# CYCLOLINOPEPTIDES IN FLAXSEED AND FLAXSEED PRODUCTS

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by

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# ABSTRACT

Cyclolinopeptides (CLPs), a group of naturally occurring, hydrophobic, cyclic peptides in flax, have attracted a great deal of attention due to their immunosuppressive activity. The purpose of this project was to increase our understanding of the occurrence of CLPs in flaxseed, flaxseed tissues and flaxseed products.

In the first study, systematic methods for CLP extraction, isolation, detection and quantification were developed. The solubility of CLPs in acetone led to its use as a preferred solvent for extraction of CLPs and other hydrophobic compounds from whole flaxseed. Solid phase extraction with a silica gel column followed by selective elution with organic solvents of increasing polarity enabled the isolation of a crude peptide-rich fraction. Reverse phase HPLC chromatography of peptide-rich fractions provided a method for separation and quantification of CLPs.

In the second study, the levels of CLPs in cultivars of flaxseed were studied to determine if there was any impact of flax genotype or environment on peptide levels. The concentration of total CLPs varied from 189 μg/g (Flanders) to 303 μg/g (Somme) in the cultivars tested. Environment, cultivar and their interaction affected the observed concentration of CLPs.

In the third study, the concentrations of CLPs in fractions produced from flaxseed were measured by HPLC in seed coat, cotyledon and oil bodies. The concentration of CLPs was higher in the cotyledon than in the seed coat. The highest CLP concentrations were found in the oil bodies.

In the fourth study, CLP levels in flaxseed oil were measured during and after oil extraction and refining. The concentration of CLPs was higher in expeller-extracted crude oil and solid foots and lower in flaxseed meal. A comparison of CLP levels in flaxseed oil extracted with a small expeller and in commercially-produced flaxseed oil was performed. Crude flaxseed oil produced with a small expeller had higher levels of peptides than were observed in commercial flaxseed oil available at a local retail health food store. The effect of oil refining processes, including acid degumming and alkali refining on CLP stability, was studied. Acid degumming using 1% $H_3PO_4$ effectively removed all CLPs. Alkali refining was also demonstrated as being effective at decreasing levels of CLPs, although it failed to remove all peptides.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin converting enzyme |
| ADF | Acid detergent fiber |
| ALA | Alpha-linolenic acid |
| CCK | Cyclic cystine knot |
| CD | Circular dichroism |
| CLA | Linolenic acid |
| CLP | Cyclolinopeptide |
| COSY | Correlation spectroscopy |
| CsA | Cyclosporin A |
| DAD | Diode array detector |
| DMSO | Dimethysulfoxide |
| DTH | Delayed type hypersensitivity |
| ESI-MS | Electrospray ionization-mass spectrometry |
| ESI-MS/MS | Electrospray ionization tandem mass spectrometry |
| ESI-TOF-MS | Electrospray ionization-time of flight-mass spectrometry |
| FLC | Flax lignan complex |
| FFA | Free fatty acid |
| FTIR | Fourier transform infrared spectroscopy |
| HDL-C | High-density lipoprotein cholesterol |
| HIR | Humoral immune response |
| HMBC | Heteronuclear multiple bond correlation |
| HMQC | Heteronuclear multiple quantum coherence |

| | |
|---|---|
| HPLC | High performance liquid chromatogram |
| HR-FABMS | High resolution fast atom bombardment mass spectrometry |
| I.V. | Intravenous administration |
| IL | Interleukin |
| IR | Infrared spectroscopy |
| LA | Linoleic acid |
| LC-MS | Liquid chromatography-mass spectrometry |
| LDL-C | Low-density lipoprotein cholesterol |
| NMR | Nuclear magnetic resonance |
| NHP | Non hydratable phospholipids |
| PA | Phosphatidic acid |
| PE | Phosphatidyl ehanolamine |
| PFC | Plaque forming cell number determination |
| P. O. | *per os* (Oral administration) |
| Q-RTPCR | quantitative real time reverse-transcriptase olymerase chain reaction |
| ROESY | Rotating frame overhause effect spectroscopy |
| SDG | Secoisolariciresinol diglucoside |
| SDG-HMG | Hydroxymethyl glutaryl ester linked complex |
| Seg-A | Segetalin A |
| TAG | Triacylglycerides |
| TC | Total cholesterol |
| TG | Triglycerides |
| TNF-alpha | Tumour necrosis factor-α |

# 1 INTRODUCTION

Flax (*Linum usitatissimum* L.), one of the oldest cultivated crops, has been and is widely grown for oil, fibre and, more recently, food (Oomah, 2001). The average worldwide flaxseed production between 1999 and 2008 was 2,220,000 tonnes (FAO, 2010). Significantly, Canada accounted for 35% of this production. Flaxseed oil can be used in paints, varnishes and inks due to its fast-drying property. Flax stems have a high fibre content, which makes them a good source of fibre for linen and paper production. With increasing demand for edible oil sources of omega-3 fatty acids, oleaginous flaxseed, the oil of which can have greater than 50% alpha-linolenic acid (ALA), is widely marketed as a functional food. Flaxseed is also added to animal feed to improve animal performance and health.

Flaxseed is widely accepted as a healthy food and numerous beneficial effects have been associated with flaxseed consumption in controlled experimental diets (Cunnane *et al.*, 1993; Jenkins *et al.*, 1999; Clark *et al.*, 1995). For instance, consumption of flaxseed flour reduces epithelial cell proliferation and nuclear aberrations in female rat mammary glands. This finding indicates that dietary flaxseed may reduce the growth rate of mammary cancer (Serraino and Thompson, 1991). It has been found that flaxseed lignan and oil components reduce mammary tumour growth in the later stages of carcinogenesis (Thompson *et al.*, 1996). Supplements of 14% flaxseed oil and 20% flaxseed meal reduce the incidence of azoxymethane-induced aberrant crypt foci formation in Fisher 344 male rats (Williams *et al.*, 2007a, 2007b). Similarly it has been shown that the substitution of corn meal with flaxseed meal (15%) or corn oil with flaxseed oil (15%) in a basal diet significantly decreased tumour multiplicity and size in the small intestine and colon in Fisher male rats. They concluded that flaxseed meal and oil may be considered as an effective chemo-preventive agents (Bommareddy *et al.*, 2009). Inclusion of 20% flaxseed in rat diets decreased plasma total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) by 21%, 33.7%

and 23%, respectively; supplementation with 30% flaxseed had a more significant effect, reducing the same factors by 33%, 67% and 23% (Ratnayake, 1992). In human studies, 15 g/d of flaxseed administered for three months was associated with reduction in serum TG and LDL-C without any alteration of high-density lipoprotein cholesterol (HDL-C) (Bierenhaum, 1993). It was also reported that consumption of 50 g flaxseed per day for four weeks lowered the plasma LDL-C by 8% in young healthy adults (Cunnane, 1995). These results support the hypothesis that flaxseed consumption has a positive effect on suppressing the development of atherosclerosis. However, it is not possible to attribute the health benefits of flaxseed consumption to a sole component present in flaxseed. Polyunsaturated fatty acids, lignan complex and CLPs are three major functional classes of compounds that might induce some or all of the observed experimental results.

The knowledge of the biological roles of flaxseed polyunsaturated fatty acids and lignan is substantial and the research about their existence, biosynthesis and metabolism in flaxseed is mature compared to that of cyclolinopeptides (CLPs). The study of CLPs has been undergoing for more than half a century and the major research has been done on the identification and conformation of CLPs, as well as their biological activities. CLPs, the main focus of this thesis, are a group of cyclic, hydrophobic peptides containing eight or nine amino acid residues with molecular masses of approximately one thousand Da. CLP-A was the first CLP identified after it was isolated from the sediments deposited from crude flaxseed oil (Kaufmann and Tobschirbel, 1959). In 1968, Weygand discovered a similar cyclic nonapeptide, CLP-B. Between 1997 and 2001, nine additional CLPs (C, D, E, F, G, H, I, J and K) were identified from the seed and root of flax (Morita *et al.*, 1997a; Morita *et al.*, 1999; Matsumoto *et al.*, 2001a). In addition, a cyclic peptide, CLP-X, containing the non-protein amino acid N-methyl-4-aminoproline, was isolated and characterized (Picur *et al.*, 1998). CLPs occur in flaxseed, but the role of these compounds is largely unknown. *In vitro* studies of CLP biological activity have been described in numerous publications (Kessler *et al.*, 1986a, 1986b; Wieczorek *et al.*, 1991; Górski *et al.*, 2001; Gaymes *et al.*, 1997; Siemion, 1999). For instance, CLP-A has the ability to inhibit cholate uptake into hepatocytes, potentially protecting the liver against poisoning (Kessler, 1986). This CLP can also inhibit the activation and proliferation of T-lymphocytes by suppressing the

activity of phosphatase in T-cell activation (Wieczorek *et al.*, 1991; Górski *et al.*, 2001). Immunosuppressive activity of CLPs described by others may be partially or wholly explained by this observation, e.g. delaying hypersensitivity response, postponing skin allograph rejection, suppressing post adjuvant arthritis and haemolytic anemia (Gaymes *et al.*, 1997; Siemion *et al.*, 1999).

None of the research has illustrated the levels of CLPs in flaxseed. The overall focus of this project was to develop a systematic method for quantifying CLPs in flaxseed and flaxseed-related materials. The objectives of this research were as follows:

**Objective 1: To establish methods for CLP extraction**, **isolation**, **detection and quantification.**

Hypothesis: An HPLC method using an internal standard may be developed that will allow accurate measurement of CLPs in flaxseed and flaxseed products.

**Objective 2: To determine CLP levels in different flaxseed cultivars.**

Hypothesis: The concentration of CLPs in flaxseed might vary among genotypes and might be affected by environmental conditions during seed development.

**Objective 3: To confirm the CLP distribution in flaxseed fractions and tissues.**

Hypothesis: CLPs concentration may vary among different flaxseed fractions such as seed coat, cotyledon and oil bodies.

**Objective 4: To compare the levels of CLPs in lab-pressed flaxseed oil and commercial flaxseed oil and investigate the effects of acid degumming and alkali refining on the levels of CLPs in lab-pressed flaxseed oil.**

Hypothesis: A portion or all of the CLPs in flaxseed oil may be removed by liquid and solid phase refining.

# 2 LITERATURE REVIEW

## 2.1 Flaxseed

Mature seed of oleaginous flax is oblong, flat and composed of an embryo consisting of two cotyledons surrounded by a thin endosperm and a smooth often shiny seed coat (hull) that varies in colour from yellow to dark brown (Figure 2-1) (Peterson, 1958). The composition of flaxseed is presented in Table 2-1 (Smith, 1958; Hadley *et al.*, 1992). Lipid, protein and fibre are three major constituents of flaxseed. An analysis of brown Canadian flaxseed conducted by the Canadian Grain Commission (2001) showed the average composition of commercial seed was 41% fat, 20% protein, 28% total dietary fibre, 7.7% moisture and 3.4% ash. Other minor components include cyanogenic glycosides, phytic acid, phenolics, trypsin inhibitor, linatine, lignans (phytoestrogens), minerals, vitamins and CLPs (Bhatty, 1995; Morita *et al.*, 1997a; Matsumoto *et al.*, 2002).

Protein content of flaxseed varies widely from 10.5-31% largely due to genetic and environmental factors (Bajpai *et al.*, 1985; Salunkhe and Desai, 1986; Oomah, 1993a). Seed protein is stored mainly in aleurone tissues. Approximately 56-70% of the protein is found in the cotyledons and about 30% in seed coat and endosperm (Dev *et al.*, 1986; Sosulski and Bakal, 1969). According to Oomah and Mazza (1993b), flaxseed meal has an essential amino acid index of 69, compared to 79 for soybean meal. The amino acid patterns of flax protein from two flax varieties are compared with that of soybean and listed in Table 2-2 (Oomah and Mazza, 1993b; Bhatty and Cherdkiatgumcha, 1990b). The essential amino acids found in flaxseed meal from both varieties are similar to those in soy flour, which makes flaxseed meal a source of one of the most nutritious plant proteins.

The major carbohydrates in flaxseed are soluble and insoluble fibre where the level of insoluble fibre is more than that of soluble fibre. Cui (2001) reported contents of insoluble and soluble fibre of 20% and 9% respectively while Hadley *et al.* (1992) reported 30% and 10% respectively. The soluble fibre mainly exists in the epidermal

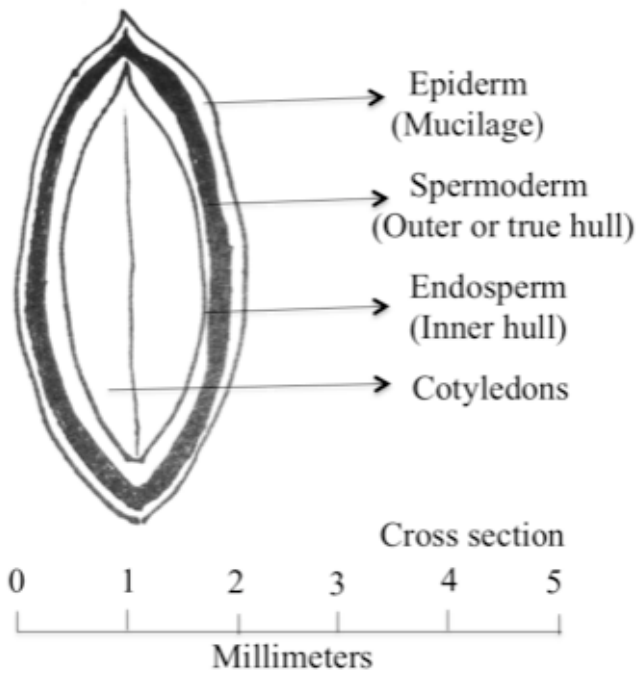



Figure 2-1 Anatomical structure of flaxseed (Modified from Peterson, 1958)

Table 2-1 Flaxseed composition (Smith, 1958; Hadley *et al.*, 1992)

| Constituent (%) | Whole seed | Embryo[1] | | Hull[2] | |
|---|---|---|---|---|---|
| | | With fat | Without fat | With fat | Without fat |
| Moisture | 7.13 | 4.31 | NR[3] | 7.89 | NR[3] |
| Nitrogen | 4.01 | 4.64 | 10.92 | 3.18 | 3.52 |
| Oil | 38.7 | 53.20 | NR[3] | 1.84 | NR[3] |
| Fiber | | | | | |
| (Soluble) | 10.2 | NR[3] | NR[3] | NR[3] | NR[3] |
| (Insoluble) | 30.4 | NR[3] | NR[3] | NR[3] | NR[3] |
| Ash | NR[3] | 3.38 | 7.95 | 2.99 | 3.31 |
| Weight fraction | NR[3] | 58.60 | 40.40 | 41.40 | 59.96 |
| % of total oil | | | 96.70 | | 3.30 |

[1] Cotyledons and embryo

[2] Seed coat

[3] Not reported

Table 2-2 Amino acid compositions of flaxseed and soy flour (Oomah and Mazza, 1993b; Bhatty and Cherdkiatgumcha, 1990)

| **Amino acid (g/100 g protein)** | **Flax Cultivar** | | **Soy flour** |
|---|---|---|---|
| | **Brown flax (NorLin)** | **Yellow flax (Omega)** | |
| Alanine (Ala) | 4.4 | 4.5 | 4.1 |
| Arginine (Arg) | 9.2 | 9.4 | 7.3 |
| Aspartic acid (Asp) | 9.3 | 9.7 | 11.7 |
| Cystine (Cys) | 1.1 | 1.1 | 1.1 |
| Glutamic acid (Glu) | 19.6 | 19.7 | 18.6 |
| Glycine (Gly) | 5.8 | 5.8 | 4.0 |
| Histidine (His)* | 2.2 | 2.3 | 2.5 |
| Isoleucine (Ile)* | 4.0 | 4.0 | 4.7 |
| Leucine (Leu)* | 5.8 | 5.9 | 7.7 |
| Lysine (Lys)* | 4.0 | 3.9 | 5.8 |
| Methionine (Met)* | 1.5 | 1.4 | 1.2 |
| Phenylalanine (Phe)* | 4.6 | 4.7 | 5.1 |
| Proline (Pro) | 3.5 | 3.5 | 5.2 |
| Serine (Ser) | 4.5 | 4.6 | 4.9 |
| Threonine (Thr)* | 3.6 | 3.7 | 3.6 |
| Tryptophan (Trp)* | 1.8 | NR[1] | NR[1] |
| Tyrosine (Tyr) | 2.3 | 2.3 | 3.4 |
| Valine (Val)* | 4.6 | 4.7 | 5.2 |

[1]NR = Not reported.

*Essential amino acids for humans

layer of the seed coat and can be extracted with water. Seed coat soluble fibre is known as mucilage that consists of both acidic and neutral polysaccharides. The acid polysaccharide fraction is largely composed of L-rhamnose (25.3%), L-galactose (11.7%), L-frucose (8.4%) and D-xylose (29.1%) while the neutral polysaccharide consists of L-arabinose (20%) and D-xylose/D-galactose (76%) (Anderson *et al*., 1947). Insoluble fibre is composed of cellulose (7-11%), lignin (2-7%) and acid detergent fibre (ADF) (10-14%) (Cui, 1994).

Oil content of flaxseed varies from 38-44% due to genotype and environment though extraction methods may contribute to some of the variation (Van Uden *et al.*, 1994; Oomah and Mazza, 1997). Oil is mainly stored in the endosperm and cotyledons in the form of cell bound microscopic droplets or oil bodies also known as oleosomes. Dorrell reported that the embryo was 45% oil while cotyledons were 51% oil and a fraction comprising seed coat and endosperm contained 23% oil (Dorrell, 1970). Fatty acid composition varies among different flaxseed types and cultivars. Most flaxseed oil (75%) is found in cotyledons, the remainder (22%) mainly exists in the seed coat and endosperm (Dorrell, 1970). The oil is primarily in the form of triacylglycerides (TAGs) with a fatty acid profile typically including linolenic (52%), linoleic (17%), oleic (20%), palmitic (6%) and stearic (4%) acids (Green, 1990). The minor lipids and lipid soluble compounds include monoacylglycerides, diacylglycerides, tocopherols, sterols and sterol-esters, phospholipids, waxes, CLPs, free fatty acids (FFAs), carotenoids, chlorophyll and other compounds. The oxidative instability of alpha-linolenic acid (ALA) present in the oil renders it unsuitable for use as edible cooking oil. In order to produce flaxseed oil with improved food properties, Australian scientists selected a new genetic variant Linola® with improved oxidative stability. In Linola varieties, the level of linoleic acid (LA) content is above 65% and ALA below 2% (Green and Dribnenke, 1994). The fatty acid composition of Linola is similar to that of oils from sunflower, safflower or corn, making Linola a more suitable edible oil source (Haumann 1990; Green and Dribnenke, 1994).

ALA, an essential polyunsaturated fatty acid in flaxseed, cannot be synthesized by human metabolism and contributes to various important physiological effects of dietary flaxseed oil. It is the intermediate in biosynthesis of hormone-like eicosanoids,

which regulate inflammation and immune function in higher animals (Mantzioris *et al.*, 1994, 1995). For example, ALA treatments exert variable effects on inflammatory mediators and markers depending on dose: ALA for 4 weeks at 14 g/d decreased the production of tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and cytokines in humans, while a lower dose did not have this effect (Caughey, 1996; Thies, 2001; Wallace *et al.*, 2003). ALA supplementation with 6% ALA depresses the levels of IL-6 and IL-10 and increases the production of TNF-α in mice (Chavali *et al.*, 1998). It was suggested that the ratio of omega-6 to omega-3 fatty acids plays an important role in suppressing atherosclerosis, in that a lower omega-6 to omega-3 fatty acid ratio decreased atherosclerosis compared to a higher ratio in apolipoprotein E, LDL receptor double knockout mice. After feeding Golden Syrian hamsters 20 g/d ALA for six weeks serum cholesterol was reduced by 17-21% (Yang *et al.*, 2005). However, no changes were found in serum TC, LDL-C or HDL-C in healthy subjects or hyperlipidemic patients (Freese and Mutanen, 1997; Sanders and Roshanai, 1983; Kestin *et al.*, 1990; Singer *et al.*, 1990). David (1983) suggested that ALA might lower the growth rate of breast and colon cancers. It is worth noting that almost all literature extolling the beneficial functions of flaxseed oil fails to confirm it is ALA itself, rather than other bioactive compounds found in flaxseed oil or their interactions, that contributes the observed health benefits.

Flax lignan and flax lignan complex (FLC) comprise a group of oil-insoluble flax compounds that are reported to have multiple physiological effects in animals and humans. FLC, which is not oil soluble, is composed of 34-38% secoisolariciresinol diglucoside (SDG), 15-21% cinnamic acid glucoside and 9.6-11% hydroxymethylglutaric acid (Westcott and Paton, 2001). The lignan complex is reported to slow the progression of atherosclerosis in humans and other mammals (Prasad, 2005; Prasad *et al.*, 2009a, 2009b; Zhang *et al.*, 2008). Treatment with FLC (40 mg/kg body wt/d) for eight weeks suppressed the development of hypercholesterolemic atherosclerosis by 34% in rabbits (Prasad, 2005). Hypercholesterolemic humans were treated with 300 mg or 600 mg of FLC for eight weeks. The 300 mg dose reduced TC and LDL-C by 15% and 17%, respectively, without any change in the ratio of TC/HDL-C, while 600 mg reduced the serum TC and LDL-C by 24% and 22%, respectively, with

a decrease in the TC/HDL-C ratio (Zhang *et al.*, 2008). Prasad *et al.* (2009b) also found that FLC was effective in slowing the progression of atherosclerosis by 31% in hyperlipidemic rabbits, along with reducing oxidative stress.

## 2.2 Flaxseed oil processing, refining and flavour chemistry

Currently, most flax is grown for industrial or food oil production. Edible flaxseed oil may be recovered by cold-pressing alone or a process of pre-pressing followed by solvent extraction (Kochhar, 2002; Goss, 1946). Cold pressing refers to a process in which no heat has been used on the oilseeds before passing through an expeller press (Fils, 2000). Prior to pressing, seeds are normally flaked then fed to the expeller press. Most of the solids are recovered from pressing as a partially defatted meal containing less than 10% oil. Partially defatted meal may be extracted with a solvent such as hexane to increase total oil recovery, but the industry avoids this process as flax meal with 10% oil content is a preferred animal feed. The crude oil is collected and settled to separate solids, gums and waxes (as "foots") from the oil before further refining. The oil-refining process is applied to the oil collected from cold-pressing and solvent extraction to obtain oil for human consumption (Figure 2-2).

### 2.2.1 Cold pre-pressing

Mechanical expeller presses can be used to extract flaxseed oil by applying pressure and shear forces on the seeds to decrease seed volume (Zheng *et al.*, 2003). While it is said to be important not to apply heat during pressing because the higher temperature will cause rapid oxidation of the oil, this is not observed in industrial processing (Reaney, Pers. Commun.). The products of pressing flaxseed are crude oil that varies from yellow to dark brown and defatted meal.

### 2.2.2 Settling and filtration

During pressing, phospholipids, wax and some fibre will dissolve in the crude oil and settling in the tanks for several days is needed to allow time for foots separation. After suspended solids have settled the oil may be filtered using a plate and frame filter and filtration improving solids (Patterson, 1989).

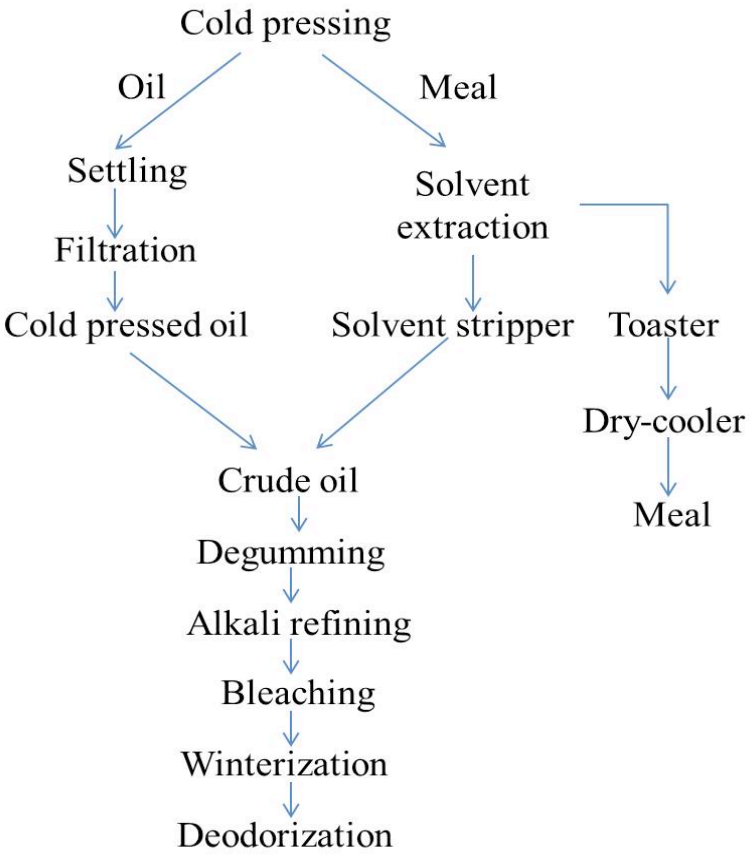



Figure 2-2 Cold pressed flaxseed oil processing (modified from Booth, 2004)

### 2.2.3 Solvent extraction

Solvent extraction is practiced to increase oil recovery after pressing (Goss, 1946). Hexane is approved for food use and efficiently dissolves triglyceride oil. It has high stability and is available in high purity commercial forms. After extraction, hexane is recovered from oil by a solvent stripper and from meal through a desolventiser-toaster. Most flaxseed meal is not extracted with solvent.

### 2.2.4 Degumming and alkali refining

Phospholipids or gums are removed from vegetable oil as part of the oil refining process. Although most phospholipids present in flaxseed oil are removed by filtration, some remain in crude oil after this treatment. Phospholipids may be removed by water degumming, which involves adding 2-4% water and mixing under vacuum at 80°C for 10-30 min (Brekke, 1975). The procedure produces sludge of hydratable phospholipids that are easily removed from the oil. The addition of phosphoric acid to oil at elevated temperature (0.13-0.53% of 75% $H_3PO_4$) can significantly improve the removal of gums (Sullivan, 1955). In some processes, gum removal immediately follows alkali refining. In such processes, the excess $H_3PO_4$ and FFAs are neutralized by mixing the oil with dilute sodium hydroxide (NaOH) forming a soap stock mixed with oil. Centrifugation of the mixture separates the soap stock from the oil. After alkali refining the oil is subject to vacuum drying to remove any water remaining in the oil.

### 2.2.5 Bleaching

Bleaching removes carotenoid and chlorophyll pigments from flaxseed oil, producing desired lighter-yellow oil colour. Acid-treated bentonite clay is activated by heat treatment to absorb the pigments, soaps from alkali refining, metals and other contaminants. Most flaxseed oil is not bleached as the oxidative stability is reduced by the removal of antioxidants during bleaching (Klein *et al.*, 1984). Flaxseed oil arising from Linola is an exception as this oil is relatively stable after bleaching.

### 2.2.6 Winterization

Cloudiness due to the trace amount of wax in vegetable oil may be reduced by

low-temperature winterization (Kreulen, 1976). Winterization involves chilling oils so that waxes crystallize. Subsequent filtration affords a separation of the solid wax from the oil. Most flaxseed oil is not winterized because it does not form precipitates on storage at cool temperatures (Reaney, Pers. Commun.).

### 2.2.7 Deodourization

Deodourization is the final step for vegetable oil refining (Tubaileh *et al.*, 2002). Deodourization involves steam distillation as a method to remove the volatile compounds which often contribute to the odour and unpleasant flavour of the oil. Steam distillation will also remove aldehydes, ketones and acids produced from peroxide breakdown after fatty acid oxidation. Tocopherol losses are a negative effect of deodourization because tocophenols are natural antioxidants which can keep highly unsaturated flaxseed oil from rapid oxidation. Most flaxseed oil is not deodourized (Reaney, Pers. Commun.). Flaxseed oil arising from Linola is an exception as this oil is fully processed and sold as a fully refined product.

### 2.2.8 Refined oil storage and oil flavour chemistry

Refined flaxseed oil has several desired qualities such as low phosphorus content and light colour compared to crude oil (Table 2-3). For Linola oil with high level of LA, oil storage conditions are similar to those suitable for sunflower oil because they are alike in fatty acid composition. However, for traditional flaxseed oil with a high content of ALA, the edible oil should be stored under cool (less than 4°C), oxygen-depleted, dark conditions with an antioxidant added to prevent rapid oxidation. Most of the flaxseed oil products sold as functional food is distributed and sold in opaque plastic or brown glass to limit degradation by light and flushed with nitrogen at the time of bottling to preserve freshness (Wiesenborn *et al.*, 2005). Additionally, it is recommended that these oils be stored at 4°C.

Fresh, unrefined flaxseed oil usually presents a mild, nutty and pleasant flavour. After short-term storage, paint-like and fishy odours and fishy and bitter flavours often arises. The flavour and odour chemistry is too complex to be attributed to a sole factor. Seed quality, variety, processing, handling and storage all contribute to the flavour of

Table 2-3 Analytical data for crude and refined flaxseed (Linola) oil (Green and Dribinenke, 1994)

| Parameters | Crude oil | Refined oil |
|---|---|---|
| Refractive index (46 ºC) | 1.4657 | 1.4665 |
| Specific gravity | 0.921 | 0.920 |
| Viscosity | 46.8 | 46.4 |
| Phosphorus (mg/kg) | 325 | <0.5 |
| Chlorophyll (mg/kg) | 0.4 | 0.0 |
| Free fatty acid (as % oleic) | 0.3 | <0.02 |
| Iodine value | 142 | 144 |
| Fatty acid composition (% wt) | | |
| 16:0 | 5.6 | 5.6 |
| 18:0 | 4.0 | 4.0 |
| 18:1 | 15.9 | 15.9 |
| 18:2 | 71.8 | 71.9 |
| 18:3 | 2.0 | 2.0 |
| Others | 0.7 | 0.6 |
| Sterols (mg/kg) | 3095 | 2324 |
| Tocopherols (mg/kg) | 507 | 172 |

flaxseed oil. Several phytochemicals, including phenolic compounds, FFAs, carbonyl products, products of oxidation and small hydrophobic peptides are believed to correlate with the off-flavour of flaxseed oil. The fishy flavoured compounds found in oxidized flaxseed oil are identified as carbonyl compounds including cis-4-heptenal (Seals and Hammond, 1970). Flaxseed oil is readily oxidized to produce peroxides which can further breakdown into smaller molecules such as aldehydes, acids and alcohols, contributing to the unpleasant rancid flavour. Arai *et al.* attributed the dark colour, bitter taste and objectionable flavour of some oils to phenolic constituents (Arai *et al.*, 1966). The phenolic compounds found in flaxseed, are mainly a complex of SDG, hydroxymethyl glutaric acid, ferulic acid glucoside and p-coumaric acid glucoside (Davin *et al.*, 1997; Ford *et al.*, 2001; Schoenrock *et al.*, 1997). However, this complex is not soluble in vegetable oil. FFAs which contribute to the rancid flavour in butter fats and certain tallows are present at low levels in most vegetable oils (Bills *et al.*, 1969) and should not be present in large amounts in good quality flaxseed or flaxseed oil.

The occurrence of a small hydrophobic peptide, more specifically, CLP-E, on the other hand, contributes a bitter flavour to flaxseed oil according to the study of Brühl *et al*. (2007). Brühl *et al*. (2007) demonstrated that the delicate nutty flavour of freshly pressed flaxseed oil is replaced by a bitter flavour during storage. The key bitter compound was isolated and identified as CLP-E by different analytical tests. Fourier transform infrared spectroscopy (FTIR), liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance (NMR) spectroscopy and amino acid analysis have all proved useful in determining the CLP-E content of flaxseed oil. The finding that CLP-E has a bitter flavour is consistent with theories that predict the bitter tasting potency of peptides. In 1971, it was first reported that peptides with hydrophobicity values more than 1400 cal/mole and molecular weight less than 6 kDa contribute a strong bitterness. Moreover, those peptides with Leu, Pro, Phe, Tyr, Ile and Trp have a tendency to be bitter (Ney, 1971). The presence of Pro residues has been found to be a major contributor to peptide bitterness (Ishibashi *et al*., 1988). It has recently been demonstrated that bitterness is determined by polarity, hydrophobicity and the spatial structure of the peptides (Kim *et al*., 2008).

## 2.3 Plant cyclopeptides

Plant cyclopeptides are cyclic compounds found in higher plants. They are often composed of 2 to 37 amino acids. While the amino acids typically found in Eukaryotic proteins are common, non-protein amino acids and D-amino acids are both found. Tan and Zhou (2006) reviewed the chemistry of plant cyclopeptides and reported structures of 455 cyclopeptides in Caryophyllaceae, Rhamnaceae and other 24 families. In their review they divided plant cyclopeptides into two classes, five subclasses and eight types according to their skeletons and distributions in plants (Figure 2-3). Among them, cyclopeptide alkaloids (Type I), Caryophyllaceae-type cyclopeptides (Type VI) and cyclotides (Type VIII) are the three largest groups due to the large numbers (185, 168 and 51 respectively) of cyclopeptides belonging in these categories.

### 2.3.1 Distribution and biological activities of plant cyclopeptides

Tan and Zhou described the distribution of cyclopeptides in plants in 2006. They stated that "455 cyclopeptides have been found in 26 families, 65 genera and 120 species; in particular, plants of the Caryophyllaceae and Rhamanaceae families commonly contain cyclopeptides. These 26 families include Amaranthaceae, Annonaceae, Araliacea, Asclepiadaceae, Asteraceae, Caryophyllaceae, Celastraceae, Compositae, Cucurbitaceae, Euphorbiaceae, Labiatae, Linaceae, Malvaceae, Myrsinaceae, Olacaceae, Pandaceae, Phytolaccaceae, Phamnaceae, Rubiaceae, Rutaceae, Schizandraceae, Solanaceae, Sterculiaceae, Urticaceae, Verbenaceae and Violaceae" (Tan and Zhou, 2006).

Literature reports of the distribution, concentration and biological activity of cyclopeptides from different plant sources show a wide range of compounds and concentrations possibly due to combined factors such as isolation methods, structures and plants genetics. The heteromonocyclopeptides, are substantially found in plant bark, root and whole seed. Other plant parts, which might contain cyclopeptides include root bark, stem bark, leaves, terminal branches, woody parts, aerial parts, flowers and fruit (Tan and Zhou, 2006). Most of these tissues and plant parts and extracts from them have biological activity. Typically these materials are cytotoxic, antimitotic, antibacterial,

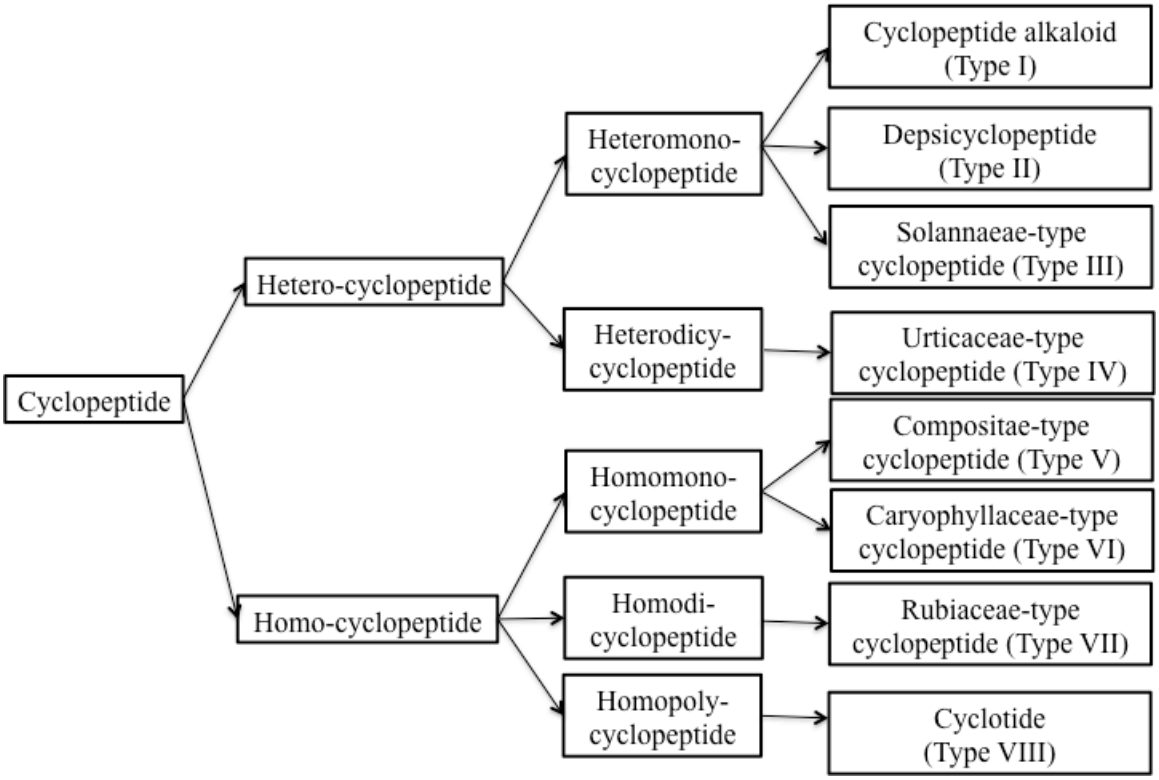
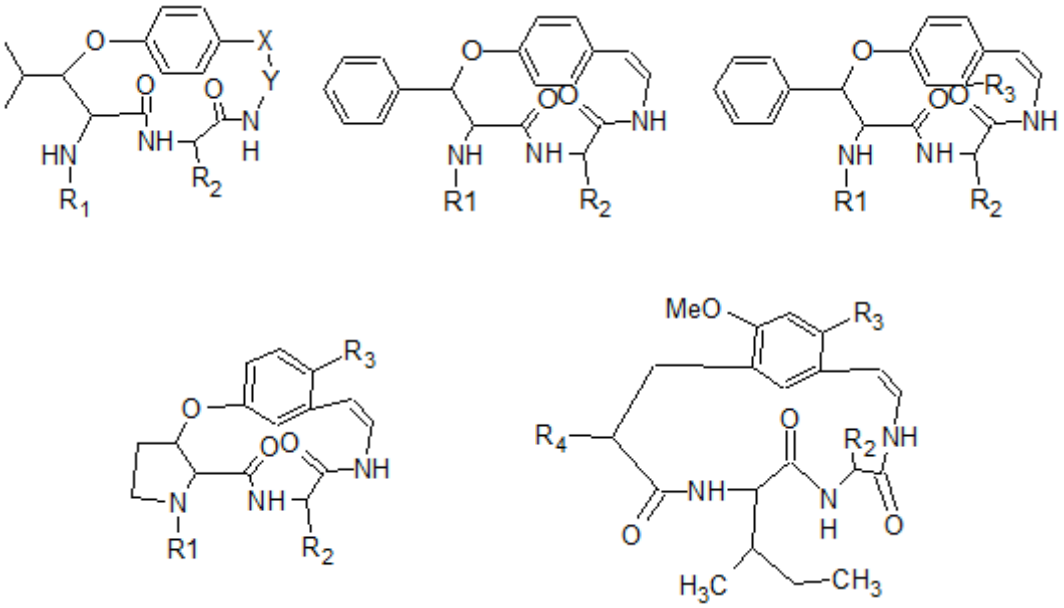
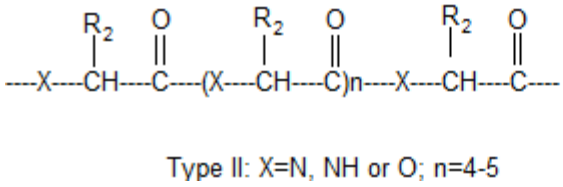



Figure 2-3 Classification of plant cyclopeptides（modified from Tan and Zhou, 2006）

antifungal, antiplasmodial or antimycobacterial. They may also act as sedatives, or immunostimulants. The alkaloid cyclopeptide (Type I, Figure 2-4. a) frangufoline (also called Sanjoinine-A), is a known 14-membered cyclopeptide alkaloid with sedative, anti-bacterial and anti-fungal activities found in the seeds of *Zizyphus jujuba* (Rhamnaceae). This plant is widely used as an herbal medicine in the Orient (Han and Park, 1987). Depsicyclopeptides (Type II, Figure 2-4. b) extracted from the whole plants of *Ardisia crenata* (Myrsinaceae) are cytotoxic, having the specific biological effects of inhibiting platelet aggregation in rabbits *in vitro*, decreasing blood pressure and causing dose-related hypotension in anaesthetized normotensive rats (Fujioka *et al.*, 1988). Solanaceae-type cyclopeptide (Type III, Figure 2-4. c), lyciumins, isolated from the root bark of *Lycium chinense* (Solanaceae) inhibit angiotensin-converting enzyme (ACE) and renin (Yahara, 1989). Urticaceae-type cyclopeptide (Type IV, Figure 2-4. d), celogentins, with antimitotic activity, are isolated from the seeds of *Celosia argenta* (Amaranthaceae) (Kobayashi *et al.*, 2001; Suzuki *et al.*, 2003). Homocyclopeptides, are found in plant roots and seeds, as well as latex, leaves, fruit and fruit peels. Astins (A, B, C) are representative Compositae-type cyclopeptides (Type V, Figure 2-4. e) derived from the roots of *Aster tataricus* (Compositae). These compounds have anti-tumour activity (Morita, 1995; Kosemura *et al.*, 1993). Yunnanins (cyclic heptapeptides) are Caryophyllaceae-type cyclopeptides (Type VI, Figure 2-4. f), that are extracted from the roots of *Stellaria yunnanensis* (Caryophyllaceae) and are found to exert cytotoxic effects on P388 leukemia cells (Morita *et al.*, 1994, 1996, 1997b; Napolitano *et al.*, 2004). In addition, some Rubiaceae-type cyclopeptides (Type VII, Figure 2-4. g), found in plant roots, stems, leaves and flowers have strong antitumour activities (Jolad *et al.*, 1977; Itokawa *et al.*, 1986, 1991; Morita *et al.*, 1992; Shen *et al.*, 1996). The most active Rubiaceae-type cyclopeptide RA-VII separated from the roots of *Rubiaceae akane* (Rubiaceae) was found to be an effective anticancer drug with low toxicity (Itokawa *et al.*, 1991). Cyclotides (Type VIII, Figure 2-4. h) are a group of plant disulfide-rich macrocyclic proteins with 28-37 amino acids with an amide head to tail cyclized peptide backbone and a cyclic cysteine knot (CCK) (Craik *et al.*, 1999). Their unique structure renders them highly chemically stable and resistant to enzymatic breakdown (Craik *et al.*, 1999; Colgrave and Craik, 2004). Some cyclotides are known for their anti-HIV

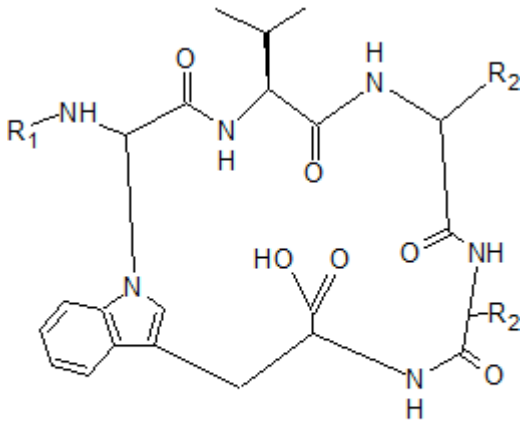


**a. Cyclopeptide alkaloids (Type I)**



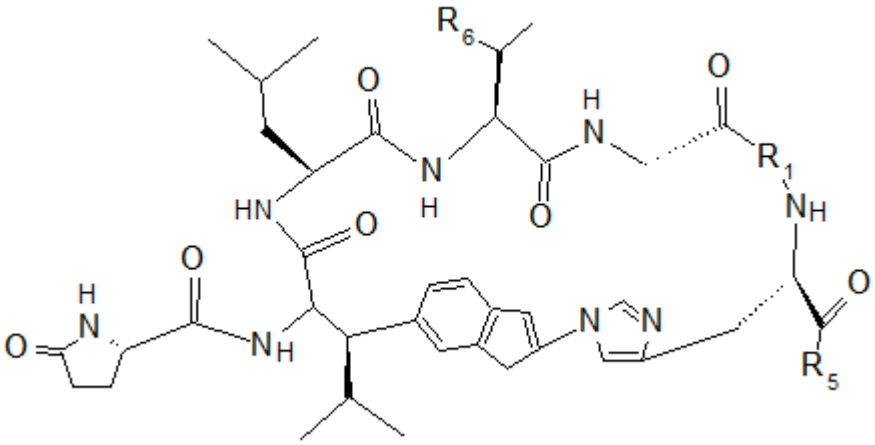
Type II: X=N, NH or O; n=4-5

**b. Depsicyclopeptides (Type II)**

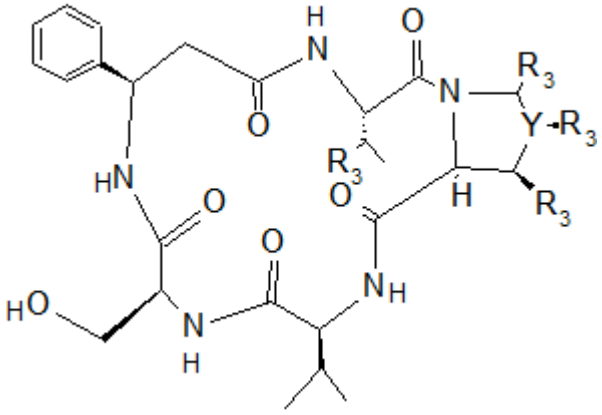


**c. Solanaceae-type cyclopeptides (Type III)**

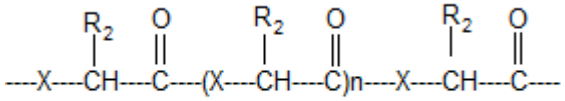
Figure 2-4 Structures of cyclopeptides（modified from Tan and Zhou, 2006）(Con't)



**d. Urticaceae-type cyclopeptides (Type IV)**



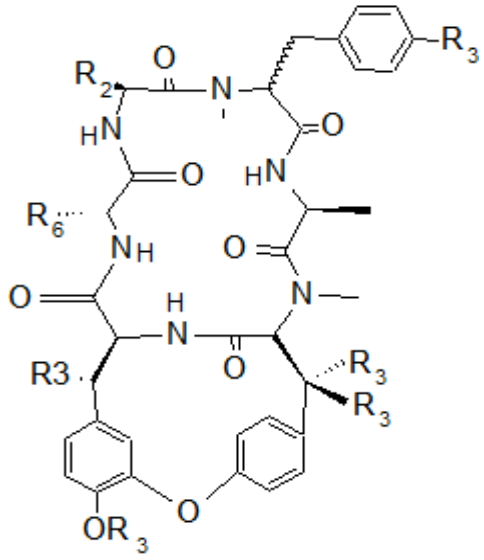
**e. Compositae-type cyclopeptides (Type V)**



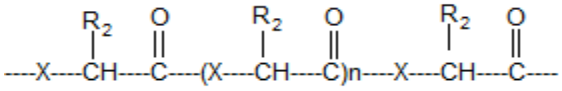
Type VI: X=N, NH; n=0, 3-10

**f. Caryophyllaceae-type cyclopeptides (Type VI)**

Figure 2-4 Structures of cyclopeptides（modified from Tan and Zhou, 2006）(Con't)



**g. Rubiaceae-type cyclopeptides (Type VII)**



Type VIII: X=N, NH; n=12, 26-29, 32,35

h. **Cyclotides (Type VIII)**

Note: (X-Y=CH=CH, CH (OH)-$CH_2$, CH ($OCH_3$)-$CH_2$, CH-CH, C ≡ C; $R_1$=amino acid residues; $R_2$=side chain of amino acids; $R_3$=H, OH, $OCH_3$, OAc, Cl, Oglc; $R_4$=$NH_2$, $NHCH_3$, N ($CH_3$)$_2$; $R_5$=OH or amino acid residues; $R_6$=$CH_3$ or $CH_2CH_3$)

Figure 2-4 Structures of cyclopeptides（modifieded from Tan and Zhou, 2006）

activities such as circulins found in the stems of *Chassalia parvifolia* (Rubiaceae) and cycloviolin from the bark of *Hybanthus parviflourus* (Violaceae) (Gustafson *et al.*, 1994; Derua *et al.*, 1996; Gustafson *et al.*, 2000; Hallock *et al.*, 1999).

### 2.4 Cyclolinopeptides

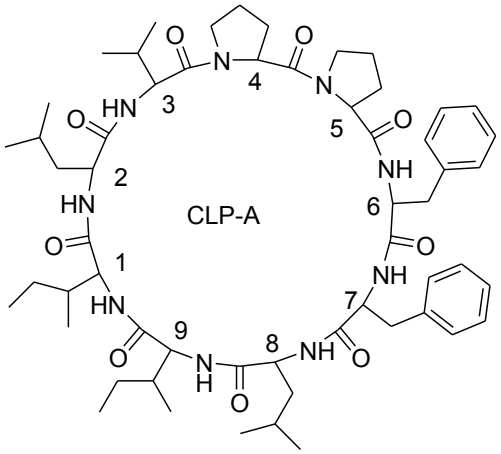
CLPs are a group of cyclic, hydrophobic peptides composed of eight or nine amino acid residues with molecular weights of approximately one thousand Da. They are Caryophyllaceae-type cyclopeptides (Type VI). After CLP-A was first isolated from the sediments deposited from crude flaxseed oil by Kaufmann and Tobschirbel in 1959, ten other CLPs were found in the seeds of *Linum usitatissimum* by 2001. The primary amino acid sequences chemical data and primary structures are summarized in Table 2-4 and Figure 2-5 (Kaufmann and Tobschirbel, 1959; Morita *et al.*, 1997a, 1999; Matsumoto *et al.*, 2001b, 2002). In addition, another cyclic peptide CLP-X with a non-proteinaceous amino acid residue (N-methyl-4-aminoproline) was isolated from *Linum album* in 1998 (Picur *et al.*, 1998).

#### 2.4.1 Biological activity of CLPs

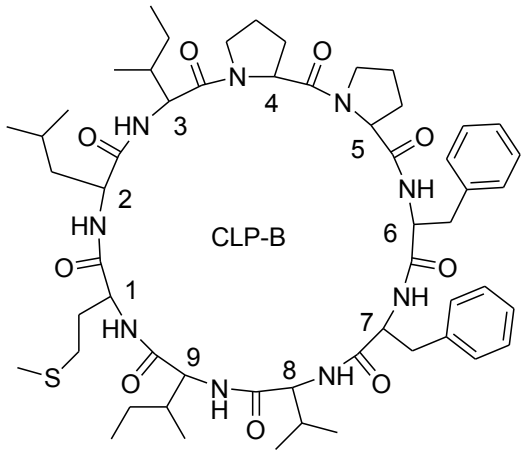
The role of CLPs in flax remains unclear, though through both *in vivo* and *in vitro* studies, it has been demonstrated that CLPs have multiple biological activities. For instance, in 1986 Kessler and co-workers reported that CLP-A inhibits cholate uptake into hepatocytes. Later, the tripeptide block -Phe-Phe-Pro- in CLP-A, which is similar to structures in antamanide and somatostatin, was proved to suppress the hepatocyte cell transport system. It is possible that this peptide sequence imparts the observed cytoprotective effects of CLP-A on hepatocytes (Kessler *et al.*, 1986a; Rossi, 1996). Immunomodulatory activity of CLP-A was studied using Jerne's plaque forming cell number determination (PFC) test for the primary and secondary humoural immune response (HIR), delayed type hypersensitivity (DTH) reaction, the skin-allograft rejection, the graft-versus-host reaction for the cellular immune response in mice, human lymphocyte proliferation test *in vitro* and the post-adjuvant polyarthritis test in rats and hemolytic anemia test in New Zealand Black mice (Wiesenborn *et al.*, 1991). The results show CLP-A affected both humoural and cellular immune response. It could also

Table 2-4 Cyclolinopeptides in *Linum usitatissimum* (Kaufmann and Tobschirbel, 1959; Morita *et al.*, 1997b; Morita *et al.*, 1999; Matsumoto *et al.*, 2001a, 2002)

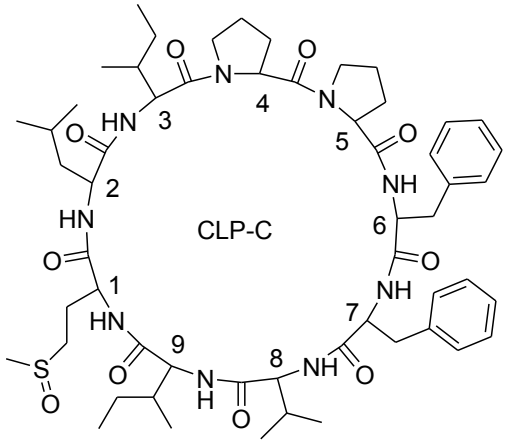
| Type | Primary structure (cyclo-) | Chemical data Formula (M.W.) |
|---|---|---|
| CLP-A | Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile | $C_{57}H_{85}N_9O_9$ (1040) |
| CLP-B | Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile | $C_{56}H_{83}N_9O_9S$ (1058) |
| CLP-C | Mso-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile | $C_{56}H_{83}N_9O_{10}S$ (1074) |
| CLP-D | Mso-Leu-Leu-Pro-Phe-Phe-Trp-Ile | $C_{57}H_{77}N_9O_9S$ (1064) |
| CLP-E | Mso-Leu-Val-Phe-Pro-Leu-Phe-Ile | $C_{51}H_{77}N_8O_9S$ (977) |
| CLP-F | Mso-Leu-Mso-Pro-Phe-Phe-Trp-Val | $C_{55}H_{73}N_9O_{10}S_2$ (1084) |
| CLP-G | Mso-Leu-Mso-Pro-Phe-Phe-Trp-Ile | $C_{56}H_{75}N_9O_{10}S_2$ (1098) |
| CLP-H | Mso-Leu-Met-Pro-Phe-Phe-Trp-Ile | $C_{56}H_{75}N_9O_9S_2$ (1082) |
| CLP-I | Met-Leu-Mso-Pro-Phe-Phe-Trp-Val | $C_{55}H_{73}N_9O_9S_2$ (1068) |
| CLP-J | Msn-Leu-Val-Phe-Pro-Leu-Phe-Ile | $C_{51}H_{77}N_8O_{10}S$ (993) |
| CLP-K | Msn-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile | $C_{56}H_{83}N_9O_{11}S$ (1090) |



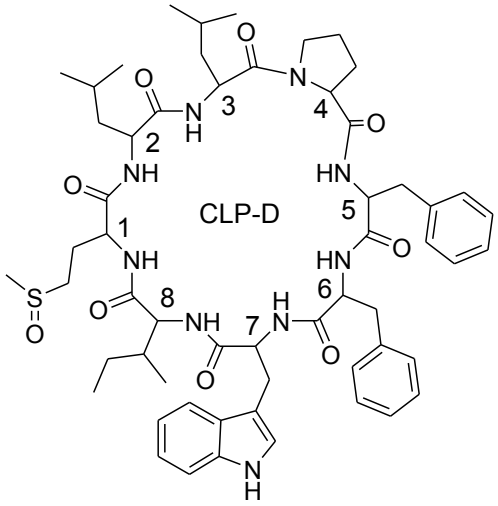

**Chemical Formula:** $C_{57}H_{85}N_9O_9$
Molecular Weight: 1040




**Chemical Formula:** $C_{56}H_{83}N_9O_9S$
Molecular Weight: 1058

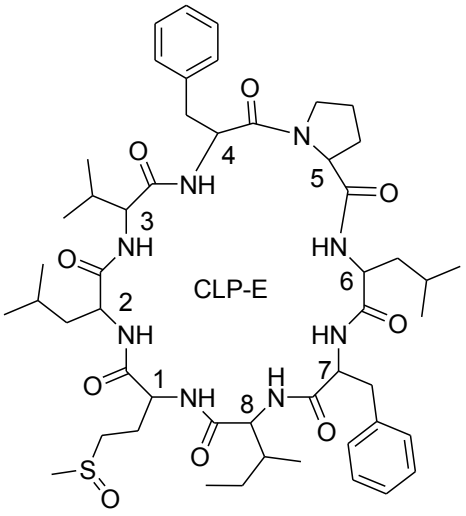



**Chemical Formula:** $C_{56}H_{83}N_9O_{10}S$
Molecular Weight: 1074

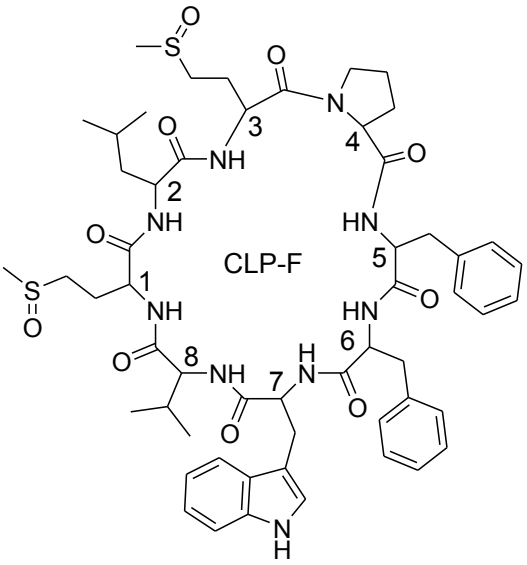



**Chemical Formula:** $C_{57}H_{77}N_9O_9S$
Molecular Weight: 1064

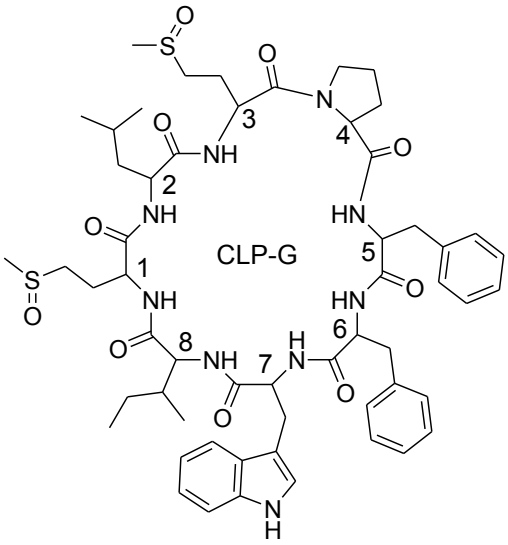
Figure 2-5 Primary structures of CLPs from the seeds of *Linum usitatissimum* (Con't)



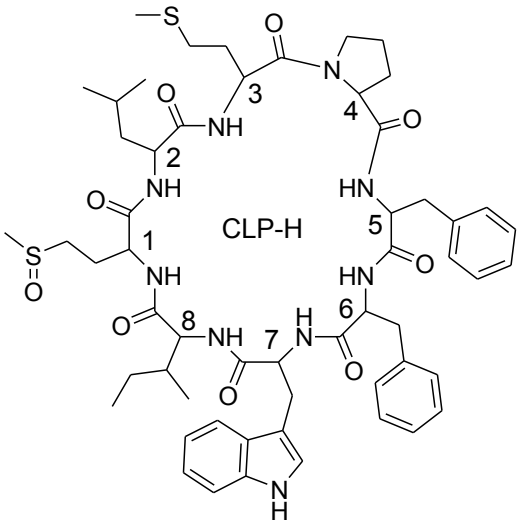

**Chemical Formula:** $C_{51}H_{77}N_8O_9S$
Molecular Weight: 977




**Chemical Formula:** $C_{55}H_{73}N_9O_{10}S_2$
Molecular Weight: 1084

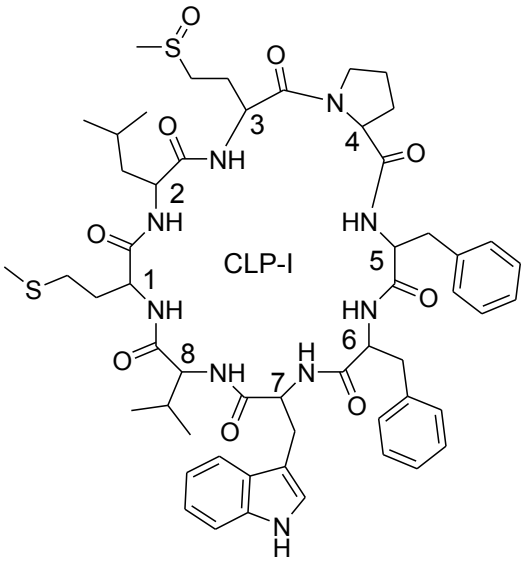



**Chemical Formula:** $C_{56}H_{75}N_9O_{10}S_2$
Molecular Weight: 1098

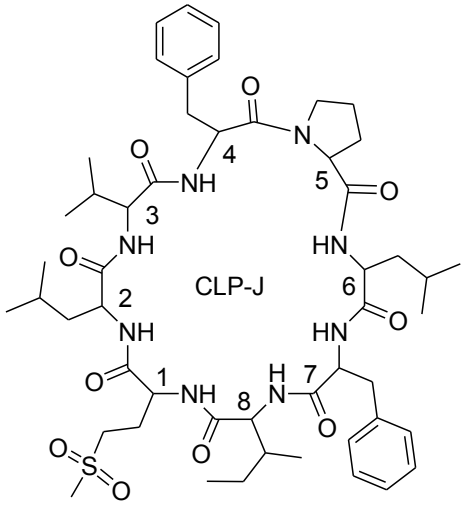



**Chemical Formula:** $C_{56}H_{75}N_9O_9S_2$
Molecular Weight: 1082

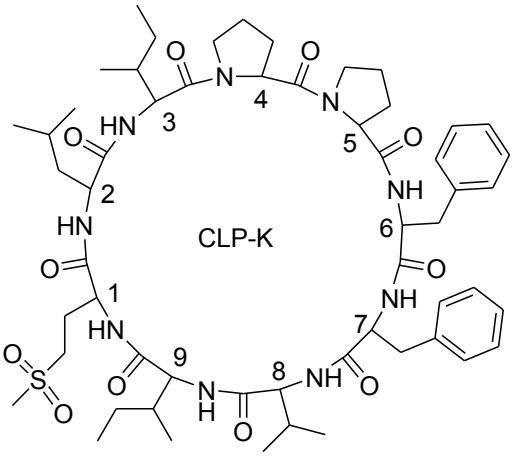
Figure 2-5 Primary structures of CLPs from the seeds of *Linum usitatissimum* (Con't)




**Chemical Formula:** $C_{55}H_{73}N_9O_9S_2$
Molecular Weight: 1068




**Chemical Formula:** $C_{51}H_{77}N_8O_{10}S$
Molecular Weight: 993




**Chemical Formula:** $C_{56}H_{83}N_9O_{11}S$
Molecular Weight: 1090

Figure 2-5 Primary structures of CLPs from the seeds of *Linum usitatissimum* (Kaufmann and Tobschirbel, 1959; Morita *et al.*, 1997b; Morita *et al.*, 1999; Matsumoto *et al.*, 2001a, 2002)

increase the skin allograft rejection time and reduce the graft-versus-host reaction index. Human lymphocyte proliferation was inhibited by CLP-A through phytohemagglutinin *in vitro* (Wiesenborn *et al.*, 1991). The symptoms associated with two immune diseases, the post-adjuvant polyarthritis in rats and hemolytic anemia of New Zealand Black mice, were alleviated. In the research of Górski *et al.* (2001), the immunosuppressive effects of CLP-A were compared with cyclosporin A (CsA), a known immunosuppressant. Both CLP-A and CsA function by inhibiting the action of Interleukin-1-alpha and Interleukin-2. This finding strongly indicates that CLP-A shares the same mechanism as CsA in the plaque-forming cells test and the autologous rosette-forming cells test. The study also compared the effects of both compounds on human lymphocytes *in vitro*. It was found that at very low concentrations, CLP-A induced the same effects as CsA on T and B cell proliferation, acquisition of activation antigens and immunoglobulin synthesis (Górski *et al.*, 2001). Overall, these studies demonstrated that CLP-A had similar biological effects to CsA.

The toxicity of CLP-A was evaluated by intravenous and oral administering to rats and mice (Siemion *et al.*, 1999). Oral administration of 4 g/kg CLP-A in olive oil, 2% gelatin solution did not harm mice while 3 g/kg to rats was also well tolerated. Intravenous administration of CLP-A at 230 mg/kg is non-toxic to mice. The combined strong immunosuppressive activity and low toxicity at relatively large doses of CLP-A makes it a potential immunosuppressive drug. The use of this compound as a drug requires additional research.

Other CLPs and their analogs were also investigated for their immunosuppressant activities. According to the research of Morita *et al.* (1997a), CLP-B inhibits concanavalin-A induced proliferation of human peripheral blood lymphocytes at treatment levels comparable to that of CsA. CLP-B and CLP-E also manifested a moderate inhibitory effect on concanavalin-A induced mouse lymphocyte proliferation (Morita *et al.*, 1997a). Many chemical analogs of CLP-A were tested for their effects on immune response (Siemion *et al.*, 1999; Benedetti and Pedone, 2005; Picur *et al.*, 2006). Many of these compounds with a structure of -Pro-Xxx-Phe- sequence (where Xxx means a hydrophobic, aliphatic, or aromatic residue) were found to exert

immunosuppressive activity though none of them exerted higher activity than CLP-A (Picur *et al.*, 2006).

The immunosuppressive activity of CLPs and its analogues make them potential value added natural products of flax and will lead to further investigations of the biological activities of CLPs. According to the patent application of Reaney *et al.* (2009), CLPs also present a biological activity of induction of heat shock protein 70A production in *Caenorhabditus elegans.*

Exposure of nematode cultures to CLP-A (0.1 μM and 10.0 μM) induced a 30% increase in the production of the HSP 70A protein, while a 3.5-fold increase was induced in the culture treated with 1.0 μM of CLP-A. Higher concentrations of CLP-A were lethal to the nematodes (Reaney *et al.*, 2009).

### 2.4.2 Isolation and separation of CLPs from flaxseed tissues

There are many published methods for isolation of CLPs from flax. The isolation procedures have depended, in part, on the tissue processed. A low concentration of CLPs in the source matrix requires the use of solvents and chromatographic columns during peptide recovery. Kaufman and Tobschirbel (1959) first isolated a cyclic hydrophobic peptide from flaxseed oil precipitates (foots), which is a slime that precipitates from flaxseed oil after extraction and settling. Later CLP-A structure was confirmed by Prox and Weygand (1967). Morita and co-workers (1997a, 1999; Matsumoto *et al.*, 2001a, 2001b, 2002) described the isolation of several additional CLPs from flaxseed, root and cake after oil pressing. Defatted flax meal (30 kg) and flax roots (30 kg) was first defatted then extracted with four volumes of hot methanol three times, followed by solvent stripping. The methanolic extract (about 4 kg) was loaded on a polystyrene column (Diaion™ HP-20). Methanol with increasing concentrations in water (0-100%) was utilized as the eluent for further separation and the bound CLPs were removed from the column with a 100% methanol. The methanol extract was then subjected to a normal phase silica gel chromatography with chloroform and methanol solvent gradient from 100:0 and the hydrophobic peptides were eluted with low concentrations (10%-15%) of methanol. Those fractions containing peptides were subjected to reverse phase HPLC with 40-75% $CH_3CN$ solvent system to yield 0.007% of CLP-A, 0.0002% of CLP-B,

0.0037% of CLP-C, 0.0015% of CLP-D, 0.0058% of CLP-E, 0.0008% of CLP-F, 0.0024% of CLP-G, 0.0002% of CLP-H and 0.00007% of CLP-I (Morita *et al.*, 1997b, 1999; Matsumoto *et al.*, 2001a, 2001b, 2002).

Stefanowicz (2001) described an isolation method for obtaining mixtures of CLPs. Ground flaxseed (5 g) was extracted overnight with 100 mL of acetone. After solvent evaporation, the remaining extract was dissolved in methanol and hydrolyzed with 10% sodium hydroxide. The resulting mixture was dried under vacuum, while the remaining fraction was mixed with ethyl acetate. The extract was shown to be a mixture of CLPs by electrospray ionization-mass spectrometry (ESI-MS) and electrospray ionization tandem mass spectrometry (ESI-MS/MS), but further separation for each cyclic peptide was not reported.

In 2007, Brühl *et al.* recovered compounds that contribute a bitter flavour to flaxseed oil. Flaxseed was first pressed using a laboratory expeller press at a temperature not exceeding 60°C. This condition produced a yield of 30% oil that was extracted at a temperature not exceeding 40°C. The "cold pressed" flaxseed oil (100 g) obtained was mixed with 100 mL heptane, then extracted three times with methanol/water (6/4; v/v, 200 mL each) and the aqueous extracts were combined, followed by solvent evaporation under vacuum. The bitter fraction identified by sensory testing was collected and dissolved in methanol/diethyl ether (1/1, v/v; 1 mL) and finally loaded onto a silica gel (20 g) column. Chromatography with a gradient of diethyl ether and ethanol from 10:0 to 0:10 was conducted. Ten fractions were collected and evaluated for bitter taste after solvent evaporation and the bitter compound was further purified by RP-C18 HPLC and proved to be CLP-E by liquid chromatography-mass spectrometry (LC-MS), electro spray ionization-time of flight-mass spectrometry (ESI-TOF-MS) and nuclear magnetic resonance (NMR) analyses. The structure was also shown to be consistent with CLP-E by Fourier transform infrared (FTIR) spectroscopy and amino acid analysis. Brühl *et al.* (2007) focused on identifying the bitter compound of flax and not efficient processes for isolating, separating or quantifying all of CLPs from flaxseed oil. In particular, no further action was taken to increase oil yield or to maximize the extraction efficiency of all cyclic peptides.

### 2.4.3 Detection, identification, confirmation of CLPs in prepared samples

Detection, identification and confirmation of CLPs from flax extracts has been achieved using ESI-MS, ESI-MS-MS, ESI-TOF-MS, LC-MS, circular dichroism (CD) spectroscopy, infrared (IR) spectroscopy, FTIR, high resolution fast atom bombardment mass spectrometry (HR-FABMS), $^{13}$C-NMR, $^{1}$H-NMR and amino acid analysis by HPLC after hydrolysis (Stefanowicz, 2001; Naider *et al.*, 1971; Brewster and Bovey, 1971; Tancredi *et al.*, 1991; Morita *et al.*, 1999; Matsumoto *et al.*, 2001a; Brühl *et al.*, 2007).

ESI-MS and ESI-MS-MS are preferred in CLP analysis due to easy sample preparation, high sensitivity and high dynamic range. In 2001, Stefanowicz described a method to detect and sequence CLPs from flaxseed by ESI-MS and ESI-MS/MS. Crude peptide extracts were dissolved in methanol containing 10 mM of ammonium acetate and injected in to a Finnigan MAT TSQ-700 MS with ESI source. The peptides CLP-B, CLP-D, CLP-E were detected in agreement with previous literature (Morita *et al.*, 1997b). The precursors of CLP-D and CLP-E were first presented as the cyclic peptide containing unoxidized Met. The sequences of CLP-F (Cyclo-(Mso-Leu-Mso-Pro-Phe-Phe-Trp-Val-)) and CLP-G (Cyclo-(Mso-Leu-Mso-Pro-Phe-Phe-Trp-Ile-)) were also proposed based on the fragmentation spectra achieved by collision induced dissociation experiments and the similarity with those of CLP-D' (Stefanowicz, 2001). The use of MS techniques has its limitations, such as the inability to provide detailed information about CLPs' conformational structures.

Conformation of CLP structures in different solutions was studied using circular dichroism (CD) and NMR. The flexibility of the peptide ring and prolyl isomers present in CLPs will allow the peptides to adopt a number of conformations in solution. The conformation of CLP-A was first investigated in several organic solvents by CD (Naider *et al.*, 1971). The results indicated that CLP-A existed in several conformations in solution with the absence of intra-molecular hydrogen bonds. In the same year, the conformation of synthetic CLP-A was illustrated by Brewster and Bovey (1971) who measured the temperature dependence of the NH chemical shifts using 100 and 200 MHz proton NMR, after exchange of peptide NH protons with deuterium. The data reveals that: 1) the main chain is cyclic, cyclo(Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile-); 2)

intramolecular hydrogen bonds are absent in dimethysulfoxide (DMSO); 3) five of the seven peptide NH protons are exposed to solvent while the remaining two might be situated in the interior of the ring (Brewster and Bovey, 1971). In another study it is noted that CLPs have the ability to form complexes with metal ions, such as $Ba^{2+}$, $K^{+}$, $Na^{+}$, $Mg^{2+}$ and $Ca^{2+}$, which make them a potential vehicle for ion delivery (Tancredi *et al.*, 1991).

Morita and coworkers were the first group to combine analytical technologies, including IR, HR-FABMS, $^{13}$C-NMR, $^{1}$H-NMR and amino acid analysis, to systematically identify the structures of CLP-B through I (Morita *et al.*, 1999; Matsumoto *et al.*, 2001b). For instance, HPLC enriched fractions containing mostly CLP-B were first dissolved in methanol then injected into HR-FABMS. A quasi-molecular ion peak at *m/z* 1058.6031 $[M+H]^{+}$ was observed, corresponding to the molecular formula of CLP-B, $C_{56}H_{83}N_{9}0_{9}S$. The IR absorptions at 3,436 and 1,659 $cm^{-1}$ indicated the presence of amino and amide carbonyl groups in CLP-B respectively. The combined application of $^{13}$C-NMR and $^{1}$H-NMR provided more structural detail regarding CLP-B. Signals from $^{13}$C-NMR spectrum (δ 173.17, 172.57×2, 171.65, 171.16, 170.722, 169.99 and 169.89) indicated the existence of nine amide carbonyl groups in CLP-B, while chemical shifts from $^{1}$H-NMR spectrum (δ 7.89, 7.82, 7.73, 7.71, 7.55, 7.45 and 7.27) showed only seven amide protons in CLP-B. Acid-hydrolysis of CLP-B yielded Leu (×1), Val (×1), Met (×1), Ile (×2), Phe (×2) and Pro (×2). The molecular weight and the lack of a terminal amino group ($^{1}$H-NMR and $^{13}$C-NMR) demonstrated that CLP-B was a cyclic peptide with nine amino acid residues. The proton signals and the corresponding carbon signals were assigned by NMR methods ($^{1}$H-$^{1}$H correlation spectroscopy (COSY) and heteronuclear multiple quantum coherence (HMQC)). The phase sensitive Rotating Frame Overhauser Effect Spectroscopy (ROESY) and heteronuclear multiple bond correlation (HMBC) experiments determined the linking between individual amino acids and the sequence of CLP-B was finally identified as cyclo-(Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile-) (Morita *et al.*, 1999). Other CLPs (CLP-C to I) are similarly determined.

A more recent study utilized LC-MS, high resolution mass spectrometry, NMR, amino acid analysis and FTIR to identify the bitter principle in stored cold-pressed

flaxseed oil (Brühl *et al.*, 2007). The isolated bitter principle (100 µg) was obtained by the method described in section 2.4.2 for structure identification. A clear pellet was produced for FTIR spectrometry by pressing a mixture of dry potassium bromide (250 mg) and an aliquot (1.5 mg) of the isolated bitter principle under vacuum. The IR spectrum was recorded from 4000 to 400 $cm^{-1}$. A broad band at 3,427.7 $cm^{-1}$ indicated the presence of a hydroxyl group with a shoulder at 3,314.0 $cm^{-1}$ for NH vibration. The weak bands of $CH_3$ and $CH_2$ groups observed at 2,959.3 and 2,928.3 $cm^{-1}$ at a ratio about 1:1 demonstrated a lack of long carbon chains and eliminated the possibility of fatty acid derivatives as bitter compounds. At the same time, C=O (vibration) and C-N, N-H (vibration) for N-monosubstituted amides were observed at 1,659.1 and 1,529.9 $cm^{-1}$, while sulfoxide (vibration) and a monosubstituted aromatic system were detected at 1,030.6, 746.0 and 701.3 $cm^{-1}$. For further structure confirmation, 100 µg of the isolated bitter compounds was dissolved in 1 mL methanol and an aliquot (5 µL) was injected into an API 4000 Q Trap LC/MS/MS by means of loop injection with methanol/water (1/1, v/v) as the solvent. A quasi-molecular ion with *m/z* 977.7 $[M + H]^+$, along with product cluster ions: ammonium with *m/z* 994.7 $[M + NH_4]^+$, sodium with *m/z* 999.7 $[M + Na]^+$, potassium with *m/z* 1,015.7 $[M + K]^+$ and double charged molecule: 489.5 $[M + 2H]^{2+}$, 500.5 $[M + H + Na]^{2+}$, 508.4 $[M + H + K]^{2+}$ and 511.5 $[M + 2Na]^{2+}$ were observed. The exact mass measurements are further confirmed by high resolution mass spectrometry. The bitter isolate was dissolved in methanol and injected into a Bruker Micro TOF using electrospray ionization in positive and negative ion modes by means of loop injection with methanol/water (1/1, v/v) as the solvent. The result showed a sodium adduction $[M + Na]^+$ (*m/z* 999.5366) in the positive ionization mode as $C_{51}H_{76}N_8O_9S + Na^+$ (*m/z* 999.5348) and quasi-molecular ion $[M - H]^-$ (*m/z* 975.7) in the negative ionization mode as $[C_{51}H_{76}N_8O_9S - H]^-$ (*m/z* 975.5390). The molecular formula $C_{51}H_{76}N_8O_9S$, therefore, was identified as the elementary composition of the isolated bitter compound. The eight nitrogen atoms in this molecular formula, the hydrophobicity of this compound and absence of amino acid fragmentation from the terminal peptide chain upon LC-MS analysis suggested a cyclic peptide structure of the bitter compound. The amino acid composition was then analyzed by hydrolysis of CLP-E followed by ion chromatography. An aliquot (300 µg) of the isolated bitter compound was mixed with

aqueous hydrochloric acid (6 mol/L; 100 µL) and heated for 17 h at 110°C under an atmosphere of nitrogen. The hydrolysate (10 µL) was injected into an ion chromatograph (0.25 mL/min) with the following gradient including deionized water (solvent A), aqueous sodium hydroxide (250 mmol/L, solvent B), aqueous sodium acetate (1 mol/L, solvent C) and aqueous acetic acid (100 mmol/L, solvent D). The retention times of the peaks from each amino acid were compared with those of authentic standards and six amino acids were identified, including L-proline, L-valine, L-leucine, L-isoleucine, L-phenylalanine and L-methionine sulfoxide (Mso). The amino acid composition corroborated the structure of the cyclic octapeptide cyclo-(Mso-Leu-Val-Phe-Pro-Leu-Phe-Ile), identified as CLP-E found previously in flaxseed (Morita *et al.*, 1999). A final structure confirmation was conducted by NMR. The bitter compound was dissolved in methanol-$d_4$ or DMSO-$d_6$ and $^1$H-COSY, NOESY, $^{13}$C, HMQC and HMBC experiments were performed. Eight α-amino acid proton signals between $\delta_H$ 3.7 and 5.0 in $^1$H-NMR spectrum were in agreement with the proposed structure of a cyclic octapeptide. Furthermore, seven amide proton resonances ($\delta_H$ 4.37, 1.83, 2.17, 1.91, 3.50, 3.56) implied the presence of one Pro in the molecule. The signal at $\delta_H$ 2.54 with an intensity of three protons indicated the presence of the methyl group of the Mso. The aromatic signal pattern of the two Phe moieties was found between $\delta_H$ 7.1 and 7.3. At the same time, $^{13}$C-NMR spectroscopy revealed eight carbonyl signals and the quaternary carbon signals of the aromatic ring in the Phe were found at $\delta_C$ shifts at 137.1 and 138.1. Correlation between neighbouring amino acids was established by nuclear overhauser effect, combined with correlations between the amide carbonyl atoms and the neighbouring amide protons as well as the amino acid protons by HMBC, to confirm the structure of the bitter compound. All the collective data were comparable to the previous report of CLP-E in the literature (Morita *et al.*, 1999).

A whole genome sequence of *Linum usitatissimum* (var. CDC Bethune) has been produced and published on linum.ca (2010). Gene sequence g24175 showed the embedded sequences of CLP-E (MLVFPLFVI), B (MLIPPFFVI) and A (ILVPPFFLI). Gene sequence of g38655 showed the embedded sequences of CLP-D (MLLPFFWI), F (MLMPFFWV) and G (MLMPFFWI) (Reaney, Pers. Commun.; Figure 2-6, 7).

**Coding sequence of g24175**

ATGGCTGTTG TGTCCTCTCT GGCTCTGACC ACTAGCCTAG TTGCTACCGC CGCCGGCCGT
AATAATAATG CCTTCCCACC ATCCTCCTCC AGGAACAACA AGGCACCAGC AGACCTTTTC
ATTACTCCCA AGACAACAAC AACAGTGAAA GCAGCAGCTG TCTCATGCAA ACGTCCCTAC
CCGAAAGGAG CAGTTGCTGC TGCTACTAGT ACCTTGTCTC CTATTTCTGG AAAGGATGGC
GGCCTCCGCA ACCAGGAGGA GAGCGATGGT ATGTTGGTCT TCCCCTTATT TATATTCGGC
AAGGAAGGTA GTCAGGACAA GTATAATGGA GCAGCTGCCC TCCGCGACCA GGAGGAGAGC
GATGGTATGT TGATCCCCCC CTTCTTTGTC ATATTCGGCA AGGAAGGTTG TCAGGATATC
GGCCACAAGT ATAATAATGC CGCAGCAGCT GGCGCCCTCC GCGACCAGGA GGAGAGCGAT
GGTATACTGG TCCCCCCCTT CTTTCTCATA TTCGGCAAGG AAGGTAGTCA GGACAAGTAT
AATGCAGCAG CAGCTGGCGG CCTCCGCGGC AAGGAGCAGC AGGGTGACAA GATGGCGGCT
GGAGCTGAGA ATTAG

**Translation of sequence g24175 into amino acid**

MAVVSSLA LTTSLVAT AAGRNNNA FPPSSSRN NKAPADLF ITPKTTTT VKAAAVSC KRPYPKGA
VAAATSTL SPISGKDG GLRNQEES DG**MLVFPL FI**FGKEGS QDKYNGAA ALRDQEES DG**MLIPPF**
**FVI**FGKEG CQDIGHKY NNAAAAGA LRDQEESD G**ILVPPFF LI**FGKEGS QDKYNAAA AGGLRGKE
QQGDKMAA GAEN_

Figure 2-6 Nucleotide and protein sequence of g24175 embedded with CLP-E (MLVFPLFVI), B (MLIPPFFVI) and A (ILVPPFFLI) (linum.ca, 2010)

**Coding sequence of g38655**

ATGGCTGTTG TGTCCTCTCT GGCTCTGACC ACTAGCCTAG TTGCTACCGC CGCCGGCCGT
AATAATAATG CCTTCCCACC ATCCTCCTCC AGGAACAACA AGGCACCAGC AGACCTTTTC
ATTACTCCCA AGACAACAAC AACAGTGAAA GCAGCAGCTG TCTCATGCAA ACGTCCCTAC
CCGAAAGGAG CAGTTGCTGC TGCTACTAGT ACCTTGTCTC CTATTTCTGG AAAGGATGGC
GGCCTCCGCA ACCAGGAGGA GAGCGATGGT ATGTTGGTCT TCCCCTTATT TATATTCGGC
AAGGAAGGTA GTCAGGACAA GTATAATGGA GCAGCTGCCC TCCGCGACCA GGAGGAGAGC
GATGGTATGT TGATCCCCCC CTTCTTTGTC ATATTCGGCA AGGAAGGTTG TCAGGATATC
GGCCACAAGT ATAATAATGC CGCAGCAGCT GGCGCCCTCC GCGACCAGGA GGAGAGCGAT
GGTATACTGG TCCCCCCCTT CTTTCTCATA TTCGGCAAGG AAGGTAGTCA GGACAAGTAT
AATGCAGCAG CAGCTGGCGG CCTCCGCGGC AAGGAGCAGC AGGGTGACAA GATGGCGGCT
GGAGCTGAGA ATTAG

**Translation of sequence g38655 into amino acid**

MAAASSLA LATASLVA TGAGGRNN AFLPSKNK TPNLFLNP NKTTSSTV KAVVSSSS CKRPYPKG
DASLFLGI DDVFGKDA VAGHDNDQ DAASGQEM AADD**MLMP FFWI**FGKE GQQQEAEE SSDD**MLMP**
**FFWI**FGKE GQQQEAES SDD**MLLPF FWI**FGKEG QQQEAESS DD**MLMPFF WI**FGKQQQ QQGESSDD
**MLMPFFWV** FGKQGDNN KGDAVEAI LKN_

Figure 2-7 Nucleotide and protein sequence of g38655 embedded with CLP-D (MLLPFFWI), F (MLMPFFWV) and G (MLMPFFWI) (linum.ca, 2010)

### 2.4.4 Quantification of CLPs in different varieties of flaxseeds

Quantification and characterization of any compound or class of compound is facilitated by the use of authentic compounds and suitable internal standards (Franke *et al.*, 1995; Balsevich *et al.*, 2009; Kanduru *et al.*, 2010). Most previous studies of CLPs do not describe precise quantification due to the lack of authentic standards and the difficult procedures required for obtaining pure individual peptides (Kaufman and Tobschirbel, 1959; Morita *et al.*, 1997b; Stefanowicz, 2001; Matsumoto *et al.*, 2002). The exceptional case was the publication by Brühl *et al.* (2007) that describes the measurement of CLP-E concentrations in flaxseed oil. Based on purified CLP-E from stored flaxseed oil obtained in a previous work of Brühl *et al.* (2007), an external calibration with a coefficient of determination $R^2$ of 0.998 was established for the range from 3-900 mg/mL. Flaxseed oil (1 g) was mixed with heptane (10 mL) and loaded onto a C18 SPE 1000-mg cartridge. The column was eluted with heptane (5 mL, 3 times) to remove non-polar compounds. Polar compounds remaining on the column were recovered with a subsequent elution with methanol (5 mL, once). After solvent removal, the extract was taken in 0.5 mL methanol and injected onto an HPLC column using a solvent gradient that started with a mixture of methanol/water (75: 25, v/v) at a flow rate of 1 mL/min and then changing to 100% methanol. The peak of CLP-E was observed at a retention time of 12.43 min. The levels of CLP-E in different flax cultivars were calculated using external calibration and the results showed the amount of CLP-E varied from 0 to 53 mg/kg in flaxseed oils with a mean of 24 mg/kg among 21 flax varieties.

## 2.5 Potential for commercial production of CLPs

### 2.5.1 Flaxseed oil as a commercial source of peptides

CLPs derived from flaxseed oil may potentially be valuable bioactive molecules with immunosuppressive and potential anti-cancer properties. Flaxseed oil could be considered as an excellent commercial source for CLP recovery as these peptides are hydrophobic and are mainly dissolved in oil after processing. However, few studies have presented the levels of CLPs in flax material except that of Brühl *et al.* (2008). In their report levels of CLP-E among 21 flax varieties were analyzed and the data showed that flax genotype might play a role in determining the level of CLP-E. However, the levels

of other peptides were not measured. It is not known if the data reflects true genotypic differences among the flax cultivars or if the variation is due to conditions of harvesting and storage. Further investigation should be conducted to evaluate the levels of CLPs among different flax varieties in order to find a good commercial source for peptide recovery.

Methods were proposed for commercial extraction and concentration of peptides from flaxseed oil using either liquid-liquid or solid-liquid extraction (Reaney *et al.*, 2009). However, the recovery from whole seed requires methods that are more costly, difficult and time-consuming. These methods usually involve the use of a great amount of solvent, labour and energy for peptide extraction and concentration (Morita *et al.*, 1997a, 1999; Matsumoto *et al.*, 2001a, 2001b, 2002; Stefanowicz, 2001; Brühl *et al.*, 2007). Chemical synthesis of CLPs, on the other hand, provides an alternative method of studying peptides. Wiezorek *et al.* (1991) described a synthetic method for CLP-A preparation on Merrifield resin using tert-butyloxycarbonyl protected amino acids. Trifluoro acetic acid and sulphuric acid are used to produce linear peptides, which were split from resin and later cyclized by Castro's agent. The final peptide was purified using HPLC. However, the synthetic method also had a very low product yield and peptide recovery was difficult.

### 2.5.2 The potential of CLPs as cryptands

Cyclic peptides containing even numbers of altering D and L amino acids are able to self-assemble and form nanotubes by intermolecular hydrogen bonding (Ghadiri *et al.*, 1993). The abundant presence of C=O and N-H functional groups of CLPs would introduce a binding cavity suitable for binding other compounds. Such compounds are called cryptands as they are members of a class of molecules with a suitably sized cavity for binding other molecules (Cramer, 1952; Pedersen, 1967). Cryptands could be widely applied in food, cosmetics, cleaning products, pharmaceuticals and agriculture products due to their multiple functions (Weber, 2005). For example, cyclodextrins, a group of cyclic oligosaccharides, can trap molecules by forming cage and channel structures to produce inclusion complex. This property could be used to modify the chemical reactivity of guest molecules, fix volatile compounds, improve solubility of substances,

solidify liquid substances and mask or preserve smell and taste. In the cosmetic industry, cyclodextrins are added into perfumes, body creams, shower gels, air refresher and detergent in order to control the release of aromatic oils (Prasad *et al.*, 1999). In the food industry, they are used for flavour delivery or protection since most of the natural or artificial flavour compounds are volatile oils which could be included into cyclodextrins to give better performance (Szejtli, 1998). Removing undesired compounds from food products is another application for cyclodextrins. They are, for example, added to milk and egg to remove cholesterol (Hedges, 1998). For drug delivery, cyclodextrins can enhance delivery efficiency by increasing the solubility of hydrophobic drug compounds in solution and increasing their availability at the surface of the biological barrier (Rajewski and Stella, 1996). They are also used to mask the bitter flavour in medicine (Frömming and Szejtli, 1994). For environmental science, organic contaminants, organic pollutants and heavy metals from environment could be dissolved and removed by cyclodextrins (Gao and Wang, 1998). The wide applications of cyclodextrins provide an indication of the potential for CLPs as cryptands.

# 3 MATERIALS AND METHODS

## 3.1 Materials

Five licensed cultivars of flaxseed (*Linum usitatissimum*) including Somme, Vimy, Flanders, CDC Bethune and CDC Valour, were grown in field plots at two locations (Saskatoon, SK and Floral, SK) in 2006 and 2008. The trials were standardized as a randomized complete block design (RCBD) with two replications. Each plot contained six rows 0.30 meters apart and 3.66 meters long. The seed was a generous gift of Dr. G. Rowland of the Saskatchewan Crop Development Centre. Commercial flaxseed was provided by Natunola Health Inc., Winchester, ON. The variety and growing conditions of the Natunola seed are unknown. Standards of CLP-A, B, C, D, E, F, G and Segtalin A (Seg-A) were prepared by Research Assistant P-G. Burnett, Food and Bioproduct Sciences, University of Saskatchewan. A quality report of each peptide is included as Appendix A. Bottles of flaxseed oil were purchased from local retail health food stores. Descriptions on the packaging of these oils included 100% organic cold pressed flaxseed oil (500 mL, Sangster Health Centers, Saskatoon, SK), flaxseed oil (448 mL, Omega Nutrition Canada Inc., Vancouver, BC), certified organic flaxseed oil (500 mL, Floral Inc, Lynden, DC), Natural Brand$^{TM}$ certified organic flaxseed oil (473 mL, General Nutrition Centres Inc., Pittsburgh, PA), certified organic flaxseed oil (500 mL, Gold Top Organics Ltd., Edmonton, AB). Chemicals used in the conduct of thesis research are listed in Appendix B.

## 3.2 CLPs in flaxseed from different varieties

### 3.2.1 Oil extraction from flaxseed

CLPs, along with other hydrophobic compounds, such as TAGs, phospholipids and carotenoids in flaxseed were first extracted for later peptide isolation. Oil extraction protocol was modified from method 960.39 (a) of the A.O.A.C (1990). In the modified process, acetone, instead of hexane, was used as a solvent for extractions. Flaxseed was ground in a coffee grinder for approximately 30 s to pass through a 1.18-mm test sieve

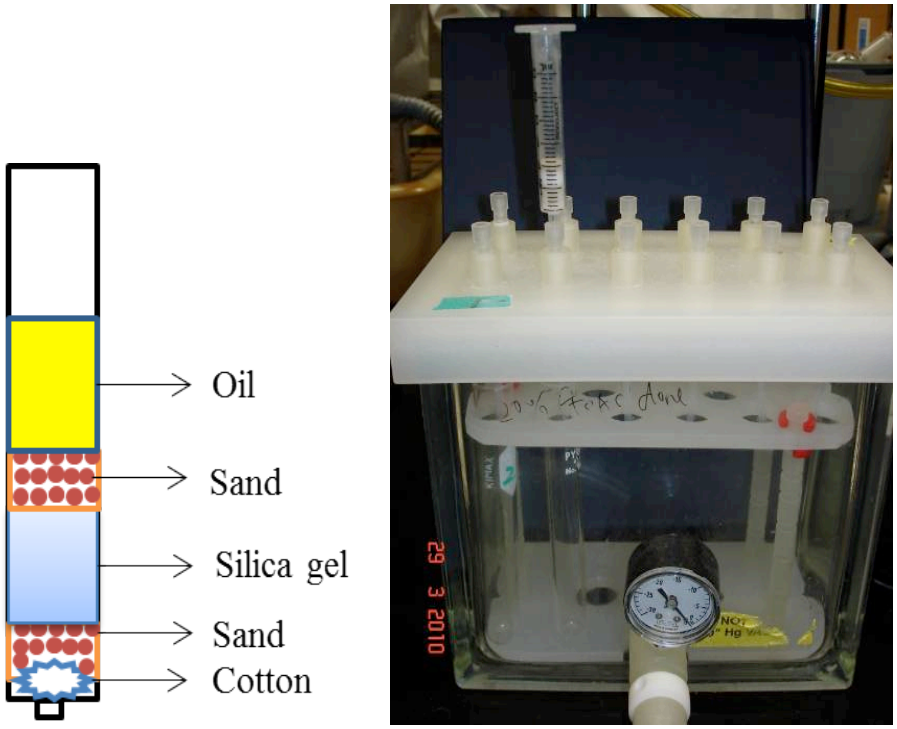
(12 inch-HH-BR-SS-US-40; VWR Company, West Chester, PA). Ground material (up to 5 g) was wrapped in filter paper (Whatman No. 4, Whatman Inc., Piscataway, NJ) and folded to fit into a cellulose extraction thimble (25 × 80 mm, Ahlstrom Atlanta LLC, Holly Spring, PA). Extraction beakers were pre-dried in the oven for one hour at temperature of 100°C and cooled in a desiccator before use. Acetone (50 mL) extraction was conducted in a Goldfisch extractor (Model 22166B, Laboratory Construction CO., Kansas City, MO.) with the heat control set at high for 5 hours. After extraction, acetone in the oil samples was recovered in a solvent recovery glass tube. Oil was purged with nitrogen for 10 s and left in the fume hood for 1 h to allow for solvent evaporation before cooling in a Pyrex® glass desiccator (2.2 L, Corning Inc., Lowell, MA). The weights of the beaker, the beaker with oil and the sample were determined using an analytical balance (Accuracy: 1 mg, Model: PB403, Mettler Toledo, Greifensee, Switzerland) and two decimal places were recorded.

The oil content of the samples was calculated according to Equation 3.1.

$$\% \text{ oil} = \frac{(\text{weight of beaker+oil})\text{-weight of beaker}}{\text{weight of sample}} \times 100 \qquad (3.1)$$

### 3.2.2 CLP isolation from acetone extracts

Silica gel 60 (230-400 mesh; column size, 0.08 × 0.5 cm; Sigma-Aldrich Canada Ltd., Oakville, ON) flash column chromatography was utilized to adsorb CLPs and other relatively polar compounds (e.g., pigment, phospholipid and wax) from acetone extracted samples obtained as described in 3.2.1. Silica gel columns (vertical) were prepared as follows: a cotton ball was placed in the bottom of a 3 mL plastic syringe with sand (50-70 mesh; 1 cm height) added on top for support. Silica gel (0.5 g) was slurried in hexane (1 mL) with a glass stirring rod in a 20 mL beaker and poured onto the sand then covered by another layer of sand (50-70 mesh; 0.5 cm height) (Figure 3-1). The plastic syringes were inserted into the Luer-Lok™ fittings (Becton, Dickinson and Co.) of a Visiprep™ solid phase extraction vacuum manifold (Model: 12-port, Supelco, Bellefonte, PA). The silica gel columns were equilibrated with 2 mL of hexane for 2 min before a mixture of oil sample (1 mL) and hexane (1 mL) was loaded onto the gel. The




(a) (b)

Figure 3-1 Silica gel column (a) and Visiprep™ solid phase extraction vacuum manifold (with one silica gel column in place) (b) used for CLP isolation

column was eluted with solvents of increasing polarity under vacuum pressure ($7.1\times10^4$ Pa) : 100% hexane (10 mL), 20% ethyl acetate (EtOAc) in hexane (10 mL), 50% EtOAc in hexane (10 mL), 100% EtOAc (10 mL) and 10% methanol (MeOH) in dichloromethane ($CH_2Cl_2$) (10 mL). CLPs were known to elute with 100% EtOAc and 10% MeOH in $CH_2Cl_2$ wash solvents according to previous studies conducted by Y-H. Jia (2008). This finding was re-confirmed in this study (Appendix C). The two peptide enriched fractions were combined in a 100 mL round bottom flask. The solvent was removed by evaporation under reduced pressure in a rotary evaporator (approximately 9,000 Pa, 40°C water bath, Rotavapor R-200, Buchi, Westbury, NY). The residue in the flasks was collected for further analysis.

### 3.2.3 HPLC method development for CLP quantification

The fractions from silica gel isolation were analyzed by reverse phase HPLC. An Agilent 1200 series HPLC system (Agilent Technologies Canada Inc., Mississauga, ON) equipped with degasser (G1322A), quaternary pump (G1311A), auto-sampler (G1316A) and diode array detector (DAD) (G1315D; wavelength range 190-600 nm) and a ZORBAX Eclipse XDB-C™ 18 column (5 µm particle size, 150 × 4.6 mm I.D.) was used for all analyses.

#### 3.2.3.1 Calibration curves of CLPs with internal standard

CLP standards and Seg-A (10 mg, each) were weighed using an analytical balance (Accuracy: 0.1 mg, Model: P/PI-214, Denver Instrument, Bohemia, NY) and dissolved in 5 mL of methanol in a three-dram vial to make stock solutions of 2 mg/mL. The vials were sealed with Parafilm™ (Pechiney Plastic Packaging, Chicago, Il) to limit solvent evaporation. A mixture of CLP-A, B, C, D, E, G each at 200 µg/mL and Seg-A, at 100 µg/mL, was prepared by adding 100 µL of each CLP, 50 µL of Seg-A stock solution and 350 µL of methanol (1 mL in total) to a 1.5 mL HPLC vial using a graduated syringe (100 µL, Model: 810 RNW, Hamilton Company, Reno, NV). The sample was filtered with 0.45 µM PTFE syringe filter (Whatman Ltd., Psicataway, NJ). The sample (15 µL) was injected onto the HPLC column and the elution gradient provided in Table 3.1 was initiated. The retention times for each peptide were

determined by three injections each day for four consecutive days. System precision was determined by relative standard deviations of signal intensity of each peptide.

Peaks of UV absorbance were detected over the wavelength range from 190-300 nm. Eluting peaks were detected at wavelengths of 214 nm and 244 nm with a 10-nm bandwidth and against a reference signal at 300 nm with a 10-nm bandwidth and at 280 nm with a 10-nm bandwidth against a reference signal at 340 nm with a 100-nm bandwidth using Chemstation for LC 3D™ system software (Agilent Technologies Canada Inc., Mississauga, ON). Area integration of eluting peaks was obtained at 214 nm with 10-nm bandwidth. UV spectra of the peptides were recorded by selecting the maximum spectrum of each LC peak in the chromatogram using Chemstation software. Calibration curves for CLP quantification were established between 10 and 500 µg/mL in the presence of Seg-A at a constant concentration of 50 µg/mL. The concentrations of CLP, x-axis and the area ratio of the peaks of CLP to Seg-A, y-axis were plotted as a standard curve. CLPs concentration was determined by calculating peak area relative to the internal standard using the standard curves. Three samples were prepared at each concentration of CLPs and injected on three consecutive days to establish the calibration curves.

No standard was available for CLP-F. The concentration of CLP-F was estimated based on CLP-G. This assumption is based on the highly similar polarity, molecular weight and chromophore composition shared by the two compounds (Fig. 2-5; Appendix D).

#### 3.2.3.2 Accuracy of HPLC methods

A set of quality control solutions (blind samples) were prepared at four concentrations in methanol (50 µg/mL, 125 µg/mL, 200 µg/mL and 500 µg/mL) by Research Assistant M. Bagonluri, Plant Sciences, University of Saskatchewan. The accuracy of the HPLC method in measurement of the concentration of these solutions was determined using the calibration curves (n=3). Recovery was calculated according to Equation 3.2.

$$\%\,\text{recovery} = \frac{\text{calculated concentration}}{\text{actual concentration}} \times 100 \qquad (3.2)$$

Table 3-1 Solvent program for CLP identification and quantification by HPLC

| **Time (min)** | **Solvent composition** (% acetonitrile)[a] | **Flow rate** (mL/min) |
|---|---|---|
| 0 | 30 | 0.5 |
| 3 | 40 | 0.5 |
| 6 | 45 | 0.5 |
| 7 | 65 | 0.5 |
| 19 | 65 | 0.5 |
| 22 | 66 | 0.5 |
| 23 | 70 | 1.0 |
| 24 | 100 | 1.0 |
| 26 | 100 | 1.0 |
| 31 | 30 | 1.0 |

[a] Gradient of acetonitrile in water

### 3.2.4 Quantification of CLPs in flaxseed from different varieties

The aforementioned five licensed cultivars of flaxseed including Somme, Vimy, Flanders, CDC Bethune and CDC Valour, which were grown in a randomized complete block design (RCBD) in two locations (Floral and Saskatoon, SK) in 2006 and 2008, were selected for CLP quantification. Oil was extracted once for each seed sample and peptide extracts were analyzed twice by HPLC as described above.

#### 3.2.4.1 Sample preparation for CLP quantification using internal standard Segetalin-A

Flaxseed oil and CLPs, were extracted by acetone from ground flaxseed as described in 3.2.1. Oil samples (1 mL) were then weighed in a 10 mL beaker using an analytical balance (Accuracy: 1 mg, Model: PB403, Mettler Toledo, Greifensee, Switzerland) and the weight was recorded to two decimal places. Seg-A solution (25 µL, 2 mg/mL) and hexane (1 mL) were added to the oil. The solution was swirled by hand before loading onto a silica gel column. After elution from the column, as described previously (3.2.2), the peptide fraction was taken to dryness and dissolved in 1 mL of MeOH which was subsequently filtered (0.45 µM PTFE syringe filter, Whatman Ltd., Piscataway, NJ) prior to HPLC analysis (as described in 3.2.3). The integrated areas of each eluting peptide observed in the chromatograms were recorded and the concentrations of CLPs in flaxseed oil were calculated using calibration curves (as described in 3.2.3). Oil content was determined according to Equation 3.1. The concentration of CLPs in flaxseed was calculated using Equation 3.3.

$$\text{concentration of CLP} = \frac{\text{calculated concentration in oil}}{\rho} \times \text{oil content} \qquad (3.3)$$

Where: calculated concentration in oil (µg/g) was obtained from the calibration curve; ρ (g/mL) was the density of the flaxseed oil; and oil content (%) was obtained from Equation 3.1.

### 3.3 CLPs in flaxseed fractions

Flaxseed (Natunola, 2008) was chosen for the study of CLP levels in flaxseed fractions. The variety and growing conditions of the Natunola seed were unknown (plot

grown pedigreed seed was not available at the time of the study). Flax fractions including gum, seed coat (called "hull"), cotyledon and oil bodies were prepared for CLP identification and quantification using the HPLC method. The seed was separated into fractions and subsequently analyzed three times beginning with the same flaxseed sample.

### 3.3.1 Water degumming and seed coat removal

Flaxseed mucilage was extracted by the method of Bhatty (1993). The seed samples (10.00 g) were added to hot distilled water (100 mL at 80°C) in a 250 mL glass beaker and extracted overnight with stirring at 400 rpm at room temperature (25°C). The mucilage was separated from the seeds using a 40 mesh screen (12 inch-HH-BR-SS-US-40; VWR Co., West Chester, PA) then freeze dried (Model 77540, Labconco Corporation, Kansas City, MO) until the weight of the dry material (called "gum") became constant. Degummed seeds were manually dissected using a stainless steel spatula (length: 17.8 cm, width: 0.3 cm, VWR International LLC., Arlington Heights, IL) by applying pressure on the seed coat. The seed coats were, thereby, separated from cotyledons. The cotyledon fraction was collected, water-rinsed twice with distilled water (10 mL) and dried overnight in a paper towel at room temperature. The seed coats from this separation were treated similarly to the cotyledons. The weight of each fraction (gum, seed coats, cotyledons and whole seeds) was determined using an analytical balance (Accuracy: 1 mg, Model: PB403, Mettler Toledo, Greifensee, Switzerland) and recorded to the nearest 10 mg. Oil was extracted from gum, seed coats, cotyledons and whole seeds with acetone using the method described in 3.2.1. The oil content of each fraction was determined according to Equation 3.1. Quantification of CLPs was performed as described in 3.2.4.1.

The gum fraction contained only a trace amount of oil (0.02 g). The beaker used for gum extraction was directly washed with MeOH (10 mL) twice and the extract was placed in a 50 mL round bottom flask to ensure the recovery of peptides from this fraction. Methanol was removed by evaporation under reduced pressure in a rotary evaporator (approximately 9,000 Pa, 40°C water bath, Buchi, Westbury, NY) and the residue in the flasks was dissolved in MeOH (1 mL). Subsequently Seg-A solution (25

μL, 2 mg/mL) was added to the MeOH and the solution was filtered (0.45 μM PTFE syringe filter) (Whatman Ltd., Piscataway, NJ) before HPLC analysis. HPLC chromatography was performed as described in 3.2.3 and the concentrations of CLPs in flaxseed fractions were calculated as described in Equation 3.3.

### 3.3.2 Oil body (oleosome) isolation

Oil bodies were isolated using the procedure of Simpson (1989). Whole seeds (20 g, Natunola) were first soaked overnight in 200 mL of 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 7.2, at 4°C. After soaking, an additional 100 mL of buffer was added and the mixture was homogenized in a blender for 3 min at 22,000 rpm (Model 8100, Eberbach Corporation, Ann Arbor, MI). The homogenate was centrifuged in a chilled rotor (4°C) at 9,000 rpm for 60 min (14,334 × *g*, Model J-E, JA-10 Rotor, Beckman Coulter, Inc., Palo Alto, CA) to form three layers: a floating fat pad, a supernatant fraction and a precipitated solid bottom residue. The floating brown-white fat pad was removed by a flat stainless spatula (VWR International LLC., Arlington Heights, IL). The fat pad was homogenized again using the same conditions in five volumes of chilled (ice bath), fresh buffer. The centrifugation-homogenization procedure was performed five times and the final fat pad was transferred to a 50 mL centrifuge tube (VWR International LLC., Arlington Heights, IL). The supernatant (also called "serum") was separated from the bottom solid residue by decanting. The fat pad and serum were freeze-dried (Model 77540, Labconco Corporation, Kansas City, MO) and fraction weights were recorded to the nearest 10 mg before oil extraction. Solid residue was stored at 4°C in a refrigerator (Model 153, Fisher Scientific, Dubuque, IA) before oil extraction.

The oils from serum and bottom residue were extracted as described in 3.2.1 except the samples were not ground. Oil was extracted from the dried oil bodies using 5 volumes of hexane by shaking the hexane-oil body mixture by hand for approximately 2 min. The hexane layer was removed to a 125 mL Erlenmeyer flask (VWR, Edmonton, Canada). The remaining solid was then mixed with 10 volume of EtOAC and shaken by hand for 2 min. After mixing the EtOAC solution was filtered (Whatman, No 2 filter paper, Whatman Ltd., Piscataway, NJ) and the filtrate was combined with

aforementioned hexane layer. Solvents (hexane and EtOAC) were removed from the fraction using a rotary evaporator under reduced pressure (approximately 9,000 Pa, 40°C water bath, Buchi, Westbury, NY). The CLPs were quantified as described in 3.2.4.1.

### 3.4 Effects of processing on the distribution of CLPs

#### 3.4.1 Crude oil extraction by expeller press

Flaxseed (1 kg, CDC Bethune, 2006, Floral) was extracted using a continuous oilseed expeller press (Komet, type CA59C, IBG Monforts Oekotec GmbH & Co., Germany) operating at 88 rpm. No heat was applied during pressing. Expeller pressed oil was allowed to settle for 2 days to produce both clear crude oil and sediment which is also known as foots. Subsequently, the upper oil layer was decanted. The foots fraction was separated from the remaining oil by filtration under vacuum (approximately 9,000 Pa) with a Buchner funnel lined with a glass-fibre filter (Whatman, Grade GF/A, Whatman Inc., Piscataway, NJ). The oil recovered by filtering the foots was combined with the crude oil that was obtained by sedimentation. Foots and pressed meal were dried in the fume hood at room temperature overnight and then stored at 4°C in a refrigerator (Model 153, Fisher Scientific, Dubuque, IA) before solvent (Goldfisch) extraction . The concentrations of CLPs in each fraction were calculated as described in Equation 3.3. Each sample was injected into the HPLC once. This experiment (including oil processing, settling, foots filtration, acetone extraction, silica gel isolation and HPLC quantification) was repeated three times and the results were presented as the average of these three replicates.

Commercial flaxseed oil from local retail health food stores including Omega (Omega Nutrition Canada Inc., Vancouver, BC), GNC (General Nutrition Centres Inc., Pittsburgh, PA), Flora (Floral Inc, Lynden, DC), Sangster (Sangster Health Centers, Saskatoon, SK) and Gold Top (Gold Top Organics Ltd., Edmonton, AB) were selected for comparison of CLPs in commercial flaxseed oils and crude oil produced in the lab (after two days settling). Peptides from three bottles of each brand from different lots (purchased on three days from one retail outlet) were utilized for this experiment. The CLPs were quantified as described in 3.2.4.1. Every sample was subject to HPLC analysis once.

### 3.4.2 Acid degumming for removal of CLPs from flaxseed oil

The effect of acid degumming on the solubility of CLPs in lab-pressed flaxseed oil (CDC Bethune) was determined by treating flaxseed oil with aqueous phosphoric acid ($H_3PO_4$). In the first study, flaxseed oil (50 mL) was heated on a hot plate until it reached 80°C after which $H_3PO_4$ (5 mL, 75%) was added. The sample was mixed vigorously with a magnetic stirrer at 600 rpm for 5 min at room temperature then centrifuged in a chilled rotor (4°C, 9,800 × *g*, Model J-E, JA-25.50 Rotor, Beckman Coulter, Inc., Palo Alto, CA) for 30 min. After centrifugation, the upper oil layer was decanted from the bottom gum. In the second, third and fourth experiments, acid degumming condition was the same as described above except 1 mL of $H_3PO_4$ (75%), 0.5 mL of $H_3PO_4$ (75%), 0.5 mL of $H_3PO_4$ (50%) were added, respectively. In the fifth experiment, 0.05 mL of $H_3PO_4$ (75%) was first added to the flaxseed oil (50 mL) and the acid degumming was performed as described above. After centrifugation, the upper oil was taken to perform a second acid degumming treatment using 0.05 mL of 75% $H_3PO_4$. The same procedure for acid degumming was followed. Experiments were repeated three times.

Untreated crude oil was used as a control. The CLPs were quantified as described in 3.2.4.1. Every sample was subject to a single HPLC analysis.

### 3.4.3 CLPs from phospholipid gum after acid degumming

After decanting oil (section 3.4.2) the phospholipid gum pellet (from 50 mL flaxseed oil), remaining in the centrifuge tube, was mixed with hexane (10 × volume) and mixed vigorously by hand. The hexane extract was filtered (Whatman, No. 2, Whatman Inc., Piscataway, NJ) and the filtrate was combined with an equivalent volume of MeOH. The mixture was transferred to a separatory funnel (125 mL) and the phases were allowed to separate. After 3 hours, the MeOH phase (upper layer) was recovered and concentrated under reduced pressure in a rotary evaporator (approximately 9,000 Pa, 40°C water bath, Rotavapor R-200, Buchi, Westbury, NY). The concentrate was washed twice with diethyl ether ($Et_2O$) (100 mL). After each wash, the upper layer was decanted and the residue was concentrated under reduced pressure using a rotary evaporator (approximately 9,000 Pa, 40°C water bath, Rotavapor R-200, Buchi, Westbury, NY).

The concentrate was re-suspended in acetone (10 × volume), followed by filtration (Whatman No. 2, Whatman Inc., Piscataway, NJ). The acetone filtrate was concentrated by a rotary evaporator under reduced pressure (approximately 9,000 Pa, 40°C water bath, Rotavapor R-200, Buchi, Westbury, NY). The collected peptides were taken into 50 mL of MeOH. A portion of the MeOH solution (1 mL) was mixed with 25 µL of 2 mg/mL Seg-A before filtering with a 0.45 µM PTFE syringe filter (Whatman Ltd., Piscataway, NJ). Each sample was injected once onto HPLC for analysis as described in 3.2.3 and the concentration of CLPs was calculated according to Equation 3.3. This experiment was repeated three times.

After elution from the column, as described previously, the peptide fraction was taken to dryness and dissolved in MeOH (1 mL), which was subsequently filtered (0.45 µM PTFE syringe filter, Whatman Ltd., Piscataway, NJ) prior to HPLC analysis (as described in 3.2.3).

### 3.4.4 Alkali refining for removal of CLPs from flaxseed oil

Lab-pressed flaxseed oil (CDC Bethune) was refined by addition of alkali into flaxseed oil to neutralize the FFAs (e.g., oleic acid). In the first experiment, flaxseed oil (50 mL) was heated on a hot plate until temperature of the oil reached 80°C and then 0.5 mL of 4M NaOH was added. The sample was mixed vigorously with a magnetic stirrer at 600 rpm for 5 min at room temperature then centrifuged (9,800 × *g*, Model J-E, JA-25.50 Rotor, Beckman Coulter, Inc., Palo Alto, CA) at 4°C for 30 min, to facilitate separation of the oil and soap that formed from the neutralization. In subsequent experiments, the conditions of alkali refining were the same as described above except 0.5 mL of 4 M KOH, 0.5 mL of 2 M $K_2CO_3$, 0.5 mL of 2 M $Na_2CO_3$, 0.5 mL of saturated $NaHCO_3$, 1.3 M $K_3PO_4$ and 0.5 mL of 1.3 M $Na_3PO_4$ were used, respectively. Experiments were repeated three times.

Crude oil without alkali treatment was used as a negative control. The CLPs were quantified as described in 3.2.4.1.

### 3.5 Statistical analysis

During extraction CLP oxidation was observed (CLP-B was readily oxidized to CLP-C, CLP-H to CLP-G, CLP-I to CLP-F). As the oxidation was not a controlled experimental variable, statistical analysis of each peptide separately was not possible. During the course of this study the genomic sequence of flax was published on the world wide web (linum.ca, 2010). Searches of this database have revealed that peptides CLP-A, B, C and E are the product of a gene sequence (g24175), additionally CLP-D, F, G, H and I were the product of gene g38655 (linum.ca, 2010). The reliability of CLP quantification was improved by grouping peptides that were products of a single gene reading frame. Products of g24175 are hereafter referred to as CLP-24175 (total CLPs expressed by gene g24175) and CLP-38655 includes all CLPs expressed by gene reading frame g38655. These groupings were used in subsequent statistical analysis.

All statistical analyses were conducted using the Statistical Analysis System (SAS for Windows®, Release 9.2, SAS Institute Inc., Cary, NC).

One way ANOVA was used to analyze the effect of cultivar on the level of CLPs in flaxseed. The following second-order polynomial equation was utilized to analyze the differences of CLPs among different flax cultivars (Equation 3.4).

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij} \qquad (3.4)$$

Where $Y_{ij}$ is the observed value for the $j^{th}$ replicate of the $i^{th}$ cultivar. $\mu$ is the grand mean. $\tau_i$ is the effect for the ith cultivar. $\varepsilon_{ij}$ is the random error associated with the $Y_{ij}$ experimental unit.

Post-hoc multiple comparison test was Tukey's test. Differences were considered significant at $P < 0.05$. Results were expressed as means ± SD (standard deviation).

In order to analyze the effects of variety, year, location and their interactions on the expression of CLPs, analysis of variance by PROC MIXED procedure, Pearson correlation (to estimate the linear relationships between the expressions of group CLP-24175 and CLP-38655) and variance components using PROC VARCOMP procedure were performed according to SAS methods. The following second-order polynomial equation was utilized (Equation 3.5).

$$Y_{ijk} = \mu + a_i + b_j + ab_{ij} + c_k + ac_{ik} + bc_{jk} + abc_{ijk} + \varepsilon_{k(ij)} \quad (3.5)$$

Where $Y_{ijk}$ is the dependent variable observed value in level *k* of the *ij* treatment. μ is the grand mean. *a* is the effect of level *i* of variety. *b* is the effect of level *j* of location. *c* is the effect of level *k* of year. $ab_{ij}$ is the effect of using level *i* of variety with level *j* of location. $bc_{jk}$ is the effect of using level *j* of location with level *k* of year. $ac_{ik}$ is the effect of using level *i* of variety with level *k* of year. $abc_{ijk}$ is the effect of using level *i* of variety with level *j* of location and level *k* of year. $\varepsilon_{k(ij)}$ is the residual.

The following second-order polynomial equation was utilized to analyze the differences of CLPs among different commercial flaxseed oils and among different processing treatments by one-way ANOVA(Equation 3.6).

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij} \quad (3.6)$$

Where $Y_{ij}$ is the observed value for the $j^{th}$ replicate of the $i^{th}$ treatment. μ is the grand mean. $\tau_i$ is the treatment effect for the ith treatment. $\varepsilon_{ij}$ is the random error associated with the $Y_{ij}$ experimental unit.

Post-hoc multiple comparison test was Tukey's test. Differences were considered significant at $P < 0.05$. Results were expressed as means ± SD (standard deviation).

# 4 RESULTS AND DISCUSSION

## 4.1 Oil content of flaxseed and flaxseed materials

Acetone (polarity index: 5.1) is a suitable solvent for extracting oil and non-oil compounds from a variety of plant materials. For example, acetone was used for extraction of fatty acids from beechwood (Demirbas, 1991). Eaves *et al.* (1952) observed that the crude oil yield of cottonseed obtained by acetone extraction was comparable to that recovered by hexane extraction. In addition, non-oil materials including pigments and gossypol were found in the acetone extract (Eaves *et al.*, 1952). Stefanowic (2001) used acetone to isolate CLPs from ground flaxseed (1:20, w/v) at room temperature. In this project, acetone was chosen for simultaneous extraction of flaxseed oil and CLPs due to the solubility of CLPs in acetone, as well as the better extraction power of acetone in the presence of water in analyzed samples. The oil contents of flaxseed and flaxseed materials including seed coat (hull), gum, cotyledon, oil bodies, serum, residue, crude oil, meal and foots, are listed in Table 4-1. The oil content of flaxseed from different varieties varied from 38.0% to 42.4%. The variance may be caused by the genetic or environmental differences among flaxseed samples, moisture difference among flaxseed samples and/or experimental errors during processing (Flax Council of Canada, 2011). It was worth noting that these data were lower than the oil contents published by the Flax Council of Canada for the same varieties (42.5%-45.7%) (2011). The difference could be explained by the fact the oil content obtained in this project was based on flaxseed determined on an “as is” basis while the reported data from the Flax Council of Canada was calculated on a dry matter basis.

In flaxseed fractions, the oil content of the cotyledon (46.3%) was higher than that of the seed coat (25.5%) or gum (1.9%). These results correspond with previous studies where 51.0% and 22.9% oil content (dry basis) were found in cotyledons and seed coat, respectively (Dorrell, 1970). The trace oil found in the gum fraction might be

Table 4-1 Oil content of flaxseed and flax materials, as is basis (n means the number of the tested samples)

| (n=4) | | Weight (g) | Oil content (%) |
|---|---|---|---|
| Somme | Mean | 5.0 | 41.5 |
| | SD | - | 2.1 |
| CDC Valour | Mean | 5.0 | 38.0 |
| | SD | - | 1.41 |
| Flander | Mean | 5.0 | 40.7 |
| | SD | - | 2.9 |
| CDC Bethune | Mean | 5.0 | 42.4 |
| | SD | - | 0.7 |
| Vimy | Mean | 5.0 | 41.7 |
| | SD | - | 1.1 |
| (n=3) | | Natunola | |
| Whole seed | Mean | 10.0 | 40.9 |
| | SD | - | 0.2 |
| Cotyledon | Mean | 5.2 | 46.3 |
| | SD | 0.1 | 0.7 |
| Seed coat | Mean | 4.1 | 25.5 |
| | SD | 0.1 | 0.3 |
| Gum | Mean | 0.7 | 1.9 |
| | SD | 0.1 | 0.8 |
| (n=3) | | Natunola | |
| Whole seed | Mean | 20.0 | 40.9 |
| | SD | - | 0.2 |
| Oil bodies | Mean | 4.9 | 88.7 |
| | SD | 0.1 | 1.2 |
| Serum | Mean | 2.7 | 24.3 |
| | SD | 0.2 | 0.6 |
| Residue | Mean | 11.0 | 28.0 |
| | SD | 0.3 | 2.0 |
| (n=3) | | CDC Bethune | |
| Whole seed | Mean | 1000.0 | 42.4 |
| | SD | - | 0.3 |
| Crude oil | Mean | 343.9 | 100.0 |
| | SD | 6.4 | - |
| Meal | Mean | 575.4 | 14.9 |
| | SD | 10.5 | 3.6 |
| Foots | Mean | 35.4 | 54.1 |
| | SD | 4.0 | 4.3 |

due to contamination during processing. Oil bodies are the main organelle for oil storage in the plant seed (Huang, 1996). In this project, the oil content of the oil body fraction was 88.7%. The oil contents of serum (24.3%) and residue (28.0%) were much lower. This is typical as the low density of the oil bodies causes them to mostly distribute in the fat pad after homogenization and centrifugation.

Flaxseed meal produced by pressing had an oil content of 14.9%. The remaining oil was found in sedimented foots (oil content of 54.1%).

### 4.2 CLP isolation

Silica gel was used by Brühl *et al.* (2007) to isolate cyclolinopeptides from flaxseed oil. In this project, silica gel columns were also used for CLP isolation from flaxseed oil at the ratio of 1:2 (w/v, silica to oil). Less polar solvent washes (such as hexane, 20% EtOAC in hexane, 50% EtOAC in hexane) were used to elute low polarity neutral compounds of flaxseed oil (e.g., TAG, wax and pigments), while 100% EtOAC and 10% MeOH in Dichloromethane (DCM) eluted CLPs from the silica gel.

### 4.3 CLP identification and quantification by HPLC

Brühl *et al.* (2007) determined the CLP-E concentration in flaxseed oil using an external standard method. In their study, an external calibration curve was established from 3-900 µg/mL CLP-E with a coefficient of determination of 0.998. However, the calibration curve or equation was not included in their publication. CLP standards have not been available to previous researchers and, therefore, there is no other literature available on the CLP concentration in flaxseed tissues. In the current study, a HPLC method for CLP detection and quantification was developed using Seg-A as an internal standard. Seven peaks were observed in HPLC chromatograms of flaxseed oil extracts after addition of the standard including Seg-A, CLP-G, CLP-C, CLP-E, CLP-D, CLP-B and CLP-A respectively, (Figure 4-1). Absorption of ultraviolet light by chromophores in the CLPs provided a signal that was readily detected by a diode array detector. These chromophores include peptide bonds (214 nm), phenylalanine (260 nm) and tryptophan (280 nm) which are useful for peptide detection, characterization and quantification (Marshak, 1996; Pace *et al.*, 1995). In HPLC chromatograms, all six CLPs at 0.2 mg/mL

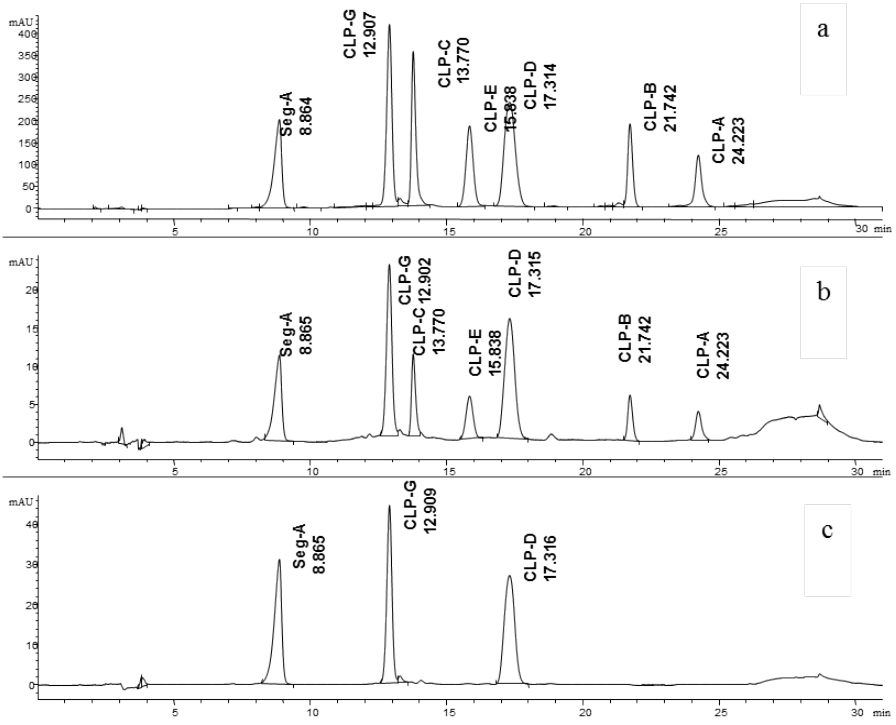



Figure 4-1 HPLC chromatograms of CLP-A, B, C, D, E, G (0.2 mg/mL, each) and Seg-A (0.1 mg/mL) at the wavelengths of (a) 214, (b) 244 and (c) 280 nm with a bandwidth of 10 nm. Defaulted reference signals were used (300 nm with 10 nm bandwidth for 214 and 244 nm, 340 nm with 100 nm bandwidth for 280 nm). HPLC conditions are provided in Table 3-1.

presented useful absorbances (over 150 mAU) at 214 nm due to the strong absorption of peptide bonds and conjugated double bonds in aromatic amino acids. Comparatively weaker absorbance (less than 20 mAU) at 244 nm was found in all peptides. Three standards (CLP-D, G and Seg-A) all had weak absorbances (around 40 mAU) at 280 nm due to the presence of the indole group of tryptophan (Trp) in their structure. The complete UV spectra of Trp-containing peptides (190 nm-300 nm) obtained from HPLC chromatograms confirmed this observation (Figure 4-2).

In a previous report of chromatographic conditions for separation of cyclolinopeptides, Brühl *et al.* (2007) separated five peptides (CLP-F, G, C, E and A) with elution times at 20-30 min. They reported that crude extracts (4.4 mg) were dissolved in water/ethanol (1:1, v/v; 1.5 mL) and 100 µL of the aliquots were injected onto a 250 mm × 4 mm, 5 µm LiChrospher 100 RP-18 column. Chromatography was performed using a mixture of methanol/water (from 75/25 to 100/0, v/v; within 25 min). In the current study, a shorter column (150 mm × 4.6 mm, 5 µm) and a different solvent system (acetonitrile/water) were employed. The same CLPs were more evenly distributed throughout the chromatogram, eluting between 12 and 25 min (Table 4-2).

The coefficient of variation (CV) of HPLC elution time was less than 3% for all CLP measurements, which indicated good reproducibility of HPLC. Calibration curves for each peptide were established with the origin (0, 0) included in each standard curve (Appendix E). The equations extracted from calibration curves were used to calculate the concentrations of CLPs of unknown samples (Table 4-3).

Quality control solutions of CLPs were made by another analyst in the lab to test the accuracy of HPLC for CLP determination. The recovery of CLPs ranged from 92% to 115% for a concentration of 50 µg/mL and from 95% to 118% at 125 µg/mL. The apparent high recovery of 115-118% could be explained by human and systematic errors when dilute samples were handled. At higher concentrations, the coefficient of variation of recovery was reduced and the range of results was consistent with 100% recovery; 94%-104% at 200 µg/mL and 95% to 103% at 500 µg/mL (Table 4-4).

### 4.4 CLPs content of flaxseed from different flax cultivars

With the exception of the report by Brühl *et al.* (2007), the concentration of

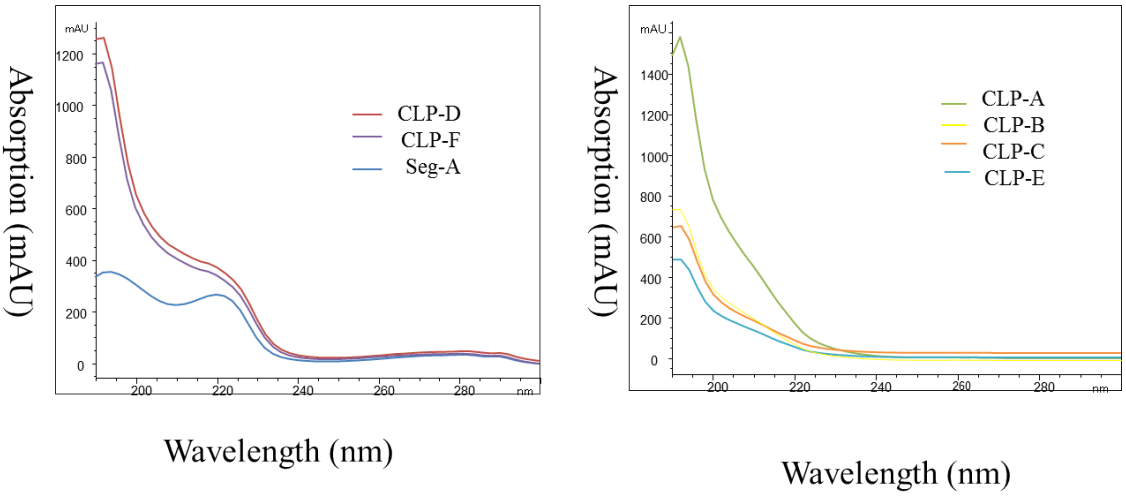



Figure 4-2 Comparison of UV spectra of Seg-A, CLP-G and CLP-D (Trp-containing peptides) and CLP-C, B, A and E (Trp-free peptides) (in methanol) extracted from HPLC chromatograms using Chemstation. HPLC conditions are listed in Table 3-1. Elution times: Seg-A (8.86 min), CLP-G (12.91 min), CLP- C (13.77 min), CLP-E (15.84 min), CLP-D (17.31 min), CLP-B (21.74 min) and CLP-A (24.22 min).

Table 4-2 Retention times of Seg-A and CLPs

| | **Retention time**[1] (min) | **CV**[2] (%) | **Area**[3] (mAU×s) | **CV**[2] (%) |
|---|---|---|---|---|
| Seg-A | 8.79 | 1.41 | 4203 | 1.49 |
| CLP-G | 12.83 | 0.46 | 5945 | 1.00 |
| CLP-C | 13.61 | 1.95 | 3875 | 1.43 |
| CLP-E | 15.72 | 0.77 | 3507 | 2.72 |
| CLP-D | 17.22 | 0.99 | 6705 | 1.32 |
| CLP-B | 21.55 | 1.02 | 2453 | 2.34 |
| CLP-A | 24.05 | 0.84 | 2080 | 2.74 |

[1] Mean of retention time for 12 runs (three runs/day for four days)

[2] Coefficient of variance=standard deviation/mean×100%

[3] Mean of peak area for 12 runs (three runs/day for four days)

Table 4-3 Quantification equations for CLPs using Seg-A as internal standard

| CLPs | Equation | R squared |
|---|---|---|
| CLP-A | $Y^b=(X^a+0.0398)/5.6897$ | 0.9984 |
| CLP-B | Y=(X-0.0104)/5.9165 | 0.9998 |
| CLP-C | Y=(X+0.0015)/9.6736 | 0.9999 |
| CLP-D | Y=(X-0.0125)/15.672 | 0.9997 |
| CLP-E | Y=(X+0.0477)/9.848 | 0.9990 |
| CLP-G | Y=(X+0.0349)/16.313 | 0.9993 |

[a]X=Area ratio of CLP/Seg-A from the HPLC chromatogram

[b]Y= Concentration of CLP (mg/mL) in analyzed sample

Table 4-4 Accuracy for assay solutions using HPLC quantification

| | **Calculated concentration (µg/mL)** | **Reported concentration (µg/mL)** | **Recovery (%)** |
|---|---|---|---|
| CLP-A | 57 | 50 | 114 |
| | 123 | 125 | 99 |
| | 197 | 200 | 99 |
| | 491 | 500 | 98 |
| CLP-B | 46 | 50 | 92 |
| | 136 | 125 | 109 |
| | 208 | 200 | 104 |
| | 475 | 500 | 95 |
| CLP-C | 58 | 50 | 115 |
| | 119 | 125 | 95 |
| | 188 | 200 | 94 |
| | 515 | 500 | 103 |
| CLP-D | 56 | 50 | 113 |
| | 147 | 125 | 118 |
| | 188 | 200 | 94 |
| | 499 | 500 | 100 |
| CLP-E | 55 | 50 | 110 |
| | 125 | 125 | 100 |
| | 190 | 200 | 94 |
| | 495 | 500 | 99 |
| CLP-G | 51 | 50 | 102 |
| | 116 | 125 | 93 |
| | 204 | 200 | 102 |
| | 473 | 500 | 95 |

CLPs in flaxseed has not been reported. In Brühl *et al*. (2007), CLP-E was determined in the oil of 25 flaxseed varieties during storage. This study provided analysis of single samples of each flax variety. A study involving repeated sampling of the same varieties grown in plots might overcome the limitations of the previous study and determine the range of flaxseed peptide content. In the current research, flaxseed samples of five cultivars grown at two locations in two growing seasons were analyzed to study the possible effects of both genotype and environment on the concentration of CLPs in flaxseed. The CLP content of flaxseed differed significantly among varieties (Table 4-5).

The one-way ANOVA analyses showed there were significant differences in single and overall CLP levels among the five varieties. Somme had the highest levels of CLP-A (65.9 μg/g), D (42.5 μg/g), F (16.6 μg/g), G (50.0 μg/g) and overall CLPs (302.9 μg/g). The peptide levels of CDC Bethune and CDC Valour were similar; CLP-A concentrations found in this study were lower than the literature report for whole seed (44-66 μg/g vs. 70 μg/g) (Morita *et al*., 1999). CLP-B, CLP-H, CLP-I were not found in any of the flaxseed samples, which may be consistent with the trace amounts (2 μg/g, 2 μg/g and 0.7 μg/g, respectively) previously reported (Matsumoto *et al.*, 2001b). Higher concentrations of CLP-C were found in oil samples than in flaxseed (54-80 μg/g vs. 37 μg/g). CLP-D concentrations varied among different varieties, in which CDC Valour and Flanders had comparable concentrations (12-43 μg/g vs.15 μg/g). CLP-E concentrations also showed significant variability, where CDC Valour shared similar results with the literature (46 μg/g-71 μg/g vs. 58 μg/g). Equal or higher concentrations of CLP-F and G were found in this study than previous literature reports (8 μg/g -17 μg/g vs. 8 μg/g, 24 μg/g -51 μg/g vs. 24 μg/g). The levels of CLP-C, CLP-F and CLP-G in all of the analyzed samples were higher than in those published previously. This observation may be due to methionine oxidation of CLP-B, CLP-H, CLP-I, or genetic and/or environmental differences among different flaxseed cultivars.

Analysis of CLPs was complicated by methionine oxidation. According to the literature, methionine can be transformed to its oxidized forms (methionine sulfoxide and methionine sulfone) by chemical and biological means (Cuq *et al.*, 1973; Shechter, 1986). Hydrogen peroxide proved to be effective in oxidizing methionine in an acid

Table 4-5 CLP levels in flaxseed from different cultivars

| (n=4)[2] | | Concentration of CLPs (μg/g)[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CLP-A | CLP-B | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | Total |
| **Somme** | Mean | $65.9^{a}$ | -[3] | $65.2^{ab}$ | $42.5^{a}$ | $61.6^{ab}$ | $16.6^{a}$ | $51.0^{a}$ | $302.8^{a}$ |
| | SD | 18.0 | - | 7.5 | 5.0 | 10.1 | 2.0 | 7.3 | 41.4 |
| **CDC Valour** | Mean | $49.2^{ab}$ | - | $61.9^{ab}$ | $12.8^{b}$ | $56.0^{ab}$ | $10.7^{bc}$ | $30.5^{bc}$ | $221.1^{ab}$ |
| | SD | 16.2 | - | 15.9 | 4.9 | 14.9 | 3.7 | 11.5 | 63.2 |
| **Flander** | Mean | $44.0^{b}$ | - | $53.5^{b}$ | $12.4^{b}$ | $46.4^{b}$ | $8.3^{c}$ | $24.0^{c}$ | $188.6^{b}$ |
| | SD | 19.2 | - | 22.9 | 6.7 | 18.9 | 4.6 | 14.8 | 81.0 |
| **CDC Bethune** | Mean | $62.3^{ab}$ | - | $68.4^{b}$ | $23.5^{b}$ | $70.1^{ab}$ | $12.4^{bc}$ | $36.2^{bc}$ | $272.9^{ab}$ |
| | SD | 15.9 | - | 25.8 | 16.1 | 20.2 | 5.3 | 16.6 | 85.6 |
| **Vimy** | Mean | $54.1^{ab}$ | - | $79.7^{a}$ | $21.1^{b}$ | $71.3^{a}$ | $14.5^{ab}$ | $41.3^{ab}$ | $282.0^{a}$ |
| | SD | 21.2 | - | 28.1 | 13.2 | 24.6 | 6.0 | 19.0 | 103.6 |

[1] Means followed by the same superscript were not significantly different by Tukey's multiple comparison test at 5% level

[2] Seed samples from two year and two locations (2×2) were analyzed

[3] "–" not detected by HPLC

environment (Shechter, 1986). The superoxide anions produced in oxidative metabolism in biological systems could oxidize methionine to the sulfoxide (Vogt, 1995). Brühl *et al.* (2007) found a rapid increase in CLP-E in flaxseed oil (from 0 mg/kg to 843 mg/kg) stored over 150 days due to oxidation. In all the samples analyzed, the methionine-containing peptides, including CLP-B, CLP-H and CLP-I, were not observed. These methionine containing peptides were possibly oxidized by the extraction procedure (Appendix F). They were likely converted to CLP-C, CLP-G and CLP-F, respectively with the exposure to heat and oxygen during oil extraction (Figure 4-3).

High intraspecific variation of secondary metabolites might be the other reason for the difficulty in measuring CLP levels in multiple samples from the same flaxseed variety. Unlike primary metabolites (such as protein, carbohydrate and lipid) that are indispensable, uniform and conserved for plant growth and development, secondary metabolites (such as flavonoids, lignan, CLPs, etc.) are often unique, diverse and adaptive to their environment (Hartmann, 1996). The variance in CLP concentration in different flaxseed varieties has not been thoroughly studied, except for the study of CLP-E changes during storage by Brühl *et al.* (2007). CLP-E levels in that study showed great variance from 0 mg/kg to 53 mg/kg among 25 flaxseed varieties at the beginning of the study. After a 150-day storage period, the levels of CLP-E increased to above 600 mg/kg without other obvious changes noticed in flaxseed oil. The large intraspecific variance of CLP-E content at the beginning of the study and the corresponding increase in CLP-E in the sample over time indicated uncertainty in measuring CLPs in flaxseed oils, especially where single samples and measurements were considered.

Due to the transformation of CLPs during processing (CLP-B could be oxidized to CLP-C, CLP-H to CLP-G, CLP-I to CLP-F), statistical analysis of each peptide separately may not be meaningful. As it was recently discovered that cyclolinopeptides are encoded in genes that have several peptides in one gene, it is proposed that CLP-A, B, C and E be grouped (CLP-24175) for statistical analysis as the products expressed by gene g24175, whereas CLP-D, F, G, H and I are also grouped (CLP-38655) as the products of gene g38655. The contents of CLP-24175 and CLP-38655 differed among varieties (Table 4-6).

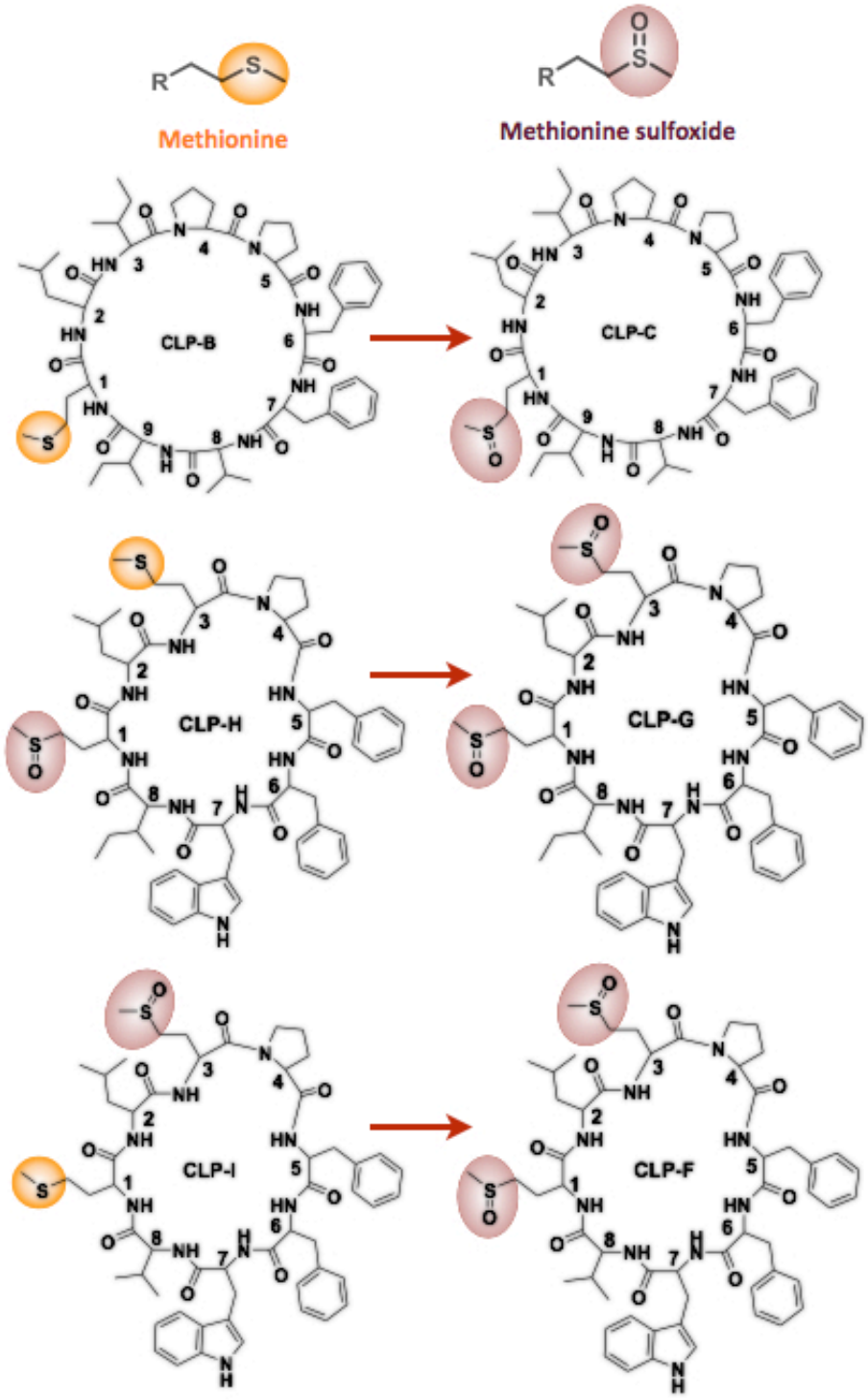



Figure 4-3 Transformation of CLP by oxidation of methionine to methionine sulfoxide

Table 4-6 CLP content (μg/g) of flaxseed varieties grown at two locations for two years

| Variety | Mean[1] of CLP-24175[2] | Range | Mean[1] of CLP-38655[3] | Range |
|---|---|---|---|---|
| CDC Bethune | 200.85[a] | 123.60-257.90 | 72.11[b] | 29.40-129.20 |
| Flanders | 152.05[c] | 89.30-208.80 | 50.45[c] | 18.20-91.10 |
| Somme | 192.73[a] | 138.90-243.10 | 110.15[a] | 88.30-124.40 |
| CDC Valour | 167.05[b] | 113.20-235.20 | 54.05[c] | 25.60-75.30 |
| Vimy | 205.11[a] | 122.30-303.50 | 76.85[b] | 52.60-140.30 |

[1] Means followed by the same superscript were not significantly different by Tukey's multiple comparison test at 5% level.

[2] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175

[3] CLP-38655 was the total CLP production (CLP-D, F, G, H and I) by gene g38655

The standard deviation obtained from Table 4-5 in the levels of CLPs and the wide range of CLP-24175 and CLP-38655 (Table 4-6) in the same flax varieties indicated that not only genotype, but the environment played a role in the concentration of CLPs observed in flaxseed. Analysis of variance was conducted to determine the effect of variety (V), year (Y), location (L) and their interaction on the concentration of CLP-24175 and CLP-38655 in flaxseed grown at two locations for two years (Table 4-7). While there was no impact of year alone on the concentration of CLP-24175 ($p=0.68$), other effects were all significant ($p<0.0001$) in contributing to variation. The interaction of V × Y × L (96%) suggested the variety responded differently to year for each location. For CLP-38655, the effects of variety, location, year and their interaction all were significant to the expression of CLP-38655. Variance of CLP-24175 was mostly caused by V × Y × L interactions, whereas the variance observed in CLP-38655 content was contributed by variety, location, V × Y, L × Y and V × Y × L, which accounted for 27%, 8%, 10%, 23% and 30% of the total variability respectively.

The complex interaction of variety and environment on CLP levels is shown in Figure 4-4. Influence of variety on the production of CLPs in each environment was plotted against four environment means. No obvious pattern was found to conclude the relationship between environmental effect (growth location, climate, etc.) on the production of CLPs in different flax varieties. This study was restrained due to the limited accessibility of flaxseed and the time-consuming processing protocols. Large scale studies of flaxseed with greater genetic differences from different locations and years should be done in the future when more rapid quantification methods have been developed.

### 4.5 Correlation between CLPs

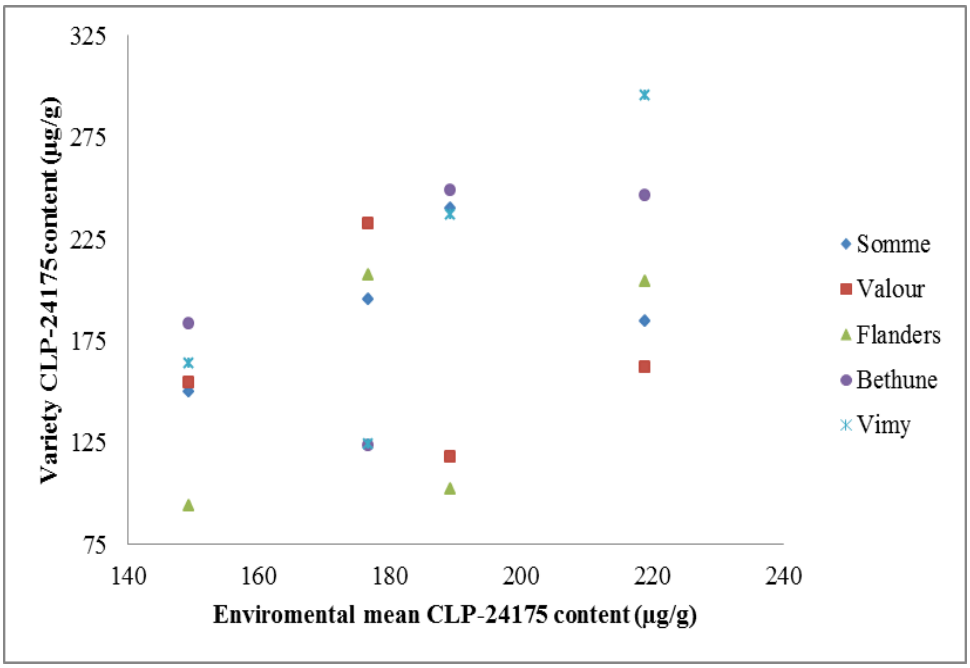
A whole genome shotgun assembly of *Linum usitatissimum* L. (var. CDC Bethune) has been published online by the University of Alberta (linum.ca, 2010). Searches of the annotated database generated from the sequences has revealed that CLP-A, B and E are expressed as motifs in a single gene sequence (g24175, Figure 2-6), while CLP-D, F and G occur as motifs in another gene (g38655, Figure 2-7) present in the flax genome (linum.ca, 2010, Reaney Pers. Commun.). There is just one copy of the

Table 4-7 Analysis of variance for CLP-24175 and CLP-38655 of flaxseed grown at two locations for two years

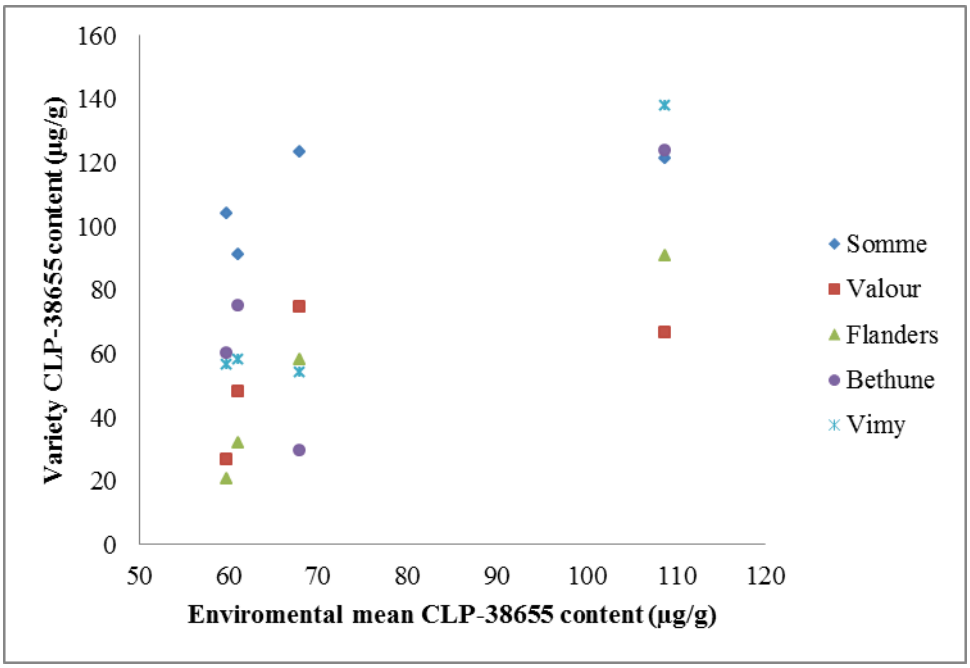
| Source | DF | Mean square | P | Variance Component (%) |
|---|---|---|---|---|
| CLP-24175[1] | | | | |
| Variety (V) | 4 | 4225.82 | < 0.0001 | 0 |
| Year (Y) | 1 | 12.88 | 0.6817 | 0 |
| Location (L) | 1 | 8079.80 | < 0.0001 | 0 |
| V × Y | 4 | 3039.04 | < 0.0001 | 0 |
| V × L | 4 | 6219.46 | < 0.0001 | 0 |
| L × Y | 4 | 16880 | < 0.0001 | 1.30 |
| V × Y × L | 4 | 9921.31 | < 0.0001 | 95.97 |
| Error | 20 | 82.42 | < 0.0001 | 2.73 |
| Total | 39 | 3083.15 | | |
| CLP-38655[2] | | | | |
| Variety (V) | 4 | 4525.90 | < 0.0001 | 27.07 |
| Year (Y) | 1 | 5605.05 | < 0.0001 | 0 |
| Location (L) | 1 | 9348.30 | < 0.0001 | 7.71 |
| V × Y | 4 | 1280.58 | < 0.0001 | 10.15 |
| V ×L | 4 | 434.17 | < 0.0001 | 0 |
| L × Y | 4 | 2710.96 | < 0.0001 | 23.21 |
| V × Y × L | 4 | 1145.93 | < 0.0001 | 30.37 |
| Error | 20 | 19.75 | < 0.0001 | 1.49 |
| Total | 39 | 1220.67 | | |

[1] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175

[2] CLP-38655 was the total CLP production (CLP-D, F, G, H and I) by gene g38655




(a)




(b)

Figure 4-4 Influence of environment (L × Y) on variety performance of (a) CLP-24175 and (b) CLP-38655 production

sequence for each of CLP-E, B and A in sequence g24175. The gene that includes the motifs for CLP-D, G and F includes one copy of D and F and three copies of the motif that encodes CLP-G. All amino acids of the peptides are given in the sequence they occur in the peptide gene. The correlation among CLP concentrations found in flaxseed was evaluated using the data from the study of genotype by environment (n=20) on peptide levels. This data may be used to determine the relationship of the expression levels of CLPs in flaxseed. The Pearson's product momentum correlation coefficients (r, p) between CLPs are listed in Table 4-8. It is worth noting that the relationship between individual and total CLPs was highly significant ($p < 0.0001$) meaning there was a linear relationship between these compounds. The highest correlations between individual CLPs occurred amongst peptides on the same gene. For example, the correlations of CLPs within g38655 were high [CLP-F and CLP-G ($r=0.996$), CLP-D and CLP-F ($r=0.851$) and CLP-D and CLP-G ($r=0.869$)] and significant (at $p<0.0001$). In comparison, the relationship between the CLPs on g38655 and g24175 was weaker [CLP-F with CLP-A ($r=0.4847$, $p=0.0015$), CLP-G with CLP-A ($r=0.5176$, $p=0.0006$), CLP-D with CLP-A ($r=0.3999$, $p=0.0106$), CLP-D with CLP-C ($r=0.5180$, $p=0.0006$)]. For peptides in g24175, CLP-C and CLP-E ($r=0.9634$, $p<0.0001$) were strongly correlated; however the correlations between CLP-A and the other peptides were weaker [CLP-A with CLP-C ($r=0.6575$, $p<0.0001$), CLP-A with CLP-E ($r=0.7019$, $p<0.0001$)]. The observed lower correlations between peptide CLP-A levels and other CLPs may be due to incomplete recovery of CLP-A, random error or a real difference in the expression of this compound. The correlation between CLP-24175 (CLP-A, B, C and E) and CLP-38655 (CLP-D, F, G, H and I) was significant ($r= 0.7439$, $p<0.0001$).

It is generally accepted that plant primary and secondary metabolites arise as products of a multitude of enzymes involved in metabolism. The concentration of any individual metabolite is controlled by a number of factors related to metabolic processes (Waterman and Mole, 1989; Rolin, 2006). These metabolites make up the metabolome. Enzymes and other protein products are produced as the result of ribosomal translation of mRNA. These compounds would be seen as belonging to the proteome. The concentration of a cyclic peptide is determined by transcription, translation and post-translational modification, typical components of the proteome

Table 4-8 Correlation coefficients between CLPs in flaxseed (Pearson's coefficients). Correlations between peptides in g24175 (CLP-A, C and E) are indicated with dark grey squares. Correlations between peptides in g38655 (CLP-D, F and G) are indicated with light grey squares.

| Variable (n=20)[3] | CLP-A | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | CLP-24175[1] |
|---|---|---|---|---|---|---|---|
| **CLP-C** | r=0.6575<br>p <0.0001 | | | | | | |
| **CLP-D** | r=0.3999<br>p=0.0106 | r=0.5180<br>p=0.0006 | | | | | |
| **CLP-E** | r=0.7019<br>p<0.0001 | r=0.9634<br>p<0.0001 | r=0.5747<br>p=0.0001 | | | | |
| **CLP-F** | r=0.4847<br>p=0.0015 | r=0.8191<br>p<0.0001 | r=0.8509<br>p<0.0001 | r=0.8173<br>p<0.0001 | | | |
| **CLP-G** | r=0.5176<br>p=0.0006 | r=0.8070<br>p<0.0001 | r=0.8694<br>p<0.0001 | r=0.8074<br>p<0.0001 | r=0.9959<br>p<0.0001 | | |
| **CLP-38655[2]** | | | | | | | r=0.7439<br>p<0.0001 |
| **Total** | r=0.7586<br>p <0.0001 | r=0.9297<br>p<0.0001 | r=0.7543<br>p<0.0001 | r=0.9484<br>p<0.0001 | r=0.9160<br>p<0.0001 | r=0.9217<br>p<0.0001 | |

[1] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175
[2] CLP-38655 was the total CLP production (CLP-D, F, G, H, and I) by gene g38655
[3] Seeds from five varieties at two locations in two years (5×2×2) were analyzed

(linum.ca, 2010). The structure of cyclolinopeptide genes could lead to as many as three peptides being produced by the post-translational modification of the pre-peptide protein g24175 and five peptides being produced from the modification of g38655 (Figure 2-6, 2-7). Although there is no reason to assume that each translated g24175 or g38655 would produce three or five cyclolinopeptides, respectively, lower numbers of cyclolinopeptides arising from sequence translation is possible. Nevertheless, the strong correlation between the cyclolinopeptides arising from the same gene is interesting. With additional research this unique discovery may shed light on the post-translational modification of peptides.

### 4.6 The levels of CLPs in flaxseed fractions

The distribution of compounds in seeds can aid in the development of processes for enrichment. Flaxseed lignan is found primarily in the seed coat and indeed isolation of flaxseed lignan from seed coat or whole flaxseed provides significant advantages over isolation from ground whole seed or seed meal (Bhatty and Cherdkiatgumchai, 1990; Bhatty, 1993). The commercial availability of a flaxseed seed coat product from Natunola (Natunola Health Inc., Winchester, ON, Canada) makes it a popular source for research of seed coat composition (Oomah and Sitter, 2009; Petit *et al.*, 2009; Kazama *et al.*, 2010).

The distribution of cyclic peptides in plant tissues is broad with many known seed borne peptides (Tan and Zhou, 2006). For instance, CLP-A was the first cyclolinopeptide isolated from seeds of *Linum usitatissimum* (Kaufmann and Tobschirbel, 1959). The bicyclic peptides Moroidin, celogentins D–H and celogentin-J were extracted with MeOH from the seeds of *Celosia argentea* (Morita *et al.*, 2000; Suzuki *et al.*, 2003). There are no reports of the distribution of CLPs in flaxseed in the scientific literature. Therefore, research was conducted to determine the distribution of CLPs in flaxseed. Natunola sells a commercial flax product that consists primarily of flaxseed seed coats. This product was chosen for studies of the concentration of CLPs in flax seed coat and seed coat free materials. Natunola flaxseed had a CLP concentration (total, 268.3 μg/g) (Table 4-9) comparable to those observed in CDC varieties (Table 4-6). The seed coat removing process used by Natunola is a trade secret. Also, we found

Table 4-9 CLP concentrations in flaxseed fractions

| (n=3)[2] | | Weight | Concentration of CLPs (µg/g)[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (%) | CLP-A | CLP-B | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | Total | CLP-24175[4] | CLP-38655[5] |
| **Gum** | Mean | 7.0 | -[3] | - | - | - | - | - | - | - | - | - |
| | SD[6] | 1.0 | - | - | - | - | - | - | - | - | - | - |
| **Seed coat** | Mean | 41.0 | 19.3$^{c}$ | - | 31.0$^{c}$ | 15.6$^{a}$ | 30.7$^{c}$ | 6.7$^{c}$ | 19.8$^{c}$ | 123.1$^{c}$ | 81.0$^{b}$ | 42.1$^{c}$ |
| | SD | 0.1 | 1.4 | - | 1.3 | 0.9 | 0.8 | 0.1 | 0.9 | 5.2 | 4.4 | 1.7 |
| **Cotyledon** | Mean | 2.0 | 25.2$^{b}$ | - | 98.8$^{a}$ | 13.1$^{ab}$ | 80.2$^{a}$ | 19.6$^{a}$ | 55.0$^{a}$ | 291.9$^{a}$ | 204.2$^{a}$ | 87.7$^{a}$ |
| | SD | 1.0 | 0.4 | - | 3.2 | 1.6 | 1.8 | 0.3 | 1.6 | 8.2 | 5.4 | 3.1 |
| **Whole seed** | Mean | 100 | 63.1$^{a}$ | - | 78.7$^{b}$ | 9.9$^{b}$ | 70.2$^{b}$ | 13.1$^{b}$ | 33.3$^{b}$ | 268.3$^{b}$ | 212.0$^{a}$ | 56.3$^{b}$ |
| | SD | 0 | 1.4 | - | 5.6 | 0.2 | 1.2 | 0.7 | 1.1 | 9.9 | 0.1 | 0.2 |

[1] Means followed by the same superscript were not significantly different by Tukey's multiple comparison test at 5% level
[2] Fractionation was repeated three times.
[3] "–" not detected by HPLC
[4] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175
[5] CLP-38655 was the total CLP production (CLP-D, F, G, H and I) by gene g38655
[6] SD=standard deviation

that the seed coat fraction contained a significant content of oil, which was likely transferred to the seed coats during the seed coat removing process. As such, the fractions (cotyledons and seed coat) provided by Natunola were not used in further studies.

Manual seed coat removal was conducted to provide more reliable results. Flaxseed gum (0.7 g) was obtained from flaxseed (10.0 g). A flaxseed seed coat fraction (4.1 g) and seed coat free seed (5.2 g) were separated from the water-degummed flaxseed (Figure 4-5). The concentration of CLPs in each fraction is presented in Table 4-9. No CLPs were detected by HPLC analysis of gum extracts (Figure 4-6). CLPs are relatively more hydrophobic than the seed gum, which is a hydrophilic mixture of polysaccharides which yield rhamnose, fucose, arabinose, xylose, galactose, galacturonic acid and glucose after acid-catalyzed hydrolysis (Erskine and Jones, 1957; Fedeniuk and Biliaderis, 1994). Similarly, previous researchers have not determined the presence of cyclic peptides in flaxseed gum.

CLP-B was not detected in any samples, likely due to the oxidation of methionine to methionine sulfoxide (Table 4-9). This oxidation is similar to the oxidation of CLP E' to E noted in bottled flaxseed oil by Brühl *et al.* (2007). The concentrations of CLP-A (25.2 μg/g), C (98.8 μg/g), D (13.1 μg/g), E (80.2μg/g), F (19.6 μg/g), G (55.0 μg/g) and overall CLPs (291.9 μg/g) were higher in the cotyledon than in the seed coat fraction, where the concentrations were CLP-A (19.3 μg/g), C (31.0 μg/g), D (15.6 μg/g), E (30.7 μg/g), F (6.7 μg/g), G (19.8 μg/g) and total CLPs (123.1 μg/g). Due to the possible oxidation of CLPs expressed by the same gene (Figure 4-3), the levels of CLP-24175 (the group of all products produced by gene g24175: CLP-A, B, C and E) and CLP-38655 (the group of all products produced by gene g38655: CLP-D, F, G, H and I) were utilized to reduce statistical variation and to obtain a measure of the products of post-translational processing of these genes. The levels of CLP-24175 and CLP-38655 in the cotyledon were more than two times those in the seed coat (204.2 μg/g vs.81.0 μg/g and 87.7 μg/g vs.42.2 μg/g, respectively). The prevalence of CLPs in the cotyledon fraction might be explained if the peptides are present in oil storage bodies as cotyledons are the major location for oil storage. The CLPs observed in the seed coat fraction were possibly contributed by the endosperm attached to the seed coats, which

**a. Flax gum after water degumming**


**b. Cotyledon**


**c. Seed coat**

**d. Whole flaxseed**

Figure 4-5 Flaxseed fractions after water degumming and manual dissection

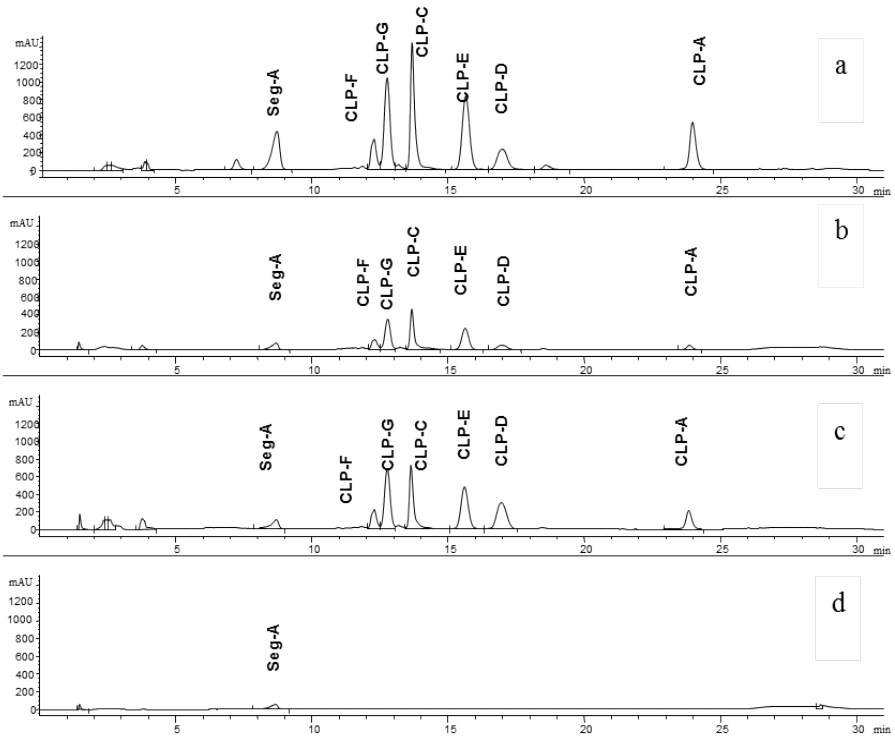



Figure 4-6 HPLC chromatograms of CLPs from flaxseed fractions at 214 $\pm$ 10 nm
Fractions: a. Whole seed, b. cotyledon, c. seed coat, d. gum

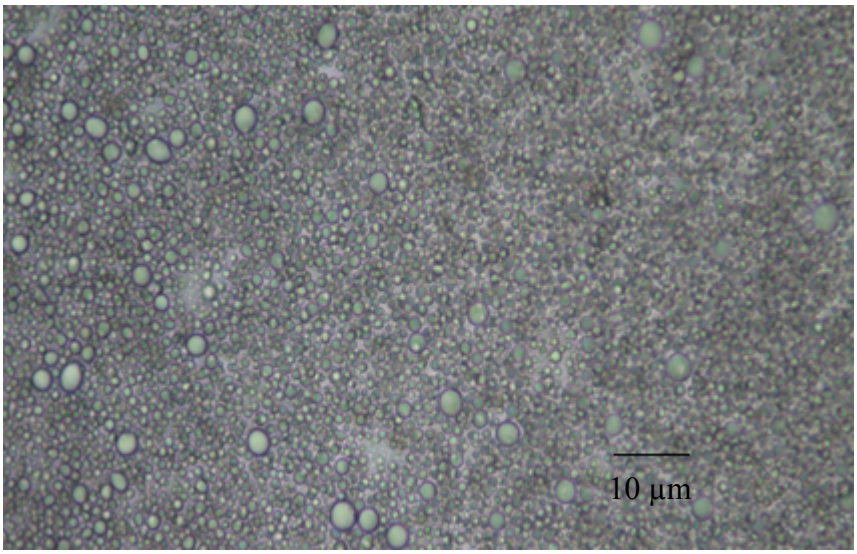
also contributed oil to the seed coats. Overall CLP recovery from seed coats and cotyledons was 75.1% of the recovery from whole seed. The loss could be explained by incomplete extraction, systematic errors and loss of material during processing, which could happen during processing without the observation of loss of total weight because the water degumming process could change the moisture content of the seed fractions. This is the first report of the distribution of CLPs in flaxseed fractions.

The distribution of CLPs in different parts of the flaxseed led us to study the location of CLP storage in flaxseed fractions. The observation that the majority of CLPs were found in flaxseed oil after conventional processing indicated the possibility that the peptides were stored in oil bodies or oleosomes, the main oil-bearing structure in flaxseed.

The diameter of oil bodies isolated from flaxseed was approximately 1.3 μm and the major components were TAG (97.7%), protein (1.3%), phospholipid (0.9%) and free fatty acids (0.1%) (Tzen *et al.*, 1993). The diameter of oil bodies obtained in this study varied from 0.5-2.0 μm, with an average of approximately 1.0 μm, which agreed with the literature (Figure 4-7, c). A brownish crude oil body pad (oil body-1, 9.3 g) was isolated from flaxseed (20.0 g) by homogenization and centrifugation. After repeated homogenization and centrifugation (six times), the oil body pad (oil body-2, 7.5 g) was substantially reduced in contaminants. The resulting fractions were freeze dried, yielding three dried fractions of oil body (4.9 g), serum (2.7 g) and residue (11.0 g) (Figure 4-7). Subsequently, the levels of CLPs were quantified by HPLC (Figure 4-8 and Table 4-10). Extraction conditions led to the oxidation of methionine containing peptides, as CLP-B, CLP-H and CLP-I were not found in any of the fractions (Figure 4-3). After the initial homogenization/centrifugation treatment, a higher concentration of CLPs are detected in crude oil bodies (643.9 μg/g) than in serum (99.5 μg/g) or residue (15.7 μg/g). After five more homogenization/centrifugation treatments, the concentration of CLPs were reduced in the crude oil body fraction (153.1 μg/g). The overall recovery of CLPs (from oil bodies, serum and residue) was 93.1% after the first time homogenization/ centrifugation and was reduced to 28.0% after the sixth homogenization/centrifugation due to the loss of CLPs associated with the oil bodies. All of the CLP-C, E, F and G and 68% of CLP-A


a. Flaxseed serum

b. Flaxseed oil body pad




c. Micrograph of oil bodies taken at a magnification of 400X

Figure 4-7 Products after oil body isolation from flaxseed

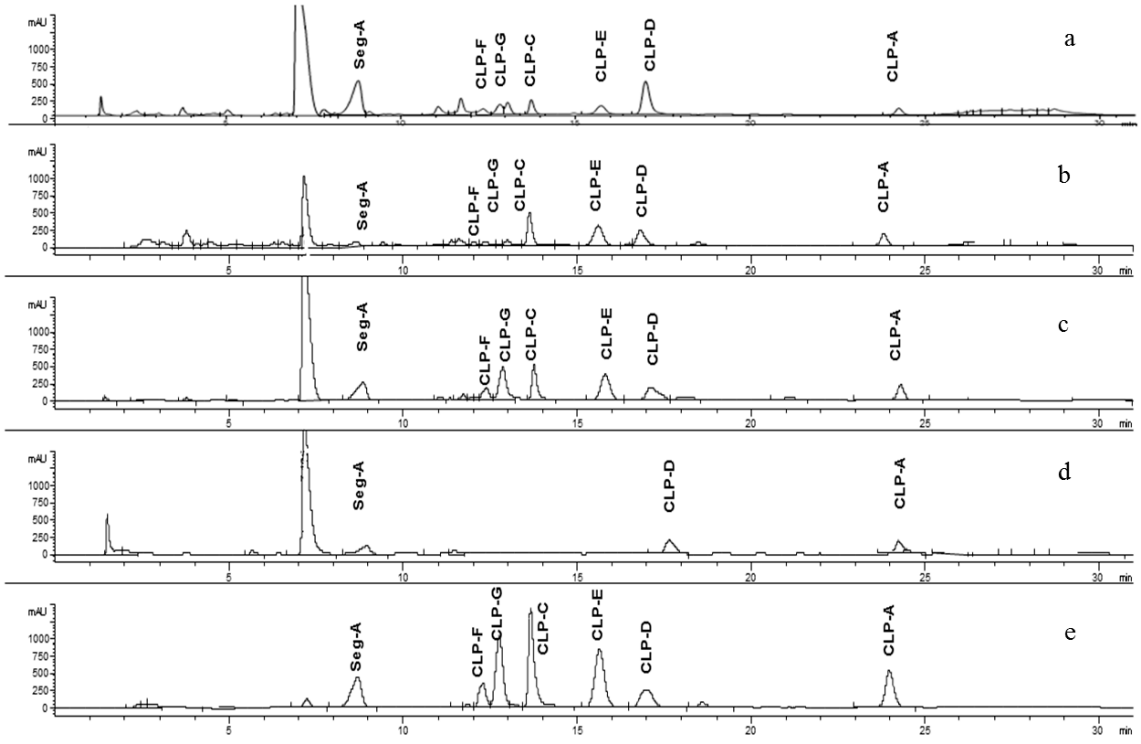



Figure 4-8 HPLC chromatograms of CLPs flaxseed fractions at 214 nm.
Fractions: a.residue, b. serum, c. oil body-1 (crude oil bodies isolated after one time homogenization/centrifugation), d. oil body-2 (pure oil bodies isolated after six times homogenization/centrifugation), e. whole seed

Table 4-10 CLPs concentrations in oil bodies, serum, and residue from flaxseed

| (n=3)[2] | | Weight | Concentration of CLPs (µg/g)[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (%) | CLP-A | CLP-B | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | Total | CLP-24175[6] | CLP-38655[7] |
| **Serum** | Mean | 13.5 | 26.1$^{c}$ | -[5] | 21.2$^{c}$ | 11.2$^{c}$ | 28.1$^{c}$ | 4.5$^{c}$ | 8.5$^{c}$ | 99.5$^{d}$ | 75.4$^{c}$ | 24.2$^{d}$ |
| | SD[8] | 1.0 | 4.3 | - | 1.5 | 3.8 | 7.1 | 1.1 | 1.8 | 12.1 | 4.5 | 3.6 |
| **Residue** | Mean | 55 | 3.4$^{d}$ | - | 2.4$^{d}$ | 4.1$^{c}$ | 3.4$^{d}$ | 1.0$^{d}$ | 1.4$^{d}$ | 15.7$^{e}$ | 9.2$^{d}$ | 6.5$^{d}$ |
| | SD | 3.0 | 0.6 | - | 0.6 | 0.3 | 0.6 | 0.1 | 0.6 | 2.6 | 0.0 | 1.9 |
| **Oil body-1**[3] | Mean | 35.0 | 163.7$^{a}$ | - | 119.1$^{a}$ | 74.5$^{b}$ | 163.7$^{a}$ | 33.5$^{a}$ | 89.3$^{a}$ | 643.9$^{a}$ | 446.5$^{a}$ | 197.3$^{a}$ |
| | SD | 8.0 | 11.9 | - | 12.1 | 17.2 | 11.9 | 0.4 | 1.2 | 34.6 | 36.0 | 17.6 |
| **Oil body-2**[4] | Mean | 29.5 | 63.0$^{b}$ | - | - | 90.1$^{a}$ | - | - | - | 153.1$^{c}$ | 63.0$^{c}$ | 90.1$^{b}$ |
| | SD | 1.0 | 5.0 | - | - | 7.0 | - | - | - | 14.2 | 8.0 | 7.0 |
| **Whole Seed** | Mean | 100 | 63.1$^{b}$ | - | 78.7$^{b}$ | 9.9$^{c}$ | 70.2$^{b}$ | 13.1$^{b}$ | 33.3$^{b}$ | 268.4$^{b}$ | 212.0$^{b}$ | 56.3$^{c}$ |
| | SD | 0 | 1.4 | - | 5.6 | 0.2 | 1.2 | 0.7 | 1.1 | 9.9 | 0.1 | 0.2 |

[1] Means followed by the same superscript were not significantly different by Tukey's multiple comparison test at 5% level
[2] Oil body isolation was repeated three times.
[3] Oil body-1 (crude oil bodies isolated after one time homogenization/centrifugation)
[4] Oil body-2 (pure oil bodies isolated after six times homogenization/centrifugation)
[5] "–" meant not detected by HPLC
[6] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175
[7] CLP-38655 was the total CLP production (CLP-D, F, G, H, and I) by gene g38655
[8] SD=standard deviation

from oil bodies were removed by homogenization/centrifugation, whereas the CLP-D in oil bodies remained basically unchanged. The lower polarity of CLP-A and CLP-D might explain their presence in oil bodies when other CLPs with higher polarity were redistributed into the aqueous layer during centrifugation. It is possible that CLPs were stored in oil bodies along with TAGs before processing and they were removed from oil bodies by homogenization/centrifugation, but further confirmation of this is not possible at this time.

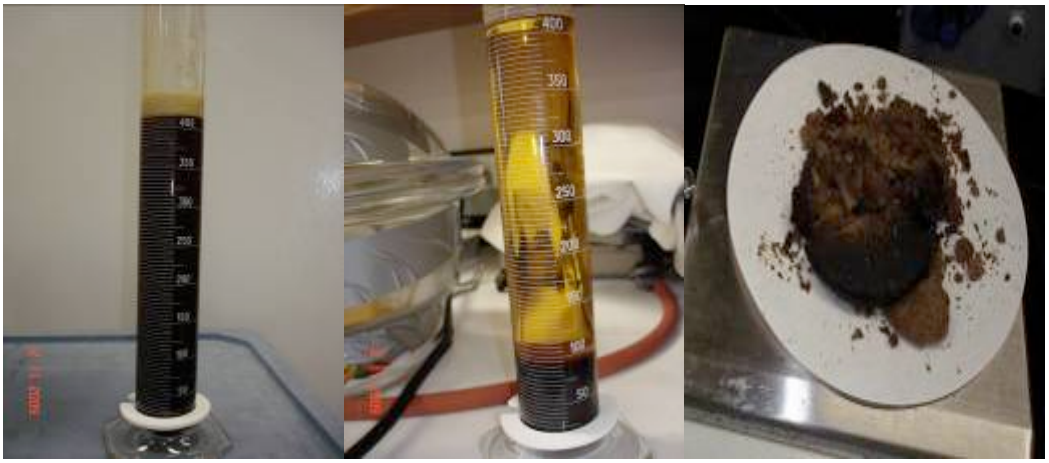
### 4.7 Effects of processing on the distribution of CLPs in flaxseed products

#### 4.7.1 CLP distribution after expeller-pressing

The extraction of natural products with vegetable oil occurs during oilseed pressing (Jung *et al.*, 1989). Phospholipids, phytosterols, tocopherols are all found in crude oil after pressing and and it is often necessary to remove these compounds with further refining processes (Verhe *et al.*, 2008). Crude oil (380.9 g) and flaxseed meal (575.4 g) were obtained after cold pressing of 1000.0 g of flaxseed. The weight loss observed (43.7 g) was likely due to residues remaining in the expeller after pressing. The oil content of flaxseed (38.1%) obtained by cold pressing was in agreement with a prior study (Dedio and Dorrel, 1977), whereas the oil content of flaxseed grown in different locations in Canada was reported to range between 25% and 41%. The crude oil was allowed to settle overnight in a 500-mL graduated cylinder to separate suspended "foots" (sediments commonly found in flaxseed oil after settling). Foots (35.4 g) were separated from oil by filtration under vacuum (Figure 4-9). The filtered oil was combined with clarified oil from the upper layer after settling, to obtain 343.9 g of oil that was used for further studies of CLP levels. The concentration of CLPs in crude oil (after settling), foots and meal are shown in Figure 4-10 and Table 4-11. CLP-B was detected in crude oil (98.0 μg/g) but not in other fractions. Met-containing peptides were oxidized during acetone extraction (Figure 4-3, Appendix F). CLP-A, CLP-C, CLP-D and CLP-E, which had lower polarity than other peptides based on their later emergence from reverse phase chromatography, were found in greater concentration in crude oil (466.7 μg/g, 368.6 μg/g, 227.5 μg/g and 462.7 μg/g, respectively), whereas the concentrations of CLP-F (92.2 μg/g) and CLP-G (266.2 μg/g), which had relatively higher polarity were higher in

(a)

(b)



(c) (d) (e)

Figure 4-9 Products after cold pressing of flaxseed

Fractions: a. whole flaxseed, b. flaxseed meal, c. crude oil without settling, d. crude oil after settling, e. foots separated from crude oil

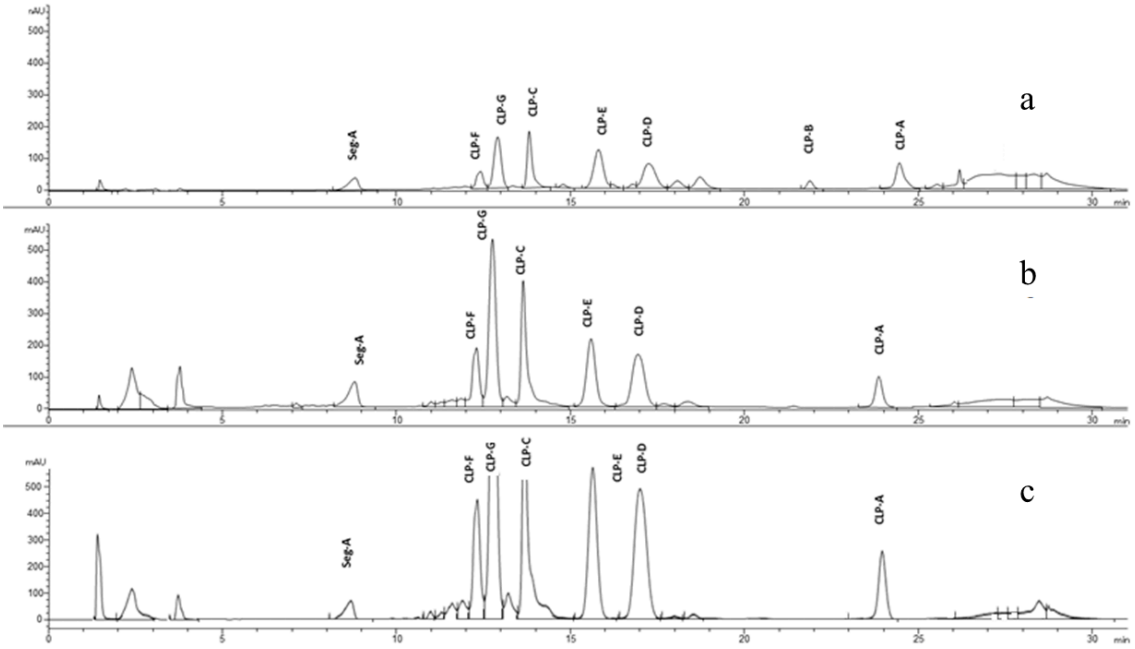



Figure 4-10 HPLC chromatograms (214 nm) of CLPs flaxseed products after processing. Fractions: a. crude oil, b. meal, c. foots

Table 4-11 CLP distribution after cold pressing

| | | Weight | Concentration of CLPs (μg/g)[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (n=3)[2] | | (%) | CLP-A | CLP-B | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | Total | CLP-24175[4] | CLP-38655[5] |
| **Crude oil** | Mean | 34.4 | 466.7[a] | 98.0 | 368.6[a] | 227.5[a] | 462.7[a] | 86.3[a] | 262.7[a] | 1972.6[a] | 1396.1[a] | 576.5[a] |
| | SD[6] | 0.6 | 6.8 | 6.8 | 27.2 | 6.8 | 35.9 | 6.8 | 6.8 | 86.7 | 66.9 | 20.4 |
| **Meal** | Mean | 57.5 | 24.3[c] | -[3] | 21.9[c] | 17.0[b] | 21.2[c] | 6.7[b] | 7.9[b] | 98.9[c] | 67.4[c] | 31.6[b] |
| | SD | 1.1 | 5.9 | - | 6.6 | 5.9 | 6.4 | 2.8 | 4.6 | 7.4 | 8.3 | 2.8 |
| **Foots** | Mean | 3.5 | 169.8[b] | - | 289.2[b] | 186.5[c] | 251.5[b] | 92.2[a] | 266.2[a] | 1255.3[b] | 710.5[b] | 544.9[a] |
| | SD | 0.1 | 10.9 | - | 16.6 | 14.5 | 16.6 | 3.6 | 13.1 | 75.0 | 44.0 | 31.0 |

[1] Means followed by the same superscript were not significantly different by Tukey's multiple comparison test at 5% level
[2] Cold pressing was repeated three times
[3] "–" meant not detected by HPLC
[4] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175
[5] CLP-38655 was the total CLP production (CLP-D, F, G, H, and I) by gene g38655
[6] SD=standard deviation

the foots fraction. A higher concentration of total CLPs produced by gene g24175 (CLP-24175) was found in crude oil (1972.6 μg/g) than in meal (67.4 μg/g) and foots (710.5 μg/g). The concentrations of total CLPs produced by gene g38655 (CLP-38655) were similar in crude oil (576.5 μg/g) and foots (544.9 μg/g), but significantly higher than that in meal (31.6 μg/g). The concentration of CLPs in meal was relatively low (total 98.9 μg/g), which was expected after cold pressing as the polarity of CLPs allowed them to dissolve in the oil during processing. It is worth noting that the overall CLPs found in crude oil, meal and foots from flaxseed (0.78 mg/g) were higher than the amount of CLPs recovered from flaxseed by Goldfisch extraction (0.37 mg/g). This observation might be a result of improved extraction of CLPs, including reduced ones (e.g., CLP-E', CLP-F' and CLP-G') after oxidation by the applied shear force and heat generated from screw-pressing. Oxidation has been noticed in the different stages of vegetable oil processing (such as soybean oil and flaxseed oil) and their co-products (such as meal and cake, etc) (Jung *et al.*, 1989; Wanasundara and Shahidi, 1998; Wiesenborn *et al.*, 2005).

A painty and bitter flavour of flaxseed oil appeared after 15 weeks of storage at 4 °C. The breakdown of ALA was suspected as the cause of the unpleasant flavor (Wiesenborn *et al.*, 2005). In 2007, Brühl *et al.* isolated the bitter compound from stored flaxseed oil and proved that oxidation of a CLP to CLP-E produced the observed bitterness. Five brands of flaxseed oil from local health stores were chosen for CLP analysis. The levels of CLPs varied among the brands (Table 4-12). Omega had the highest levels of CLP-B (112.2 μg/mL) among all the flaxseed oils tested whereas Sangster and Gold Top had none. Flora had the highest amounts of CLP-C (263.1 μg/mL), CLP-D (165.3 μg/mL) and CLP-F (29.1 μg/mL), but they all had lower peptide levels compared to that of flaxseed oil prepared in the lab, where CLP-A (397.7 μg/mL), CLP-B (83.3 μg/mL), CLP-C (310.6 μg/mL), CLP-D (197.0 μg/mL), CLP-E (393.9 μg/mL), CLP-F (70.8 μg/mL), CLP-G (219.7 μg/mL) and total CLPs (1,673.0 μg/mL) were found. Flora had the highest level of the bitter peptide CLP-E (270.1 μg/mL) among five commercial flaxseed oils, which implied more bitter intensity in this oil. Lab-produced crude oil contained more CLP-E (393.9 μg/mL) than any of the commercial flaxseed oils. Significant differences of CLP-24175 were found among the

Table 4-12 Comparison of CLP levels in commercial and lab-produced crude flaxseed oils

| (n=3)[2] | | Concentration of CLPs (µg/mL)[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CLP-A | CLP-B | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | Total | CLP-24175[4] | CLP-38655[5] |
| **GNC** | Mean | 179.2$^{c}$ | 34.6$^{b}$ | 172.1$^{bc}$ | 29.0$^{b}$ | 194.5$^{b}$ | 32.2$^{a}$ | 93.1$^{b}$ | 734.6$^{c}$ | 580.4$^{b}$ | 154.2$^{b}$ |
| | SD[6] | 10.9 | 18.1 | 22.7 | 5.6 | 20.3 | 3.8 | 6.8 | 48.8 | 36.7 | 13.7 |
| **Sangster** | Mean | 251.9$^{b}$ | -[3] | 167.7$^{bc}$ | 44.0$^{b}$ | 161.0$^{b}$ | 20.7$^{a}$ | 15.1$^{c}$ | 660.5$^{c}$ | 580.7$^{b}$ | 79.8$^{c}$ |
| | SD | 31.2 | - | 13.2 | 6.8 | 9.7 | 8.1 | 8.3 | 45.3 | 45.2 | 8.6 |
| **Gold top** | Mean | 155.6$^{c}$ | - | 192$^{bc}$ | 20.2$^{b}$ | 189.8$^{b}$ | 16.5$^{a}$ | 29.5$^{c}$ | 603.5$^{c}$ | 537.3$^{b}$ | 66.2$^{c}$ |
| | SD | 20.4 | - | 15.6 | 3.1 | 9.8 | 2.7 | 1.5 | 46.7 | 44.8 | 6.8 |
| **Omega** | Mean | 276.8$^{b}$ | 112.2$^{a}$ | 103.4$^{c}$ | 19.3$^{b}$ | 159.8$^{b}$ | 13.4$^{a}$ | 48.5$^{bc}$ | 733.5$^{c}$ | 652.2$^{b}$ | 81.3$^{c}$ |
| | SD | 28.9 | 20.9 | 4.9 | 2.7 | 10.1 | 2.1 | 4.7 | 56.8 | 53.2 | 3.6 |
| **Flora** | Mean | 285.7$^{b}$ | 62.6$^{b}$ | 263.1$^{ab}$ | 165.3$^{a}$ | 270.1$^{a}$ | 29.1$^{a}$ | 84.1$^{b}$ | 1160.0$^{b}$ | 881.5$^{a}$ | 278.5$^{a}$ |
| | SD | 22.1 | 4.4 | 58 | 34.7 | 24.7 | 9.6 | 13.9 | 59.5 | 103.0 | 56.5 |
| **Crude oil** | Mean | 397.7$^{a}$ | 83.3$^{a}$ | 310.6$^{a}$ | 197.0$^{a}$ | 393.9$^{a}$ | 70.8$^{a}$ | 219.7$^{a}$ | 1673.1$^{a}$ | 1185.5$^{a}$ | 487.6$^{a}$ |
| | SD | 14.8 | 11.2 | 17.3 | 4.0 | 17.5 | 4.1 | 3.4 | 59.5 | 56.9 | 17.3 |

[1] Means followed by the same superscript were not significantly different by Tukey's multiple comparison test at 5% level
[2] Three samples of each oil were analyzed
[3] " –" meant not detected by HPLC
[4] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175
[5] CLP-38655 was the total CLP production (CLP-D, F, G, H, and I) by gene g38655
[6] SD=standard deviation

commercial oils. Crude oil and Floral flaxseed oil had the highest concentrations (1,185.5 μg/mL and 881.5 μg/mL respectively), whereas GNC, Sangster, Gold Top and Omega samples had lower concentrations of these peptides (580.4 μg/mL, 580.7 μg/mL, 537.3 μg/mL and 652.2 μg/mL, respectively). The concentration of CLP-38655 among different oils also differed significantly. A higher concentration was found in crude oil and Flora (487.5 μg/mL and 278.5 μg/mL), whereas the remaining products were lower in these peptides. The lower levels observed in some commercial products might have been caused by additional processing of the commercial products such as acid degumming and alkali refining. The effects of processing on CLP removal are described below.

### 4.7.2 Effects of acid degumming on removal of CLPs from flaxseed oil

Crude flaxseed oil contains minor components such as phospholipids, FFAs and metal containing compounds (Green and Drimbinenke, 1994). Concentrations of minor constituents of vegetable oils, such as phospholipids, phytosterols, tocopherols and phytosterol esters, are reduced during refining processes (Ferrari *et al.*, 1996). Degumming of crude soybean oil removed 76.4% of phosphorus, 73.1% of iron and 51.4% of FFAs (Jung *et al.*, 1989). The phosphorus in degummed flaxseed oil was reduced from 325 mg/kg to 0.5 mg/kg without obvious loss of sterols and tocopherols (Green and Drimbinenke, 1994; Hosseinian *et al.*, 2004). Other minor compounds from vegetable oil could also be partly removed by acid degumming. For instance, 1.1% of oryzanol was removed from degummed rice bran oil (Krishna *et al.*, 2001).

Acid degumming with $H_3PO_4$ effectively removed CLPs from crude flaxseed oil (Table 4-13). Degumming with 1%, 2% or 10% (v/v) of 75% $H_3PO_4$ to flaxseed oil removed all of the peptides. Lower concentrations of acid are commonly used in industrial settings for degumming. Sullivan (1955) reported the use of 0.13-0.53% of 75% $H_3PO_4$ in industry. Two-stage degumming was tested using two treatments with just 0.1% of 75% $H_3PO_4$ (v/v) to mimic an acid-conserving degumming protocol (Reaney, Pers. Commun.). The two-stage acid degumming treatment removed all of CLP-B, C, D, F and G, leaving trace amounts of CLP-A (8.6%) and E (5.8%) in crude oil. Acid degumming with a lower concentration of acid [1% (v/v) of 50% $H_3PO_4$]

Table 4-13 $H_3PO_4$ acid degumming effects on CLPs from flaxseed oil

| Method[1] (n=3)[2] | | Concentration of CLPs (μg/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CLP-A | CLP-B | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | Total | CLP-24175[4] | CLP-38655[5] |
| **A** | Mean | -[3] | - | - | - | - | - | - | - | - | - |
| | SD[6] | - | - | - | - | - | - | - | - | - | - |
| **B** | Mean | - | - | - | - | - | - | - | - | - | - |
| | SD | - | - | - | - | - | - | - | - | - | - |
| **C** | Mean | - | - | - | - | - | - | - | - | - | - |
| | SD | - | - | - | - | - | - | - | - | - | - |
| **D** | Mean | 34.1 | - | - | - | 22.7 | - | - | 56.8 | 56.8 | - |
| | SD | 19.7 | - | - | - | 0.1 | - | - | 19.7 | 17.3 | - |
| **E** | Mean | 178.0 | - | - | - | - | - | - | 178.0 | 178.0 | - |
| | SD | 6.6 | - | - | - | - | - | - | 6.6 | 6.6 | - |
| **F** | Mean | 397.7 | 83.3 | 310.6 | 197.0 | 393.9 | 70.8 | 219.7 | 1673.1 | 1185.5 | 487.6 |
| | SD | 14.8 | 11.2 | 17.3 | 4.0 | 17.5 | 4.1 | 3.4 | 59.5 | 56.9 | 17.3 |

[1] Method A: 10% (v/v) of 75% $H_3PO_4$; Method B: 2% (v/v) of 75% $H_3PO_4$; Method C: 1% (v/v) of 75% $H_3PO_4$; Method D: Acid degumming twice with 0.1% (v/v) of 75% $H_3PO_4$; Method E: 1% (v/v) of 50% $H_3PO_4$; Method F: Crude oil without treatment

[2] Each method was repeated three times

[3] "–" meant not detected by HPLC

[4] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175

[5] CLP-38655 was the total CLP production (CLP-D, F, G, H, and I) by gene g38655

[6] SD=standard deviation

removed all peptides from the oil except CLP-A (44.8%).

Phospholipids found in crude soybean oil are not dissolved but are mostly in micelles that encapsulate sugars and metals (Sengupta, 1986). Non-hydratable phospholipids (NHP), phosphatidic acid (PA) and part of the phosphatidyl ethanolamine (PE) are present as $Ca^{2+}$ and/or $Mg^{2+}$ salts, which could be removed by addition of strong acid into crude oil at elevated temperature (Sullivan, 1955; Young *et al.*, 1994). In this experiment, acid degumming using $H_3PO_4$ proved to be an effective way of removing CLPs from crude flaxseed oil. The absence of CLPs after degumming may indicate that CLPs are entrained in phospholipid micelles that are removed during acid degumming treatment. It is also possible that CLP solubility in oil may require binding to metals that are also removed by acid degumming. These observations may also explain why CLPs were primarily found in the serum of the oleosome extracts, as both phospholipid micelles and metal complexes are more likely to be present in the aqueous phase. Degumming treatments with $H_3PO_4$ would be considered a safe and practical approach for CLP removal from flaxseed oil as it is commonly used to remove the phospholipids from crude oil in industrial oil refining. Larger scale testing of acid degumming on the peptides in flaxseed oil should be conducted in the future.

### 4.7.3 CLPs from gum after acid degumming

It was not certain if the gum from acid degumming could be used as a source of peptides or the amide bonds of peptides are susceptible to hydrolysis by acid. It is possible that acid degumming of flaxseed oil leads to the hydrolysis of CLPs, as the concentration of acid and temperature of processing were relatively high (up to 75% $H_3PO_4$ at 80°C). Therefore, gums from acid degumming treatments were tested to determine CLP levels (Figure 4-11 and Table 4-14). CLP-B was not found in the gum. The yield of CLPs from extracted gums varied by peptide type. Peptides CLP-A, CLP-C and CLP-E (54.5%) were recovered with reasonable efficiency (55%, 74% and 55%, respectively). The trp-containing peptides CLP-D, CLP-F and CLP-G were recovered in low yields of 0%, 17% and 10%, respectively. Overall, 71.6% of CLP-24175 and 10.5% of CLP-38655 were recovered. The partial loss of these peptides and the absence of CLP-D might be caused by hydrolysis under the strongly acidic conditions.

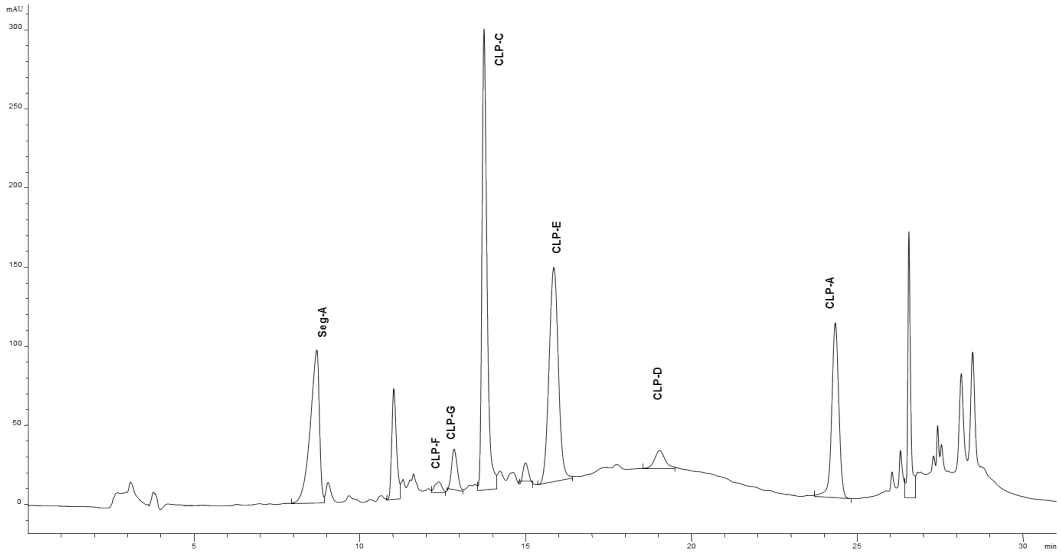



Figure 4-11 HPLC chromatogram of CLPs in gum from 1 mL flaxseed oil

Table 4-14 CLP recovery from gum after acid degumming of flaxseed oil

| (n=3)[1] | | Concentration of CLPs (µg/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CLP-A | CLP-B | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | Total | CLP-24175[6] | CLP-38655[7] |
| **A[2]** | Mean | 219.4 | -[5] | 230.2 | - | 214.7 | 12.3 | 22.1 | 698.8 | 666.7 | 30.00 |
| | SD[4] | 3.6 | - | 7.1 | - | 20.8 | 1.4 | 2.4 | 33.9 | 28.9 | 0.1 |
| **B[3]** | Mean | 397.7 | 83.3 | 310.6 | 197 | 393.9 | 70.8 | 219.7 | 1673.1 | 931.0 | 285.2 |
| | SD | 14.8 | 11.2 | 17.3 | 4.0 | 17.5 | 4.1 | 3.4 | 59.5 | 54.2 | 7.8 |

[1] Acid degumming was repeated three times
[2] A: CLPs recovery from the gum of 1mL oil
[3] B: Crude oil without treatment
[4] SD=standard deviation
[5] "–" meant not detected by HPLC
[6] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175
[7] CLP-38655 was the total CLP production (CLP-D, F, G, H, and I) by gene g38655

The recovery of CLPs from gums was difficult, requiring significant amounts of solvent and labour. Recovery procedures could be improved in the future by using liquid-liquid partitioning of CLPs in different solvents. However, this study did prove the gum from acid degumming was a good source of CLP-24175 and by future engineering, it could be used for industrial scale isolation of CLPs.

### 4.7.4 Effect of alkali refining on CLPs in flaxseed oil

The solubility of non-polar peptides in oil and organic solvents was reported by Iqbal and Balaram (1982). A non-polar decapeptide Boc-Aib-Pro-Val-Aib-Val-Ala-Aib-Ala-Aib-Aib-OMe (Aib=α-aminoisobutyric acid), aggregates and adopts a $3_{10}$ helical conformation in organic solvents [$CDCl_3$ and $(CD_3)_2SO$] stabilized by eight intramolecular hydrogen bonds. They also found that peptides containing the hydrophobic amino acid residue, leucine, are very soluble in vegetable oils (commercial olive oil or safflower oil), as well as in mineral oil.

Free fatty acids (FFAs) are amphiphilic and may have the potential to stabilize CLPs in flaxseed oil or even increase their oil solubility. These compounds may be removed from oil by alkali refining (Handrix, 1990; Markley and Feuge, 1954). Alkali refining reduced FFAs from 0.74% to 0.02% in crude soybean oil (Jung *et al.*, 1989).

The FFAs of flaxseed oil were neutralized by a number of alkali treatments to evaluate the impact of alkali refining on CLPs in oil. HPLC data suggested that the alkalinity and chemistry of the alkali used in alkali refining influenced CLP removal from flaxseed oil (Table 4-15). All alkaline solutions removed substantial amounts of CLPs. However, none of them removed all of the peptides. CLP-B was not found in any sample because of its oxidation to CLP-C during heating. The stronger alkalis, sodium hydroxide (NaOH), potassium hydroxide (KOH), potassium carbonate ($K_2CO_3$) and sodium carbonate ($Na_2CO_3$), appeared more effective at removing CLP-D, F, G whereas sodium bicarbonate ($NaHCO_3$), tripotassium phosphate ($K_3PO_4$) and trisodium phosphate ($Na_3PO_4$) removed only CLP-D and F. Alkali refining removed most of CLP-38655 (0-4.1% recovery) and some CLP-24175 (21.1%-51.9% recovery). Therefore, Trp-containing peptides (CLP-D, F and G) were effectively removed during alkali refining, whereas Trp-free CLPs (CLP-A, C and E) were not. The higher polarity of

Table 4-15 Alkali refining effects on CLPs from flaxseed oil

| **Method**[1] | | **Concentration of CLPs (μg/mL)**[2] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| n=3 | | CLP-A | CLP-B | CLP-C | CLP-D | CLP-E | CLP-F | CLP-G | Total | CLP-24175[4] | CLP-38655[5] | Recovery (%) |
| A | Mean | 124.9 | -[3] | 45.9$^{e}$ | - | 79.0$^{c}$ | - | - | 249.8$^{c}$ | 249.8$^{c}$ | - | 14.9 |
| | SD | 2.4 | - | 0.5 | - | 1.0 | - | - | 3.0 | 3.0 | - | |
| B | Mean | 216.8$^{b}$ | - | 30.9$^{e}$ | - | 43.6$^{d}$ | - | - | 291.3$^{c}$ | 291.3$^{c}$ | - | 17.4 |
| | SD | 4.8 | - | 0.5 | - | 0.8 | - | - | 5.0 | 5.0 | - | |
| C | Mean | 222.0$^{b}$ | - | 66.0$^{de}$ | - | 55.3$^{cd}$ | - | - | 343.3$^{bc}$ | 343.3$^{bc}$ | - | 20.5 |
| | SD | 3.0 | - | 0.4 | - | 0.9 | - | - | 3.1 | 3.1 | - | |
| D | Mean | 208.3$^{b}$ | - | 72.8$^{de}$ | - | 82$^{cd}$ | - | - | 363.1$^{bc}$ | 363.1$^{bc}$ | - | 21.7 |
| | SD | 2.7 | - | 1.7 | - | 3.0 | - | - | 4.8 | 4.8 | - | |
| E | Mean | 220.1$^{b}$ | - | 103.5$^{cd}$ | - | 68.6$^{cd}$ | - | 11.2$^{b}$ | 403.4$^{bc}$ | 392.2$^{bc}$ | 11.2$^{b}$ | 24.1 |
| | SD | 7.2 | - | 2.8 | - | 1.8 | - | 0.1 | 11.8 | 11.8 | 0.1 | |
| F | Mean | 255.0$^{b}$ | - | 141.0$^{c}$ | - | 93.8$^{c}$ | - | 17.8$^{b}$ | 507.6$^{b}$ | 507.6$^{b}$ | 17.8$^{b}$ | 30.3 |
| | SD | 6.0 | - | 2.8 | - | 2.7 | - | 0.2 | 13.3 | 11.3 | 0.2 | |
| G | Mean | 220.0$^{b}$ | - | 233.3$^{b}$ | - | 213.3$^{b}$ | - | 20.0$^{b}$ | 686.6$^{b}$ | 666.6$^{b}$ | 20.0$^{b}$ | 41.0 |
| | SD | 0.1 | - | 5.8 | - | 23.1 | - | 0.1 | 28.9 | 7.4 | 0.1 | |
| H | Mean | 397.7$^{a}$ | 83.3 | 310.6$^{a}$ | 197.0 | 393.9$^{a}$ | 70.8 | 219.7$^{a}$ | 1673.1$^{a}$ | 1185.5$^{a}$ | 487.6$^{a}$ | |
| | SD | 14.8 | 11.2 | 17.3 | 4.0 | 17.5 | 4.1 | 3.4 | 59.5 | 56.9 | 17.3 | |

[1] Method A: 1% (v/v) 4 M NaOH; Method B: 1% (v/v) 4 M KOH; Method C: 1% (v/v) 2 M $K_2CO_3$; Method D: 1% (v/v) 2 M $Na_2CO_3$; Method E: 1% (v/v) saturated $NaHCO_3$; Method F: 1% (v/v) 1.3 M $K_3PO_4$; Method G: 1% (v/v) 1.3 M $Na_3PO_4$; Method H: Crude oil without treatment. Each method was repeated three times.

[2] Means followed by the same superscript were not significantly different by Tukey's multiple comparison test at 5% level

[3] "–" means not detected by HPLC

[4] CLP-24175 was the total CLP production (CLP-A, B, C and E) by gene g24175

[5] CLP-38655 was the total CLP production (CLP-D, F, G, H, and I) by gene g38655

the indole group of Trp compared to other CLP amino acids (e.g., Val, Leu, Ile and Phe) may have increased the water solubility of CLP-38655. The total CLPs remaining after NaOH, KOH, $K_2CO_3$, $Na_2CO_3$, $NaHCO_3$, $Na_3PO_4$ and $K_3PO_4$ alkali refining were 14.9%, 17.4%, 20.5%, 21.7%, 24.1%, 30.3% and 41.0%, respectively.

The experiment showed stronger alkalis were more efficient than weaker ones (NaOH > KOH > $K_2CO_3$ > $Na_2CO_3$ > $NaHCO_3$ > $Na_3PO_4$ > $K_3PO_4$) at CLP removal. The removal of only a portion of the CLPs during alkali refining process might be caused by: 1) a change in the solubility of CLPs in the presence of ions; 2) a change in the solubility of CLPs in the presence of soap; and/or 3) release of CLPs from FFAs. In the future, the solubility of CLPs in soap solutions, binding between CLPs and different ions and binding between CLPs and FFAs can be studied to test these hypotheses.

# 5 SUMMARY AND CONCLUSIONS

Flax is widely grown in western Canada. CLPs in flaxseed, along with lignan and linolenic acid, are drawing increased attention due to their potential health benefits (Cunnane *et al.*, 1993; Jenkins *et al.*, 1999; Clark *et al.*, 1995; Wieczorek *et al.*, 1991). The study of CLPs, in particular, has been increasing since their immunosuppressive activity was first discovered (Wieczorek *et al.*, 1991). The present project investigated: 1) methods for CLP extraction, isolation, detection and quantification, 2) the concentration of CLPs in flaxseed from different flaxseed cultivars, 3) the distribution of CLPs in different parts of the flaxseed, 4) the concentration of CLPs in lab-pressed flaxseed oil and commercial flaxseed oils and 5) the effects of acid degumming and alkali refining on the level of CLPs in cold-pressed flaxseed oil.

CLP-A was first found in the sediment of standing flaxseed oil. The low polarity of the peptides is responsible for their tendency to concentrate in seed oil after crushing. In this study, acetone was chosen for CLP extractions from flaxseed due to their high solubility in this solvent. Solid phase extraction (silica gel column) followed by solvent elution was used to separate crude peptides from other low polarity hydrophobic compounds. HPLC was utilized for CLP separation, isolation, identification and quantification, whereas MS was used as an alternative method for CLP identification.

Prior to this study, the level of CLPs in domestic flaxseed cultivars and the effect of the growth environment on the concentrations of CLPs in flaxseed had not been reported. The concentration of peptides in five cultivars grown in two locations over two years were analyzed. The concentration of CLPs varied, with Somme having the highest levels of CLP-A, D, F, G and total CLPs. Cultivar, environment and their interaction proved to be significant variables that influenced the production of CLPs in flaxseed even though no pattern was found in the relationship between the effect of environment and the concentration of CLPs in different flax cultivars.

The distribution of CLPs in flaxseed was studied using the HPLC quantification method that was developed in this work. It was found that the water-soluble gum contained no peptides. The cotyledon had the highest concentration of CLPs, whereas seed coat had lower levels. Oil bodies, the main oil storage organelles, were found to be the main location for CLPs after one time homogenization/centrifugation; while residues and serum also contributed a small proportion of the CLPs. More homogenization/centrifugation steps caused large losses of CLPs, which might be due to the redistribution of CLPs in the aqueous solution. The main organelle for CLP storage was not determined in this study and further study is required to determine the distribution of peptides in flaxseed tissues.

Compared to the meal, CLPs were found in greater amounts in crude oil and solid foots from expeller-pressed flaxseed. The concentration of CLPs in crude flaxseed oil produced on a lab scale was much higher than those in commercial oils, which led us to test the effect of oil refining on the removal of CLPs. Acid degumming using $H_3PO_4$ proved to be effective for removal of all CLPs from crude flaxseed oil. Alkali refining was also effective at removing CLPs, even though this treatment failed to remove all peptides equally.

This work developed systematic methods for CLP extraction, isolation, separation, detection and identification. It increased our knowledge of CLPs from flaxseed, including their levels in flaxseed and flaxseed fractions, their distribution after oil processing and methods for removing and recovering peptides from flaxseed oil. This work has demonstrated the presence of CLPs in flaxseed and flaxseed oil, which led to the consideration of flaxseed as a good source for CLP recovery. The presence of CLPs in flaxseed assures that CLPs will also be found in flax-related food products (flaxseed oil, flaxseed meal, flaxseed bread, etc.). This work also illustrated ways that CLPs may be extracted from flaxseed oil and could lead to large-scale industrial extraction processes. The ability to extract CLPs at a larger scale should allow faster exploration of the potential applications of these molecules and provide the flaxseed industry with potential value-added co-products.

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# 7 APPENDIX A

## Quality reports for standard CLP-A, B, C, D, E, F, G and Seg-A

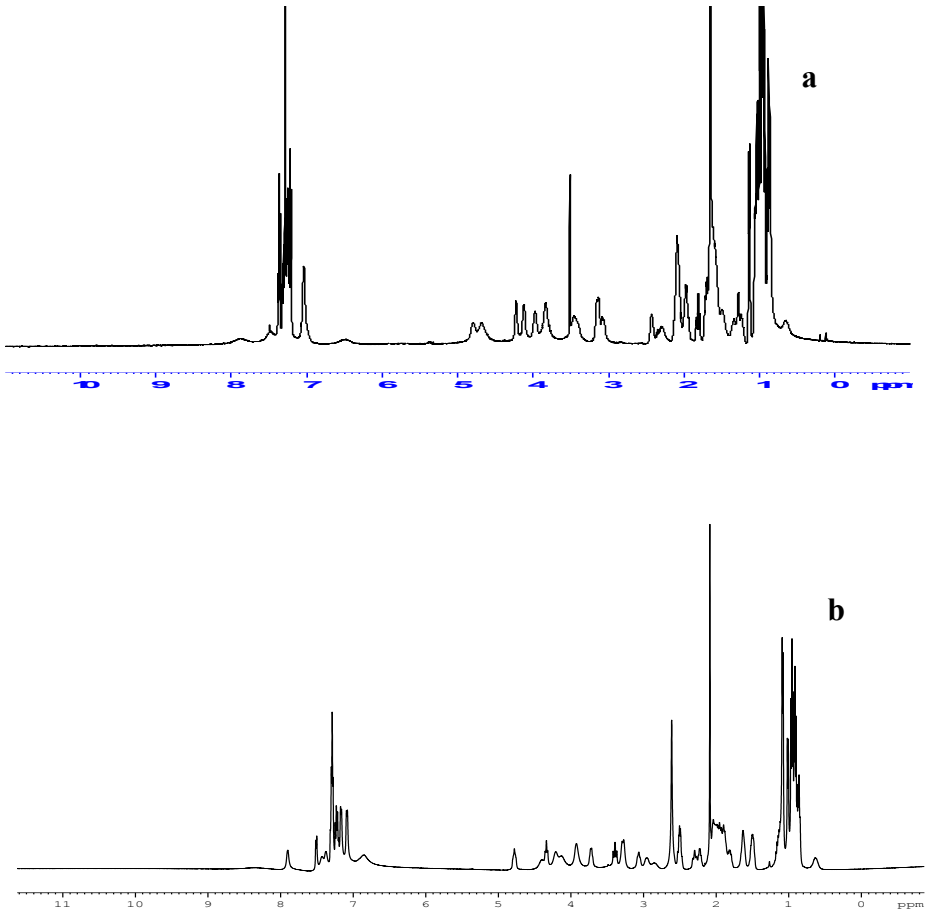



Figure 7-1 NMR spectra of standard CLPs and Seg-A. a. The 1H NMR of CLP-A in $CDCl_3$, b. The 1H NMR of CLP-B in $CDCl_3$ (Con't).

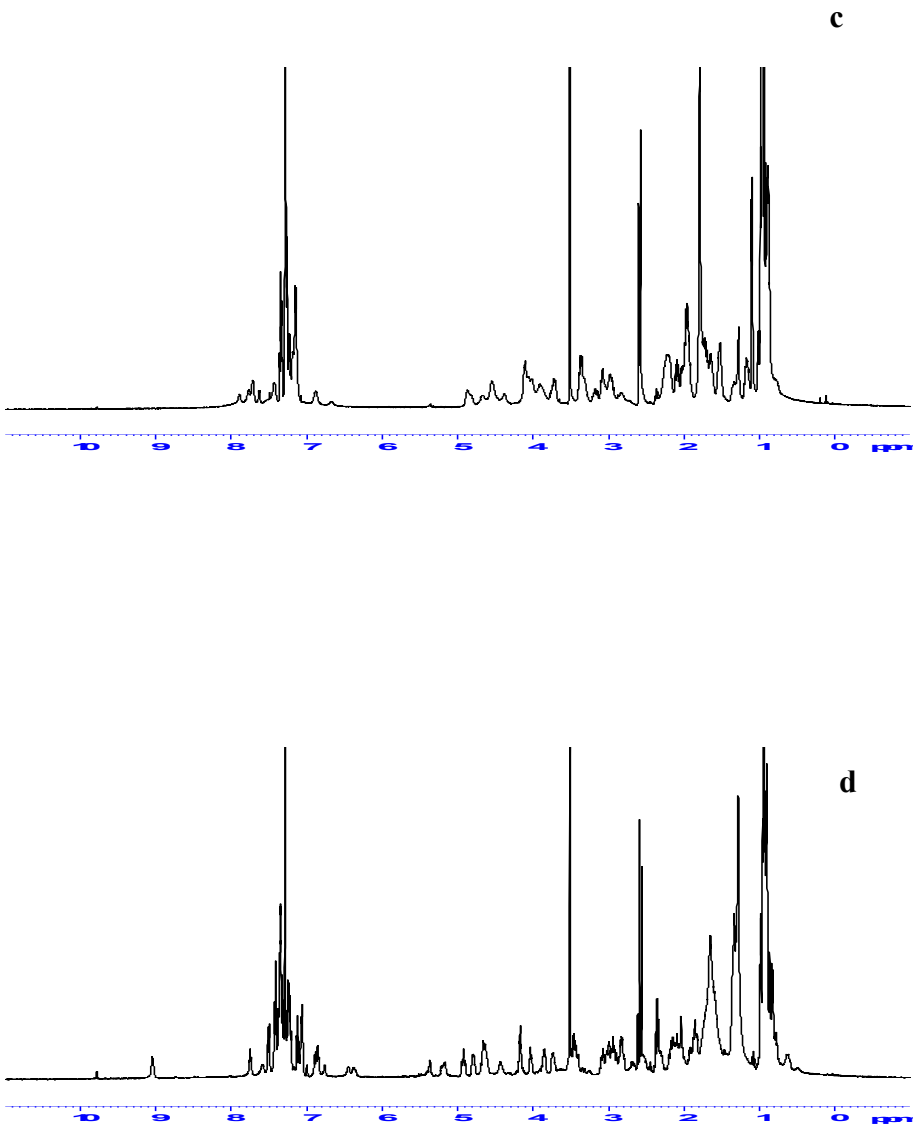



Figure 7-1 NMR spectra of standard CLPs and Seg-A. c. The 1H NMR of CLP-C in $CDCl_3$, d. The 1H NMR of CLP-D in $CDCl_3$ (Con't).

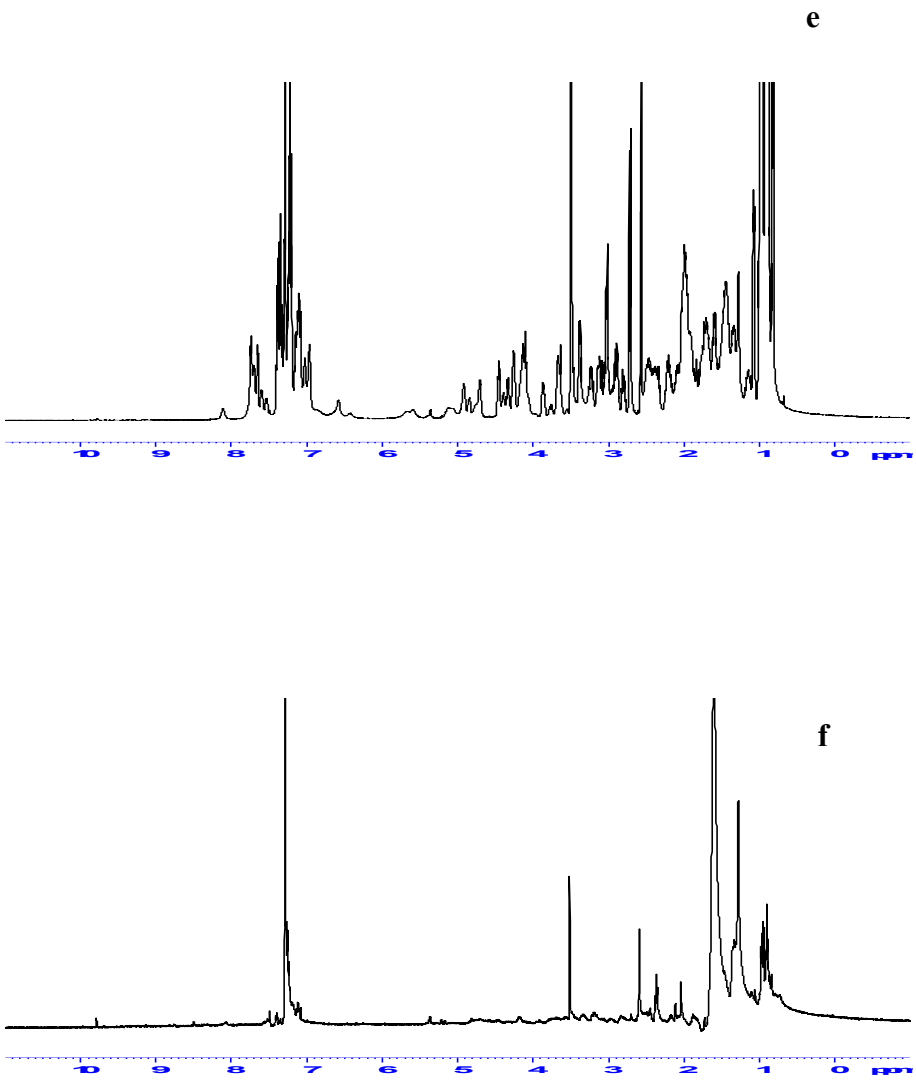
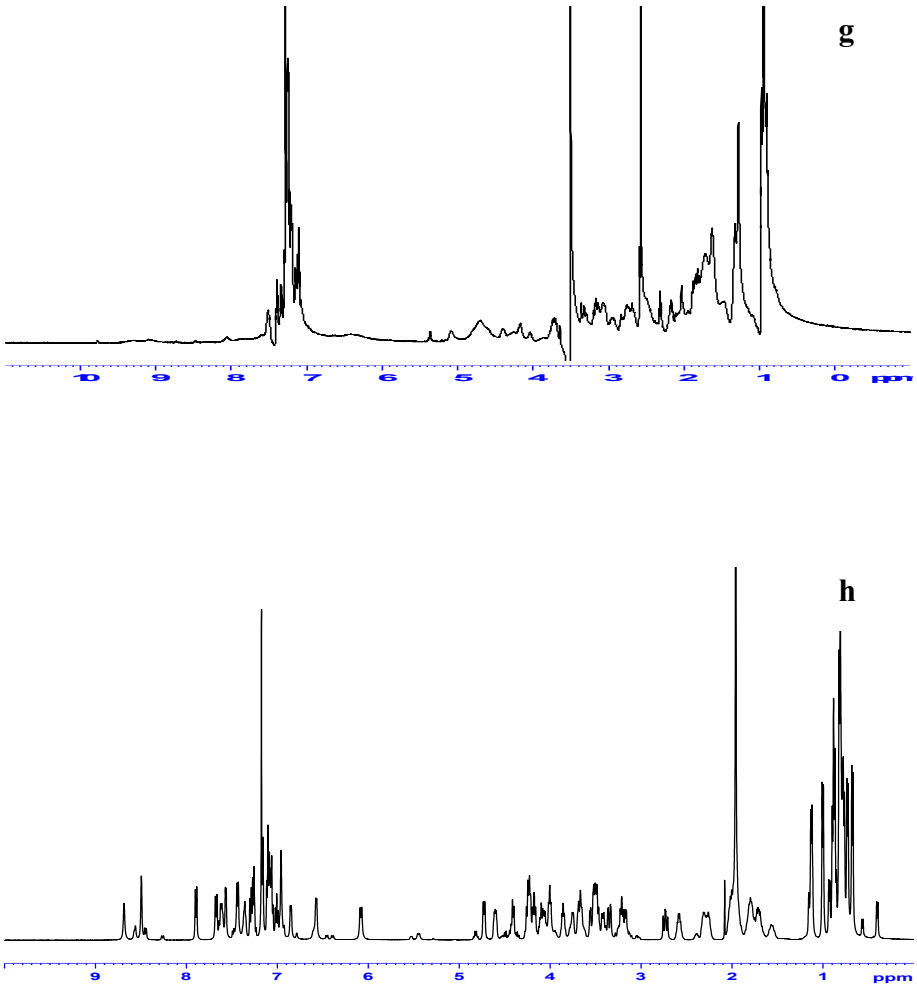



Figure 7-1 NMR spectra of standard CLPs and Seg-A. e. The 1H NMR of CLP-E in $CDCl_3$, f. The 1H NMR of CLP-F in $CDCl_3$ (Con't).




Note: The standard peptides CLP-A, CLP-B, CLP-C, CLP-D, CLP-E, CLP-F, CLP-G and Seg-A were diluted in deuterated chloroform (CDCl3) respectively and measurements were performed using a 500 MHz NMR equipped with TXI and BBO probe (Bruker, Bremen, Germany, SSSC, Saskatoon). All spectra were measured in solution 500 MHz for 1H NMR. Manual baseline correction and integration were applied in the software of XWIN-NMR 3.0.

Figure 7-1 NMR spectra of standard CLPs and Seg-A. g. The 1H NMR of CLP-G in $CDCl_3$, h. The 1H NMR of Seg-A in $CDCl_3$.

ESI-MS spectra for CLP-A, B, C, D, E, F, G and Seg-A

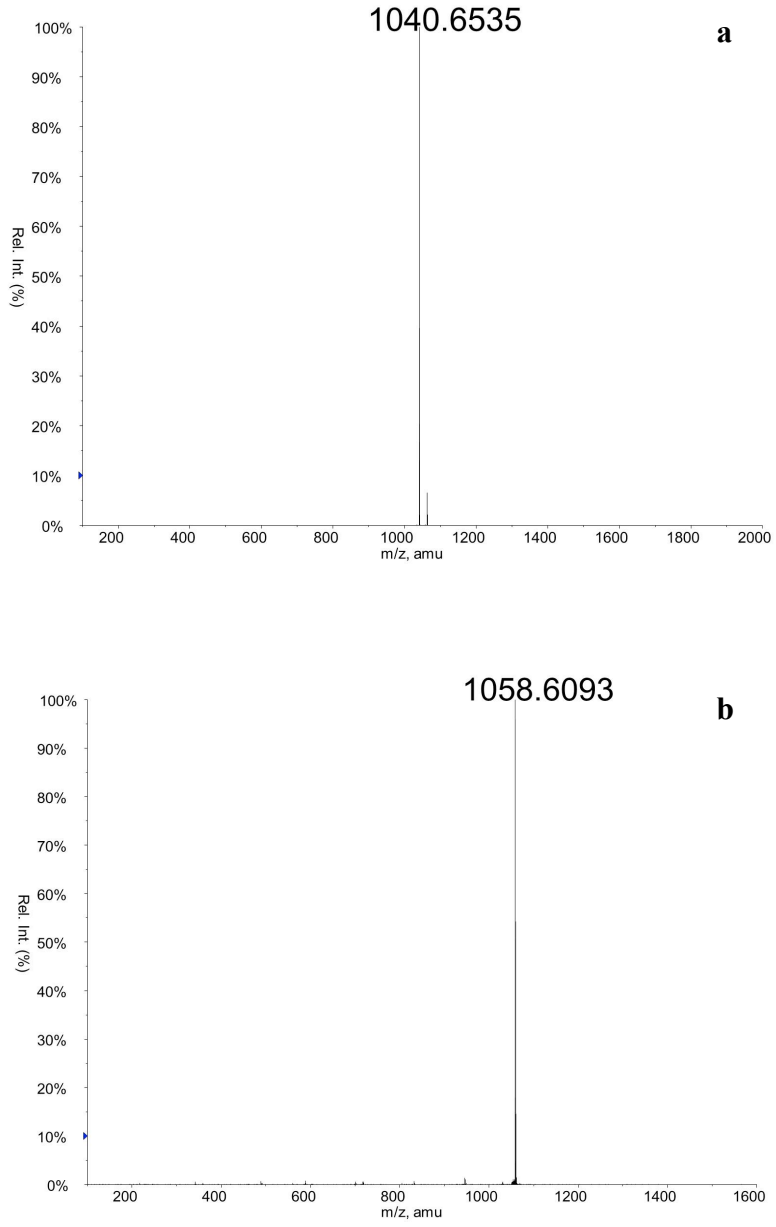



Figure 7-2 ESI-MS spectra of standard CLPA, B, C, D, E, F, G and Seg-A. a. ESI-MS of CLP-A, b. ESI-MS of CLP-B (Con't)

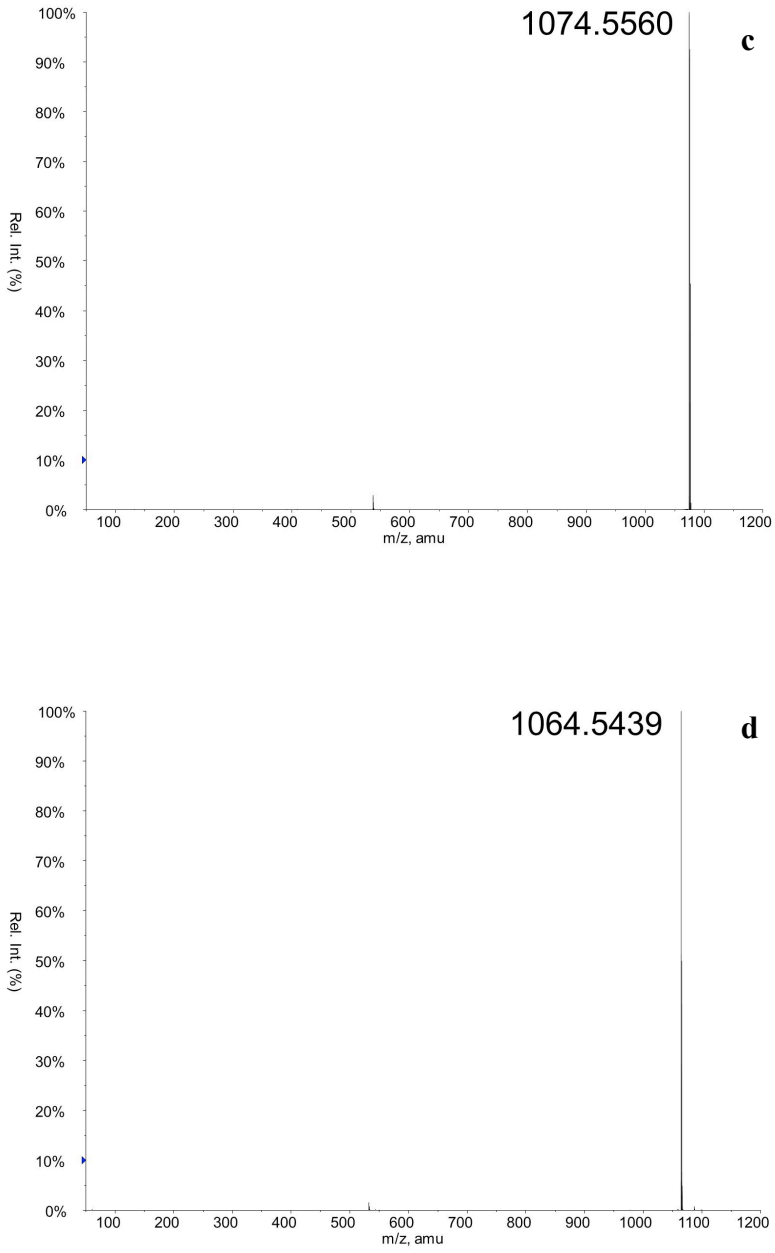
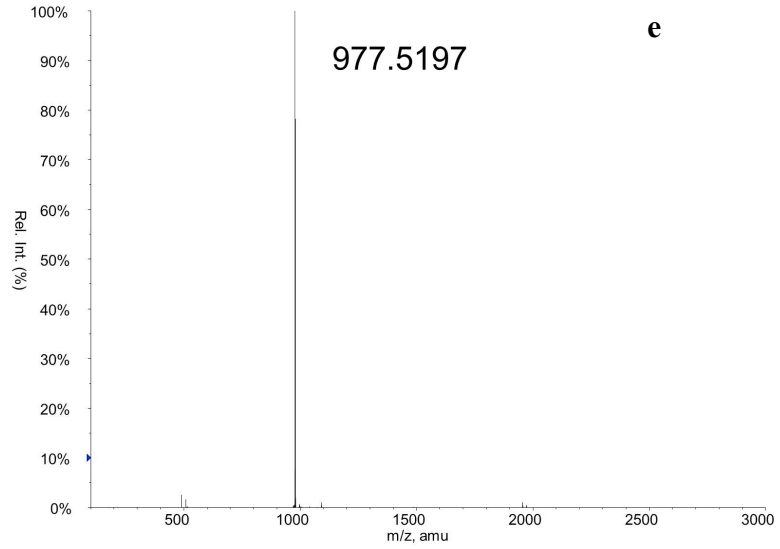



Figure 7-2 ESI-MS spectra of standard CLPA, B, C, D, E, F, G and Seg-A. c. ESI-MS of CLP-C, d. ESI-MS of CLP-D (Con't)



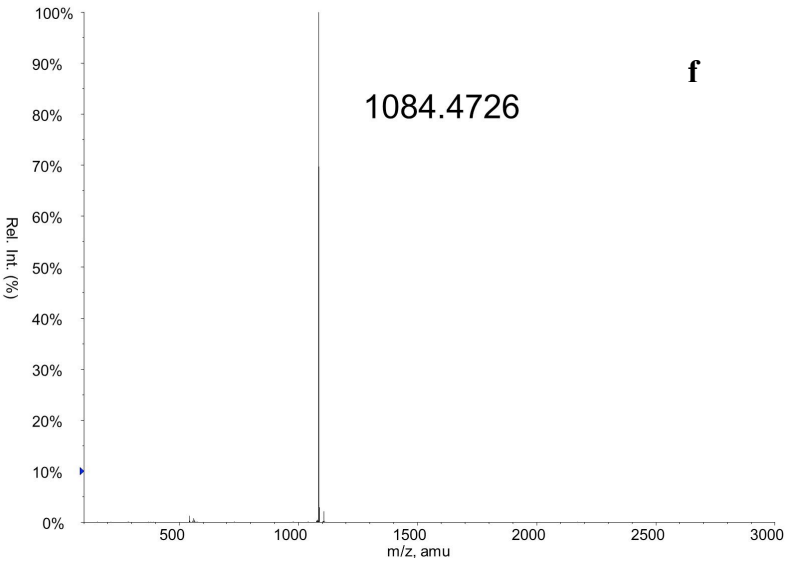
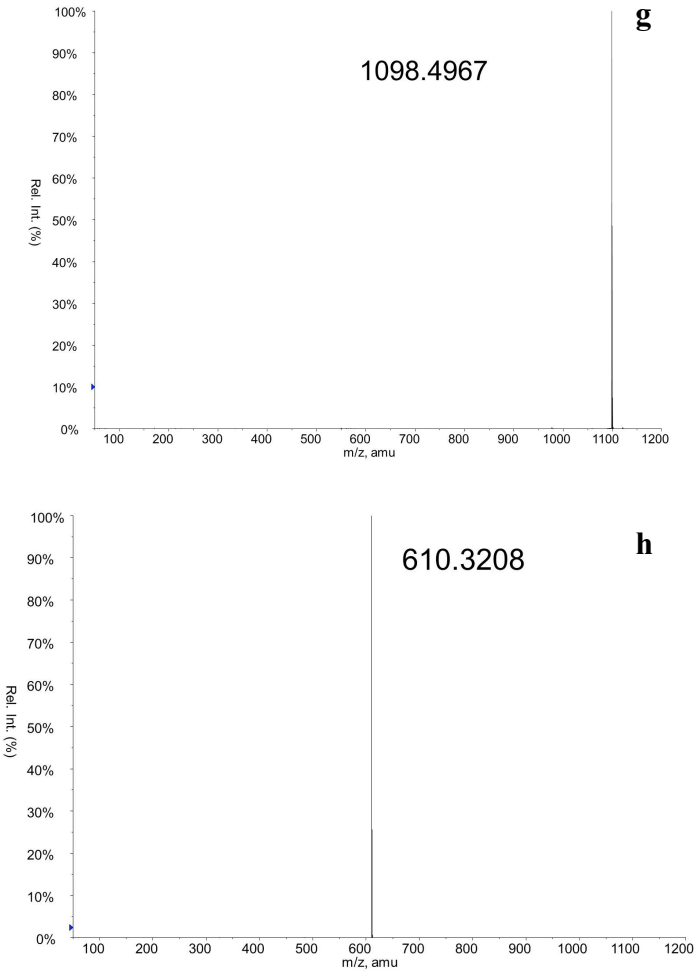




Figure 7-2 ESI-MS spectra of standard CLPA, B, C, D, E, F, G and Seg-A. e. ESI-MS of CLP-E, f. ESI-MS of CLP-F (Con't)




Note: The standard peptide was prepared in a solution containing 90% methanol, 10% water and 0.1% formic acid in a total volume of 1 mL for MS analysis. MS analysis was performed on a Hybrid Quadrupole-TOF LC/MS/MS system. The solution was introduced into the turbo ion electrospray spectrometer source by loop injection at a rate of 5 μL per min. Ion scanning experimental data was acquired with the pulsing function turned on, using a dwell time of 50 ms and the step size of one Dalton. All signals were created and analyzed by the Analyst QS 1.1 software.

Figure 7-2 ESI-MS spectra of standard CLPA, B, C, D, E, F, G and Seg-A. g. ESI-MS of CLP-G, h. ESI-MS of Seg-A

# 8 APPENDIX B

# LIST OF CHEMICALS

**Purchased from EMD chemicals Inc** (Gibbstown, NJ)

| | |
|---|---|
| Acetone | GR ACS grade |
| Acetonitrile | HPLC grade |
| Dichloromethane | GR ACS grade |
| Diethyl ether | GR ACS grade |
| Ethyl acetate | GR ACS grade |
| Hexane | GR ACS grade |
| Methanol | GR ACS grade |
| Methonal | GR ACS grade |
| Phosphoric acid | GR ACS grade |
| Potassium carbonate | GR ACS grade |
| Potassium hydroxide | GR ACS grade |
| Sodium bicarbonate | GR ACS grade |
| Sodium carbonate | GR ACS grade |
| Sodium hydroxide | GR ACS grade |
| Sodium chloride | GR ACS grade |
| Tripotassium phosphate | GR ACS grade |
| Trisodium phosphate | GR ACS grade |

**Purchased from Sigma-Aldrich** (St. Louis, MO)

| | |
|---|---|
| Sand | |
| Silica gel 60 | Flash chromatography/preparative liquid chromatography grade |

**Purchased from Bio-Rad laboratories** (Hercules, CA)

| | |
|---|---|
| Tris-HCL buffer | 1.5 M, PH 8.8 |

# 9 APPENDIX C

## Solvent fractions from silica gel isolation

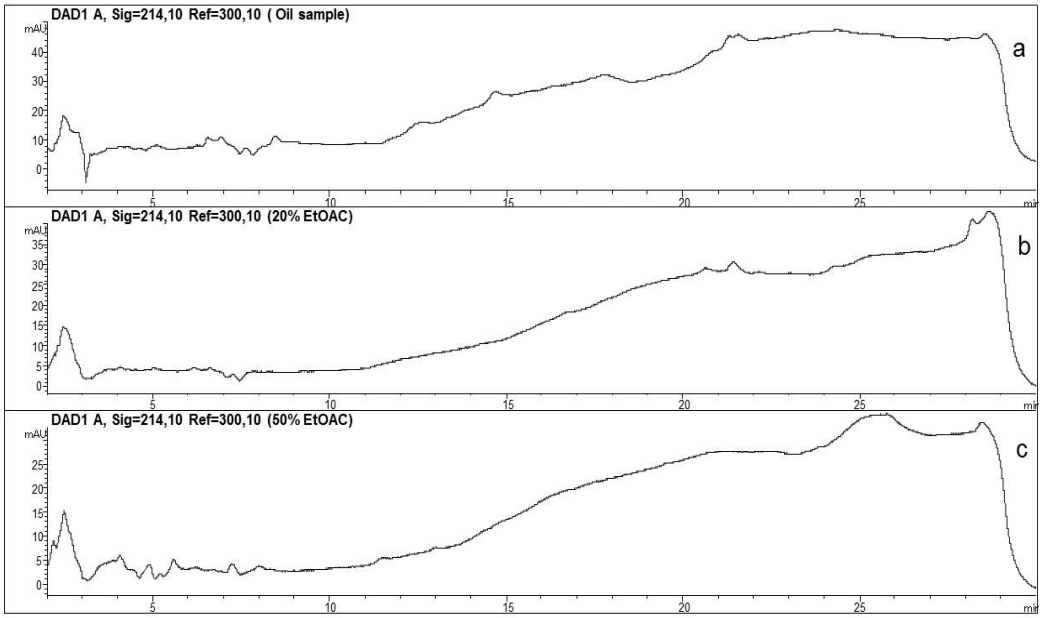
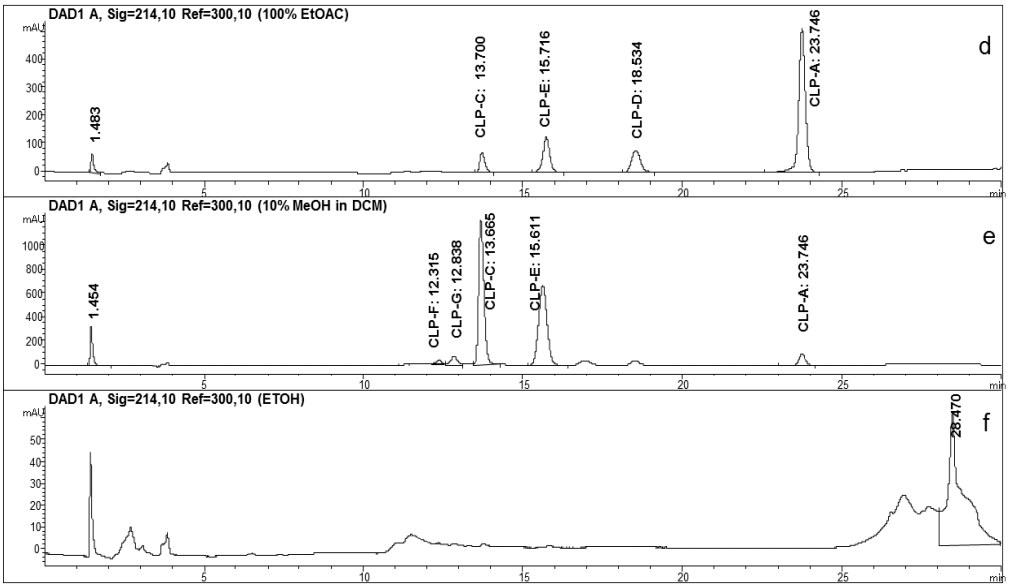



Figure 9-1 Fractions from peptide isolation using Silica gel column. Fraction a, oil, fraction b, 20% EtOAC in hexane, fraction c, 50% EtOAC in hexane (Con't)




Note: Silica gel isolation of CLPs from flaxseed oil was conducted as described in 3.2.1 and 3.2.2. HPLC separation was followed as described in 3.2.3. CLPs were enriched in Fraction d (100% EtOAC) and e (10% MeOH in DCM) while other Fraction a (oil), b (20% EtOAC), c (50% EtOAC), or f (EtOH) did not contain any of the peptides.

Figure 9-2 Fractions from peptide isolation using Silica gel column. Fraction d, 100% EtOAC, fraction e, 10% MeOH in DCM, fraction f, EtOH

# 10 APPENDIX D

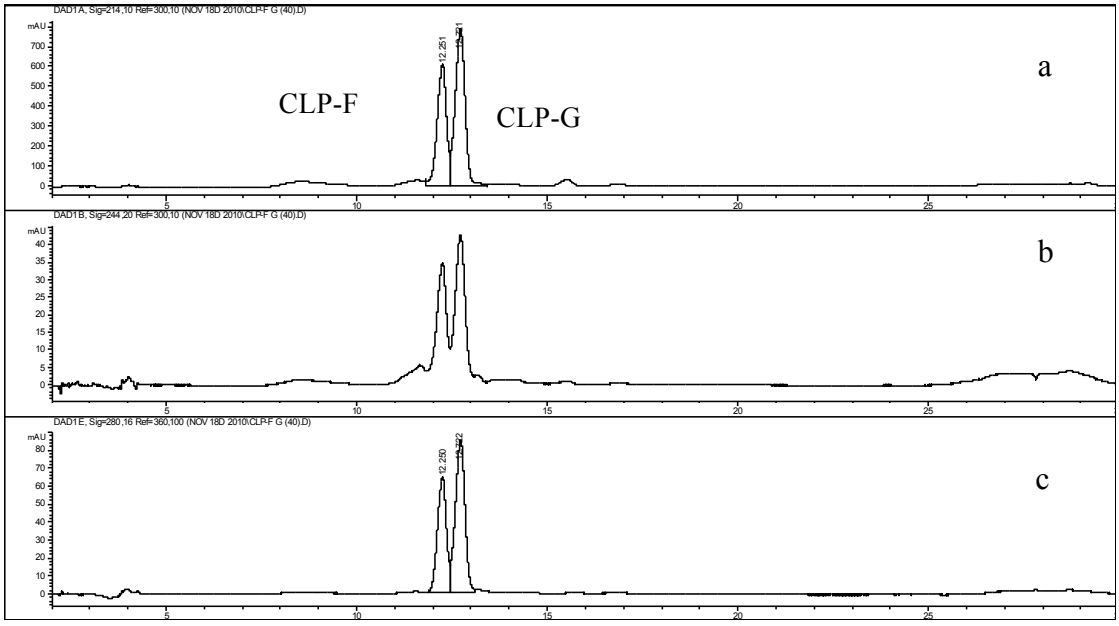



Figure 10-1 HPLC chromatogram of CLP-F (0.6 mg/mL) and CLP-G (0.7 mg/mL) under the wavelengths of (a) 214, (b) 244 and (c) 280 nm

# 11 APPENDIX E

## Calibration curves of CLPs using internal standard Seg-A

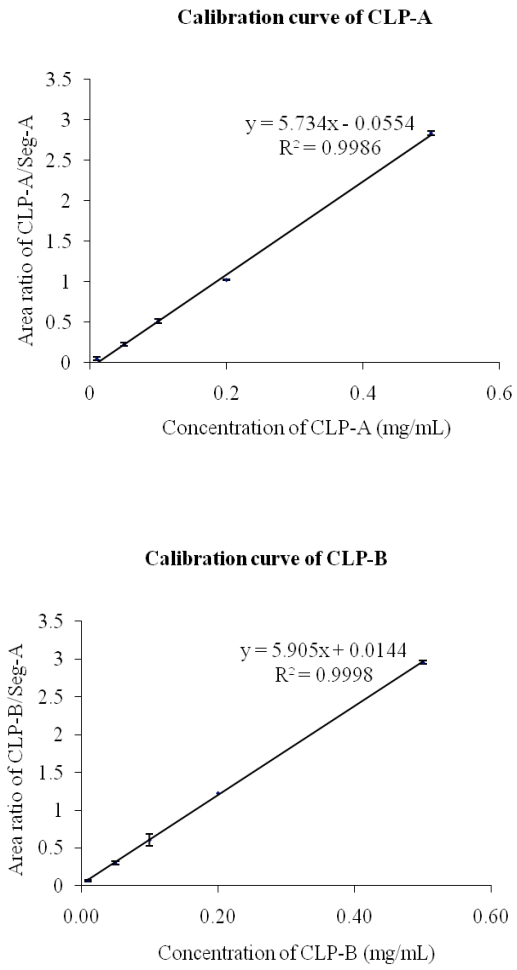
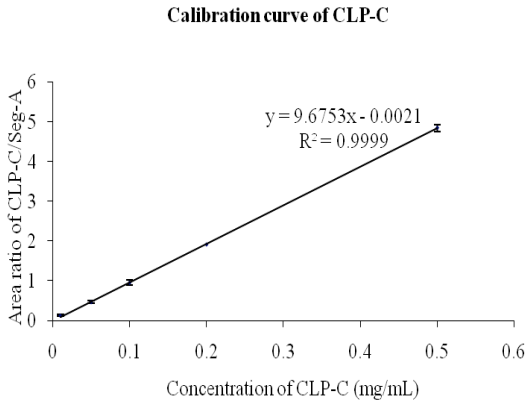



Figure 11-1 Calibration curves of CLPs using internal standard Seg-A (0.05 mg/mL) (Con't)



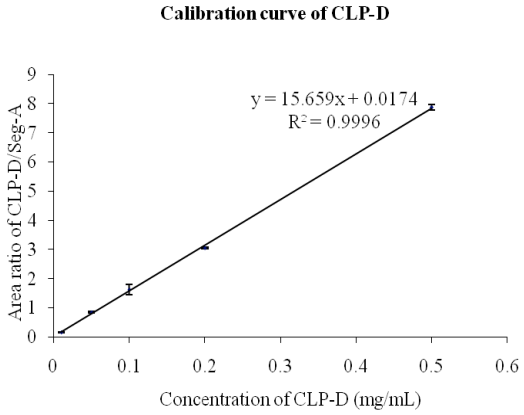
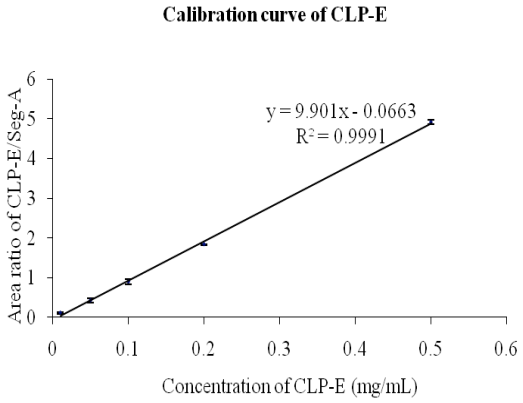




Figure 11-1 Calibration curves of CLPs using internal standard Seg-A (0.05 mg/mL) (Con't)



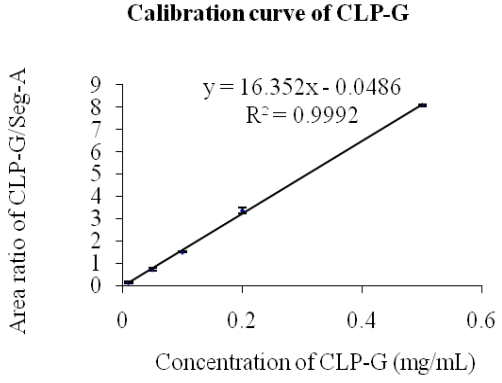




Figure 11-1 Calibration curves of CLPs using internal standard Seg-A (0.05 mg/mL)

# 12 APPENDIX F

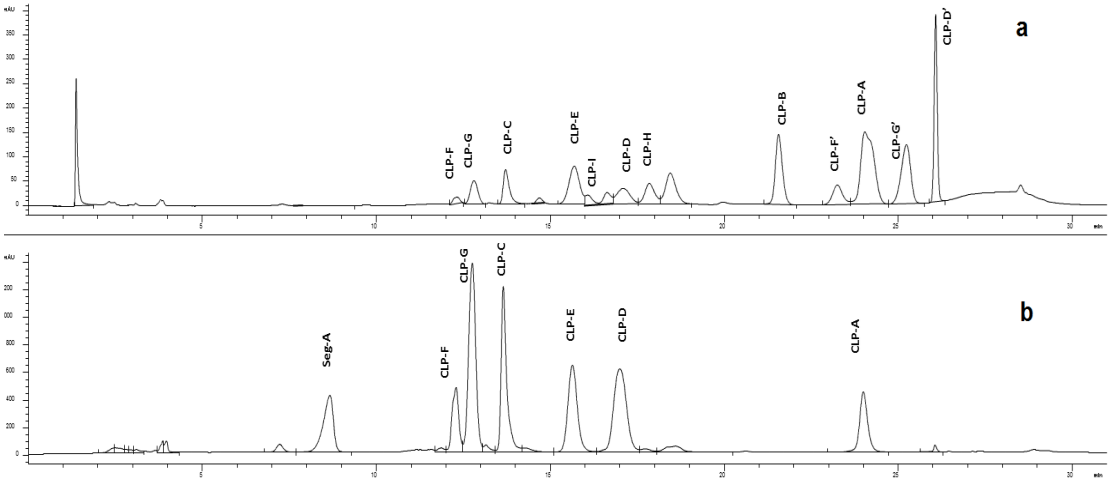



Figure 12-1 Transformation of CLPs during processing

a: CLPs isolated from degummed flaxseed by directly stirring in 10 × (W/V) 70% methanol in water for 2 hrs at room temperature

b: CLPs isolated from flaxseed oil extracted by goldfisch extraction