinpoint the responsible components with these active biological activities. Another useful approach for further experiment is to use normal cell line rather than cancerous cell line like THP-1. The results from current experiments were based on cancerous cell lines, it is not very clear if the protective effect of protein extract from flaxseed is equally applicable to normal cell lines.

Further research should be carried out based on the current findings in this study, eg. to further characterize the Cd binding components; to scale up the established method for purification of low Cd content major storage proteins for industry production, and to investigate the biological functions of flaxseed proteins. It is anticipated that further studies could lead to promising applications of flaxseed proteins in food, medicinal research, and industry.

5.3   REFERENCES

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APPENDICES

Appendix 1: Effects of THP-1 Cell Concentration on Color Formation.

The ability to convert the tetrazolium salt MTS into the formazan product varies among different cell types and depends on their metabolic capacity (Promega Corporation, 2005). In order to establish the appropriate cell concentration for subsequent cytotoxicity experiments, cells were harvested and resuspended in fresh medium without FBS, and then serially diluted, and incubated for 6 to 8 hours in a 24-well plate, prior to analysis of cell viability by the cell proliferation assay with MTS/PMS.

The results of color formation in the cell proliferation assay, after varying incubation times with MTS/PMS, are shown in Figure A-1. Color formation (absorbance at 492 nm) was linearly related to cell concentration over the tested range of 0 to 10^6 cells/mL. The regression coefficients were higher than 0.99. The color reaction also increased with incubation time after addition of MTS/PMS. For example, at a cell concentration of 5 x 10^5 cells/mL, the absorbance value after half-hour incubation was 0.3, while the absorbance after 4-hour incubation was 0.79. In the subsequent cell culture experiments, cell concentration of 5 x 10^5 cells/mL and incubation time of 4 hours after addition of MTS/PMS were used for the cell proliferation assay.

Reference:

Promega Corporation. 2005.
   The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay. Printed in USA, Part # TB 169. Available online at: www.promega.com.
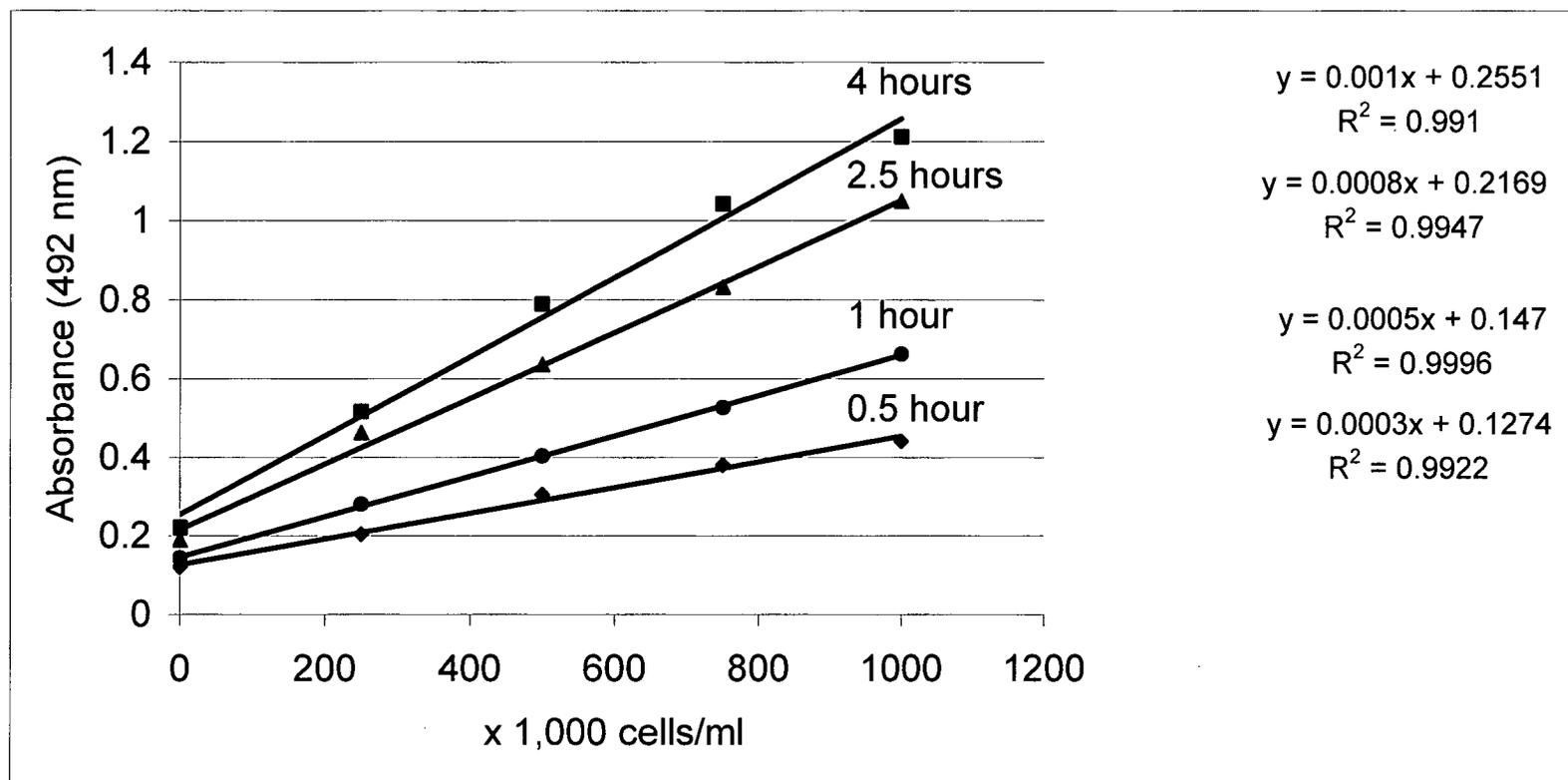
Figure A-1. Effect of cell concentration on color formation (absorbance at 492 nm) in the cell proliferation assay, measured at 0.5, 1, 2.5 and 4 hrs after addition of MTS/PMS. Each point represents the mean of 3 determinations by MTS/PMS, with S.D. of less than 5% of the mean value. The regression equations on the right of the figure is corresponding to each line on the left of the figure, R^2 is the regression coefficient.

Appendix 2: H_2O_2, Fenton System and THP-1 Cell Death

The effects of increasing concentrations of H_2O_2 on the cell death were tested, as shown in Figure A-2. No obvious cell death was observed when H_2O_2 concentration was less than 0.006%. A marked dependence of cell death on H_2O_2 concentration was observed from 0.006 to 0.03% H_2O_2. At H_2O_2 concentration higher than 0.03%, the curve flattened indicating death rate (decrease in absorbance / increase in H_2O_2 concentration) approaching zero. These results indicated that H_2O_2 concentration of 0.03% is enough to cause extensive cell death in THP-1 cell culture.

Because of this result, it is unclear whether the cell death caused in the Experiment of Appendix 2 was in fact the result of Fenton reaction, or to H_2O_2 per se. In the Fenton reaction, the reaction of H_2O_2 with $[Fe^{2+}]$ produces hydroxyl radicals, which lead to the cell death. However, as shown in Figure A-2, 0.03% H_2O_2 used in Fenton reaction in Experiment 2 was too high, since H_2O_2 can not only react with $[Fe^{2+}]$ to produce hydroxyl radical, but it also can itself cause cell death directly. A further experiment was conducted aiming to find out if there was a difference in cell growth between Fenton system and H_2O_2 alone (Figure A-3). The results showed that there was no difference between H_2O_2 treatment (0.03% H_2O_2) and the treatment with Fenton system (0.03% H_2O_2 + 25 μM $FeCl_2$). It could be concluded that the cell death caused in the previous experiment using Fenton system was probably actually caused by direct toxicity from H_2O_2.

Further experiments were conducted using 0.003% H_2O_2, which does not itself cause cell death according to the result mentioned above (Figure A-2); this concentration was also the one used by Halliwell (1987) in a model Fenton system for generating

hydroxyl radical. The results shown in Figure A-4 indicate that this Fenton system (0.003% H_2O_2 + 25 mM [Fe^{2+}]) had no obvious effect to induce THP-1 cell death. This result further confirmed that the cell death in the experiment of Appendix 2 was not caused by this Fenton reaction. However, the Fenton system used by Halliwell (1987) could cause THP-1 cell death, even though the decrease in absorbance was small (Figure A-4). Halliwell's Fenton system used [Fe^{3+}] instead of [Fe^{2+}], and at the same time ascorbic acid was introduced to this system in order to convert [Fe^{3+}] to [Fe^{2+}], which was needed to produce hydroxyl radical. The mechanisms for Fenton reaction are not clear, but mostly it is believed that the following reactions are involved:

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complexes} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

$$Fe^{3+} + H_2O_2 \rightarrow \text{intermediate complexes} \rightarrow Fe^{2+} + O_2^{\bullet -} + 2H^{+}$$

$$Fe^{3+} \xrightarrow{\text{Ascorbic acid}} Fe^{2+}$$
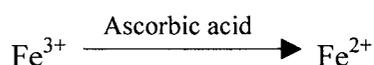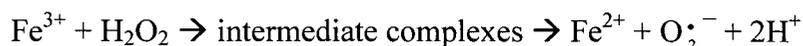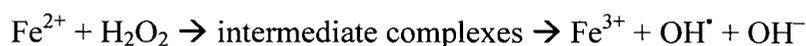
The Fenton system used by Halliwell et al. (1987) only produced 20% decrease in the absorbance of the treated cell culture. This was not enough to be used for testing the protective effect of protein samples against acute toxicity caused to THP-1 cells.

Trolox is a water soluble analogue of vitamin E, which is widely used as an antioxidant in cell culture experiments. Trolox was chosen as an antioxidant calibrator for these experiments because it has been used previously by many researchers with the cell proliferation assay employing MTT (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan). In this experiment, MTS was used instead of MTT. The MTS assay was developed from MTT, and both assays are based on bioreduction of MTS or MTT into a formazan by viable cells. However, further experiment showed that Trolox is not a correct calibrator for antioxidant activity when used with MTS assay and probably for MTT too, because

MTS can be reduced by Trolox itself, causing a strong color reaction. Thus the increase in color reaction in the presence of Trolox can not be correlated with the number of viable cells (Figure A-5). According to Bruggisser et al. (2002), antioxidants such as ascorbic acid, vitamin E and N-acetylcysteine interfered with the MTT tetrazolium assay, and may lead to false positive results.

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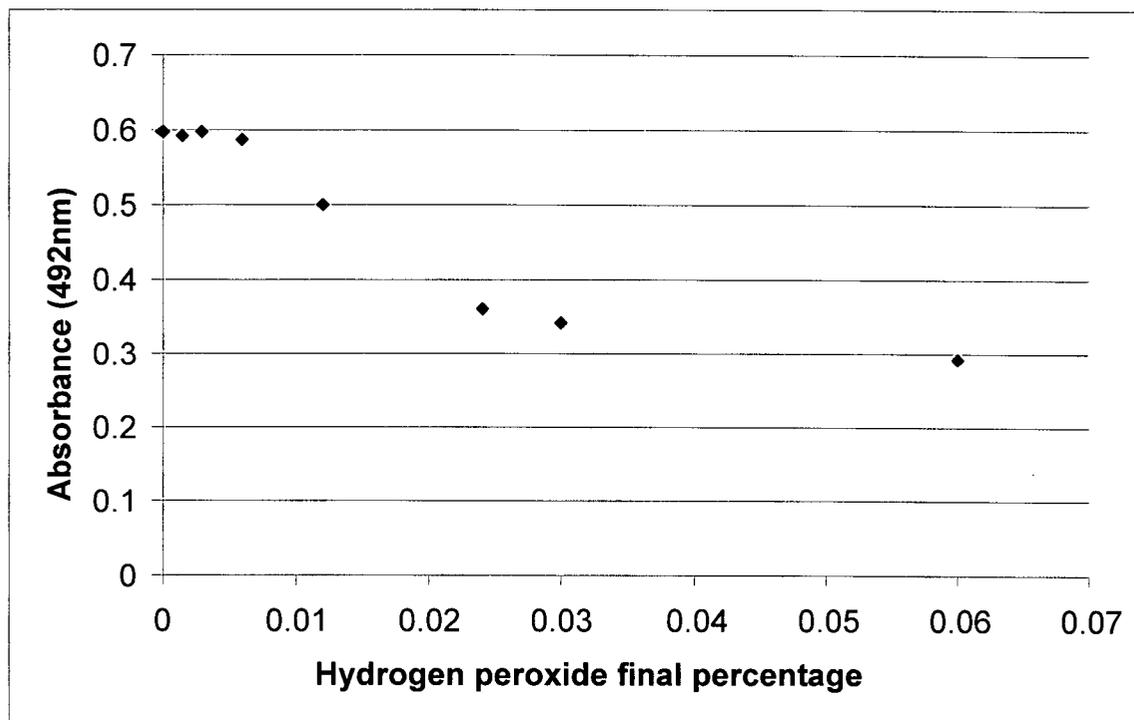
Figure A-2. Effect of hydrogen peroxide concentration on THP-1 cell death as observed by the decrease in absorbance at 492 nm in the cell proliferation assay (0.45×10^6 cells/mL used).
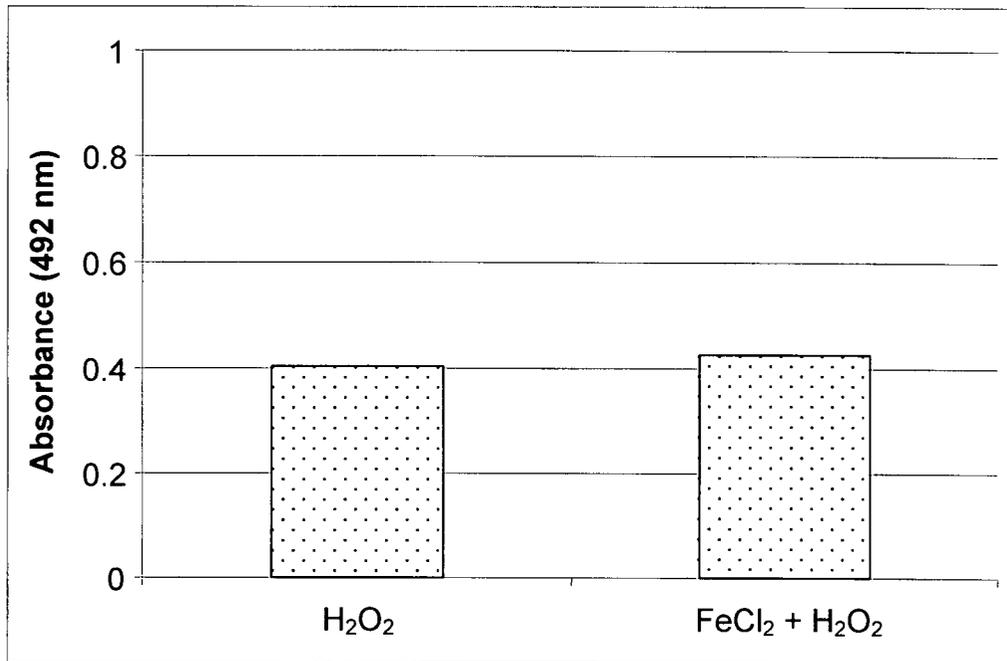
Figure A-3. Comparison of H_2O_2 (0.03%) and Fenton reaction (0.03% H_2O_2 + 25 μM $FeCl_2$) on cell growth as observed by the absorbance at 492 nm in the cell proliferation assay.

1.0

0.8

Absorbance (492 nm)

0.6

0.4

0.2

0.0

Control   H₂O₂   FeCl₂+ H₂O₂   FeCl₃+ H₂O₂
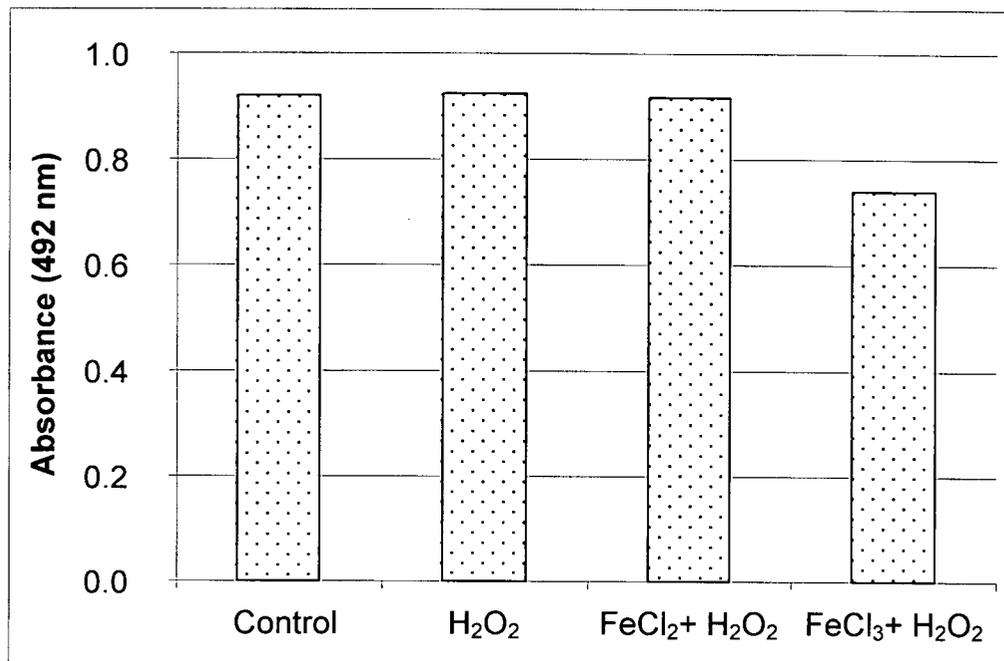
Figure A-4. Absorbance at 492 nm in the cell proliferation assay as a function of H_2O_2 & Fenton system treatments. Treatment of H_2O_2 contained only 0.003% H_2O_2, treatment of $FeCl_2$ + H_2O_2 contained same amount of H_2O_2 and 25 uM $FeCl_2$, treatment of $FeCl_3$ + H_2O_2 contained same amount of H_2O_2 and 25 uM $FeCl_3$ as well as ascorbic acid and EDTA as defined by Halliwell (1987). Control treatment contained no Fenton chemicals.
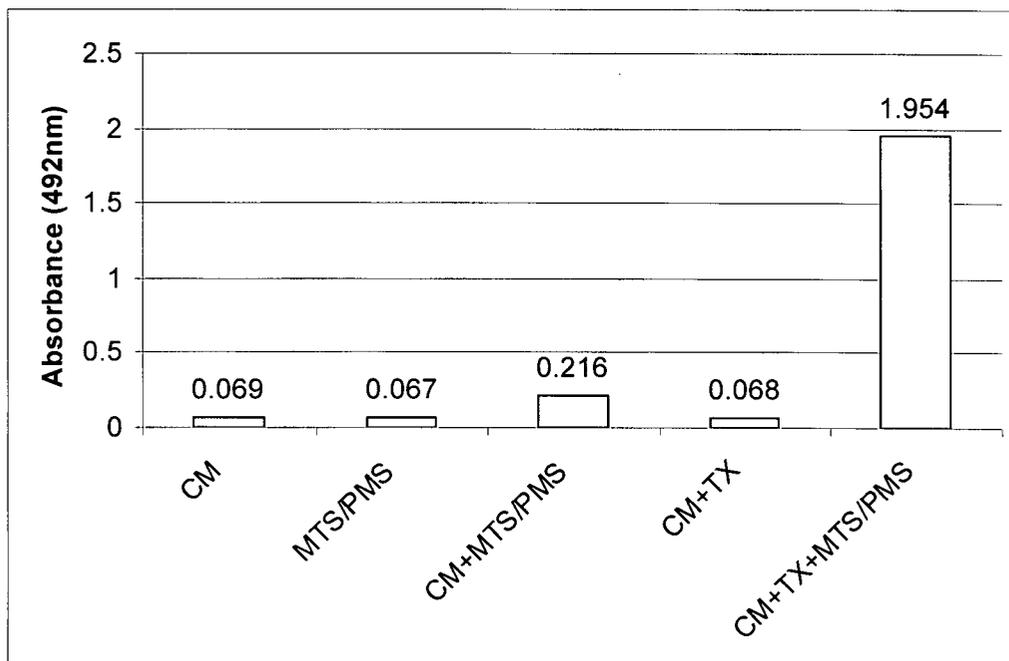
Figure A-5. Background evaluation of the color reaction related to cell proliferation assay. CM represents culture medium without cells, MTS/PMS is the color reagents for cell proliferation assay, TX is Trolox. The number on the top of each bar is the absorbance at 492 nm listed out for easy comparison.

Appendix 3: H_2O_2 and THP-1 Cell Death

H_2O_2 induced THP-1 cell death was investigated, and a dose dependent reaction curve was obtained. In order to clearly express the effect of H_2O_2 toxicity to THP-1 cells, the data were expressed as % relative absorbance (Figure A-6). The negative control without addition of H_2O_2 represent 100% viable cells in the cell culture, while the positive control representing 0% viable cells was measured in the cell culture with addition of highest concentration of H_2O_2 (0.1%), conditions under which all cells were dead as observed microscopically. The level of cell viability was determined using equation (1).

The cell death caused by H_2O_2 was trivial at concentrations not exceeding 0.003%, which is the concentration usually used for the Fenton reaction (Halliwell et al., 1987). Over 90% cell death was observed in the presence of 0.06% H_2O_2, whereas 50% lethality was observed at 0.018% H_2O_2. In subsequent cell culture experiments, 0.06% H_2O_2 was used to test protective effects of protein samples against direct oxidative stress.

References:

Halliwell, B.; Gutteridge, J. M.; Aruoma, O. I. 1987.
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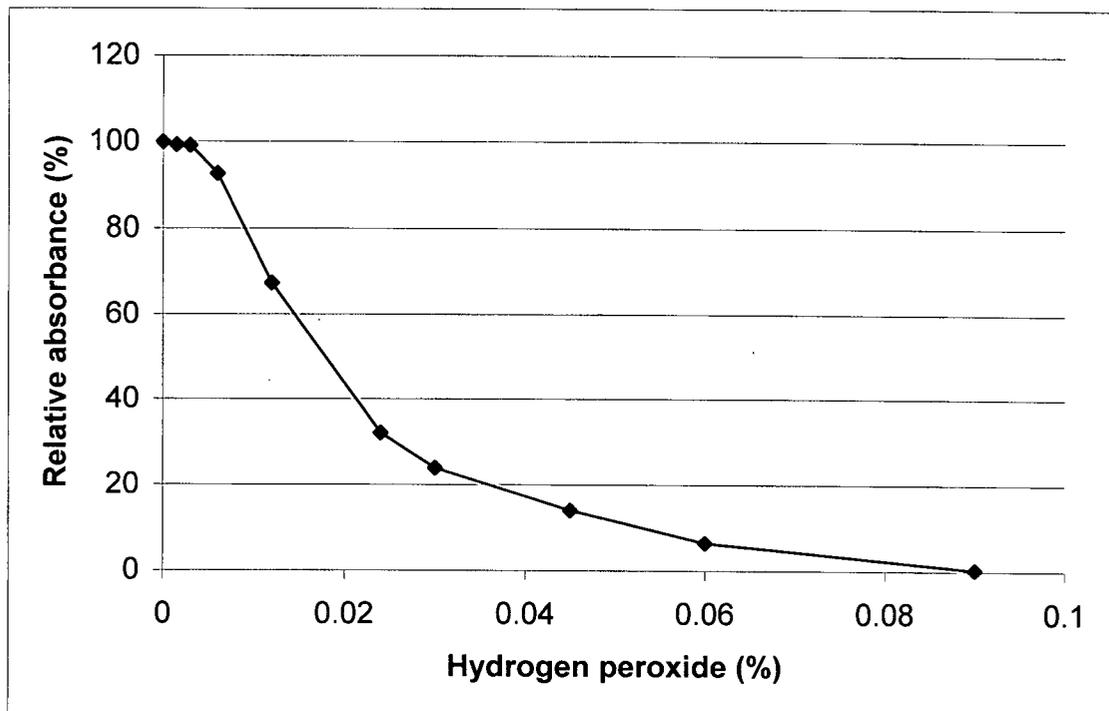
Figure A-6. Influence of hydrogen peroxide on THP-1 cell death as observed by the absorbance at 492 nm in the cell proliferation assay.

Appendix 4: Cd and THP-1 Cell Death

Cd-induced cell death of THP-1 cells was investigated with increasing concentrations of $CdCl_2$ in the range from 0 to 1000 µM (Figure A-7). Cell death was dependent on $CdCl_2$ concentration in the range of 0 to 600 µM. The death curve was sigmoidal in shape, with 50% cell death at 150 µM $CdCl_2$. A concentration of 600 µM $CdCl_2$ was required to kill over 90% of the cells. About 70% cell death occurred at $CdCl_2$ concentration of 300 µM. This concentration was used in subsequent experiments to evaluate protective effects of flaxseed proteins against Cd-induced cytotoxicity.
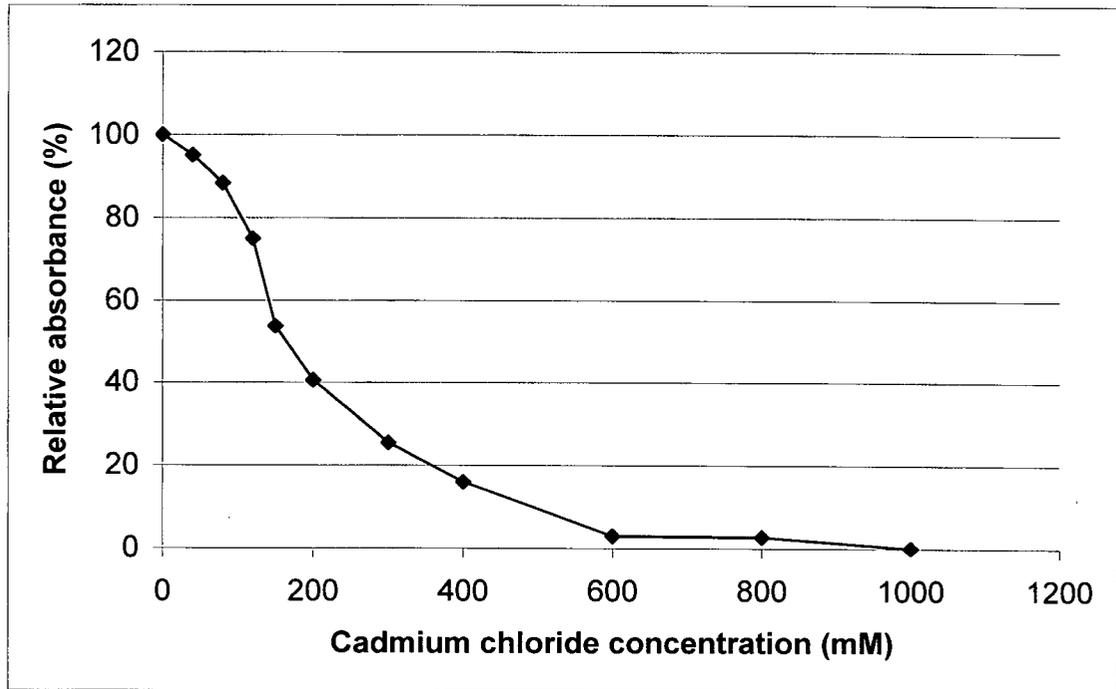
Figure A-7. Cytotoxic effect of various concentrations of cadmium on THP-1 cells. Each point represents the mean of 3 determinations with S.D. value less than 5% of the mean value.