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Distribution of Cyclolinopeptides in Flaxseed Fractions and Products

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Supporting Information

ABSTRACT: Hydrophobic cyclic peptides, termed cyclolinopeptides, found in flaxseed are known for their immunosuppressive activity. This study is the first report of the occurrence of cyclolinopeptides in flaxseed fractions and products produced by aqueous processing and cold pressing. The distribution of cyclolinopeptides in flaxseed was determined after processing of flaxseed by various industrial and laboratory processes. Extracts of the water-soluble mucilage did not contain cyclolinopeptides. The cotyledon had the highest concentration of cyclolinopeptides, whereas seed coat had lower levels. An oil body fraction separated from seed after homogenization in water, followed by centrifugation, had the highest concentration of cyclolinopeptides of the fractions produced by this method. Further washing of the oil body fraction led to a loss of cyclolinopeptides. When oilseed was extruded using an expeller press, cyclolinopeptides were found in greater concentrations in crude oil and the solid sediment present in the oil fraction than in meal or the unprocessed seed. The concentration of cyclolinopeptides in crude flaxseed oil immediately after pressing was much higher than that observed in flaxseed oils purchased from a retail outlet. The effect of oil refining treatments on the removal of cyclolinopeptides was also tested. Acid degumming using aqueous H_3PO_4 removed cyclolinopeptides from crude flaxseed oil. Alkali refining was less effective as this treatment failed to remove all peptides equally. This work illustrates ways that cyclolinopeptides may be extracted from flaxseed oil that could be developed for large-scale industrial extraction. The ability to extract cyclolinopeptides on a larger scale would allow faster exploitation of commercial applications of these molecules and provide the flaxseed industry with value-added coproducts.

KEYWORDS: *Linum usitatissimum L., cyclolinopeptide, HPLC quantification, flaxseed*

INTRODUCTION

Flax (*Linum usitatissimum* L.) seed oil contains cyclic hydrophobic peptides, known as cyclolinopeptides, that are composed of eight or nine amino acid residues.[1] Cyclolinopeptides derived from flaxseed oil have known bioactivity with immunosuppressive and anticancer effects.[2] Flaxseed oil is an excellent commercial source of cyclolinopeptides as these compounds are hydrophobic and are dissolved in oil after seed processing. The only previous report of cyclolinopeptides in a flaxseed product was by Brühl et al.[3] Flaxseed oil has a bitter flavor that limits its use as a food product. Brühl et al.[3] noted that the extent of the bitter flavor of flaxseed oil correlated with the concentration of cyclolinopeptide E (**5**) in the oil. In addition to the negative impact of peptides on the use of flaxseed oil as a food substance, the peptides may also have a negative impact on the use of flaxseed oil as a drying oil. Treatments that removed the cyclolinopeptides from flaxseed oil also accelerated the rate of viscosity increase that occurs when flaxseed oil is heated.

Recently, liquid and solid phase extractions were proposed for commercial scale extraction and concentration of peptides from flaxseed oil.[4] However, recovery of cyclolinopeptides from whole seed requires the use of large volumes of solvent, and such procedures may be costly, laborious, and time-consuming.[5−11] Cyclolinopeptides may also be produced by chemical synthesis. Wieczorek et al.[2] described a synthetic method for preparing cyclolinopeptide A (**1**) from *tert*-butyloxycarbonyl protected amino acids on Merrifield resin. Trifluoroacetic acid and sulfuric acid are used to release linear peptides from the resin. The linear peptides were subsequently cyclized by Castro's reagent. The final peptide was purified using high-performance liquid chromatography (HPLC). However, the synthetic method had a low product yield, and peptide recovery was difficult. Knowledge of the distribution of compounds in flaxseed can aid in the development of processes for enrichment. Flaxseed lignan is found primarily in the seed coat; thus, isolation of flaxseed lignan from seed coat or whole seed provides significant advantages over isolation from ground whole seed or seed meal.[12,13] The commercial availability of a seed coat product makes it a practical source for research on seed coat composition and commercial lignan production.[14−16] Although oil was isolated from flaxseed hulls, the concentration of peptides in this oil in this fraction were not determined.[14] Cyclic peptides are widely distributed in plant tissues with many known seedborne peptides.[17] Cyclolinopeptide **1** was the first cyclolinopeptide isolated from seeds of *L. usitatissimum* L., but it was not determined where the peptide was located in the seed.[1] The bicyclic peptides moroidin[18] and celogentins D−H and J[19] were extracted with methanol (MeOH) from the seeds of *Celosia argentea*; again, the location of the peptide in the seed was not reported.

Knowledge of the distribution of cyclic peptides in seed tissues may aid in understanding the role these compounds play in the plant, and this information may also lead to improved methods for extraction for research or commercial purposes.

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Table 1. Cyclolinopeptide Levels in Flaxseed Fractions

		concn of cyclolinopeptides[a] (μg/g)						
fraction (n = 3)[b]	wt (dry basis) (%)	1	3	4	5	6	7	total
mucilage	7.0 ± 0.8	ND[c]	ND	ND	ND	ND	ND	ND
cotyledon	52.0 ± 1.0	25.2 ± 0.4b	98.8 ± 3.2a	13.1 ± 1.6ab	80.2 ± 1.8a	19.6 ± 0.3a	55.0 ± 1.6a	291.9 ± 8.2a
seed coat	41.0 ± 1.1	19.3 ± 1.0c	31.0 ± 1.3c	15.6 ± 0.9a	30.7 ± 0.8c	6.7 ± 0.1c	19.8 ± 0.9c	123.1 ± 4.8c
whole seed	100.0 ± 1.0	63.1 ± 1.4a	78.7 ± 3.6b	9.9 ± 0.2b	70.2 ± 1.2b	13.1 ± 0.7b	33.3 ± 1.1b	268.3 ± 7.9b

[a]Cyclolinopeptide **2** was not detected by HPLC. Values in the same column followed by the same letter are not significantly different at $p < 0.05$. [b]Fraction separation was repeated three times. [c]Not detected by HPLC.

Table 2. Cyclolinopeptide Concentrations in Fractions Derived by Aqueous Processing of Flaxseed

		concn of cyclolinopeptides[a] (μg/g)						
fraction (n = 3)[b]	wt (dry basis) (%)	1	3	4	5	6	7	total
pellet	55.0 ± 2.9	3.4 ± 0.2d	2.4 ± 0.1d	4.1 ± 0.4c	3.4 ± 0.3d	1.0 ± 0.1d	1.4 ± 0.1d	15.7 ± 1.0e
supernatant	13.5 ± 1.0	26.1 ± 2.3c	21.2 ± 1.5c	11.2 ± 3.8c	28.1 ± 2.1c	4.5 ± 0.1c	8.5 ± 1.8c	99.6 ± 10.6d
oil body-1[c]	35.0 ± 2.6	163.7 ± 11.9a	119.1 ± 12.1a	74.5 ± 7.2b	163.7 ± 11.9a	33.5 ± 0.4a	89.3 ± 1.9a	643.8 ± 34.6a
oil body-2[d]	29.5 ± 1.0	63.0 ± 5.0b	ND[e]	90.1 ± 7.0a	ND	ND	ND	153.1 ± 11.2c
whole seed	100.0 ± 1.0	63.1 ± 1.4b	78.7 ± 3.6b	9.9 ± 0.2c	70.2 ± 1.2b	13.1 ± 0.7b	33.3 ± 1.1b	268.3 ± 7.9b

[a]Cyclolinopeptide **2** was not detected by HPLC. Values in the same column followed by the same letter are not significantly different at $p < 0.05$. [b]Fraction separation was repeated three times. [c]Oil bodies isolated after one homogenization/centrifugation. [d]Oil bodies isolated after six homogenizations/centrifugations. [e]Not detected by HPLC.

No papers detailing the distribution of cyclolinopeptides in flaxseed have appeared in the scientific literature. The present research was directed to the determination of (1) the cyclolinopeptide distribution in flaxseed fractions including seed coat, cotyledon, and oil bodies; (2) the distribution in flaxseed during cold pressing and subsequent oil sedimentation and filtration; (3) the comparative levels of cyclolinopeptides in commercial flaxseed oils; and (4) the effects of acid degumming and alkali refining on the levels of cyclolinopeptides in pressed flaxseed oil.

MATERIALS AND METHODS

General. Natunola Health Inc. (Winchester, ON, Canada) provided whole flaxseed that was used to determine the cyclolinopeptide levels in flaxseed. Flaxseed (var. CDC Bethune) used for crude oil extraction by expeller press was grown in Floral, SK, Canada. Five commercial organic flaxseed oils from local retail health food stores (Saskatoon, SK, Canada) were obtained for cyclolinopeptide analysis. Two samples of crude flaxseed oil (laboratory-pressed flaxseed oil, var. CDC Bethune) were prepared for comparisons made at the time of each study. One sample was compared with the commercial organic samples, whereas the other was used as the oil for alkali and acid refining studies. Flaxseed samples were extracted with acetone and the extracts subjected to HPLC analysis using segetalin-A (Seg-A) as an internal standard according to the method of Gui et al.[20] Each sample was analyzed three times. The structures of cyclopeptides are shown in Table S1 and Figure S1 of the Supporting Information, respectively. Solvents were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). All recovered extracts were weighed and stored below 4 °C until further analysis.

Cyclolinopeptides in Flaxseed Fractions. *Water Degumming and Seed Coat Removal.* Flaxseed mucilage was extracted as described by Mazza and Biliaderis[21] with some modification. The flaxseed samples (10 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 solution was separated from the flaxseeds using a 40-mesh screen (12 in.; VWR, West Chester, PA, USA) and then freeze-dried (Labconco Co., Kansas City, MO, USA) until the weight of the dry mucilage became constant. Degummed flaxseeds were manually dissected using a stainless steel spatula by applying pressure on the seed coat and separating the seed coats from cotyledons. The cotyledon fraction was collected, rinsed twice with distilled water (10 mL), and freeze-dried. The seed coats were treated similarly to the cotyledons. Dried mucilage, cotyledon, seed coat, and whole seed were extracted with acetone using the method described previously.[20] The mucilage fraction contained only a trace amount of oil (0.02 g). The beaker used for mucilage extraction was 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. The MeOH solvent from the extract-dried mucilage was removed using a Büchi R-200 rotary vacuum evaporator (Brinkmann Instruments, Inc., Westbury, NY, USA) at 40 °C, and the residue was dissolved in MeOH (1 mL). Subsequently, a solution containing Seg-A (25 μL, 2 mg/mL) as an internal standard was added to the MeOH extract, and this solution was then filtered through a 0.45 μM PTFE syringe filter (Whatman Ltd., Piscataway, NJ, USA) before HPLC analysis. Quantitative HPLC was performed as described previously,[20] and the resulting data are included in Table 1.

Oil Body (Oleosome) Isolation. Oil bodies were isolated using the procedure of Simpson and Nakamura.[22] Whole seeds (20 g, Natunola) were 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 22000 rpm (Eberbach Co., Ann Arbor, MI, USA). The homogenate was subjected to centrifugation at 14000g for 60 min at 4 °C in a chilled rotor (Beckman Coulter, Inc., Palo Alto, CA, USA) to form three layers: a floating fat pad, a supernatant fraction, and a precipitated solid bottom residue. The floating oil body (fat pad) was removed using a flat stainless spatula. A portion of the oil body was extracted for peptide analysis. The remaining oil body was homogenized again using the same conditions in 5 volumes of chilled, fresh buffer. The centrifugation−homogenization procedure was performed five times. The supernatant was separated from the pellet by decanting. The supernatant and pellet were freeze-dried (Labconco Co.) and weighed before oil extraction. The oils from dried supernatant and dried pellet were extracted as described previously[20] except the samples were not ground. Oil was extracted from the freeze-dried oil bodies using 5 volumes of hexane by shaking the hexane−oil body mixture by hand for 2 min. The hexane layer was removed completely, and the remaining solid was mixed with 10 volumes of ethyl acetate (EtOAc) and then shaken by hand for 2 min. After mixing, the EtOAc solution was filtered (Whatman no. 2, Whatman Ltd.), and the filtrate was combined with the aforementioned hexane layer. Solvents (hexane and EtOAc) were removed from the fraction by a rotary vacuum evaporator in a 40 °C water bath. Quantitative

HPLC was performed as described previously,[20] and the resulting data are included in Table 2.

Effects of Processing on the Distribution of Cyclolinopeptides. *Crude Oil Extraction by Expeller Press.* Flaxseed (1 kg, var. CDC Bethune) was extracted using a continuous Komet oilseed expeller press (CA59G, IBG Monforts Oekotec GmbH & Co. KG., Mönchengladbach, Nordrhein-Westfalen, Germany) operating at 88 rpm. No heat was applied during pressing. Expeller pressed oil was allowed to settle for 2 days at room temperature to produce both crude oil and sediment. Subsequently, the upper oil layer was decanted. The sediment was separated from the remaining oil by filtration under vacuum pressure (9000 Pa) with a Buchner funnel lined with a glass-fiber filter (Whatman grade GF/A, Whatman Ltd.). The oil recovered by filtering the sediment was combined with crude oil that was obtained by sedimentation. The sediment and pressed meal were stored at 4 °C before solvent (Goldfisch) extraction. The concentration of cyclolinopeptides in each fraction was calculated as described previously,[20] and the resulting data are included in Table 3.

Commercial Flaxseed Oils. Three bottles of each brand from retail outlets were utilized for this project. The cyclolinopeptides were quantified as described previously.[20] Untreated crude oil was used as a control. The concentrations of cyclolinopeptides in acid-treated oils were determined, and results are included in Table 4.

Acid Degumming of Flaxseed Oil. Oil was obtained after pressing and settling to remove sediment as described above. The effect of acid degumming on the solubility of cyclolinopeptides in crude flaxseed oil (var. CDC Bethune) was determined by treating the 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 1% (v/v) of 75% H_3PO_4 was added. The sample was mixed vigorously with a magnetic stirrer at 600 rpm for 5 min at room temperature and then centrifuged in a chilled rotor (4 °C, 9800*g*) for 30 min. After centrifugation, the upper oil layer was decanted from the bottom sediment. In the second and third experiments, the acid degumming conditions were varied. Smaller amounts of acid were used. For example, 0.1% (v/v) of 75% H_3PO_4 were studied as well as degumming twice with 0.1% (v/v) of 75% H_3PO_4. Finally, a lower concentration of phosphoric acid 1% (v/v) of 50% H_3PO_4 was used for degumming as described above. Untreated crude oil was used as a control. The concentrations of cyclolinopeptides in acid-treated oils were determined, and the results are included in Table 5.

Cyclolinopeptides in Phosphoric Acid Separated Residues. After decanting, the oil above the phospholipid gum pellet (from 50 mL of flaxseed oil), remaining in the centrifuge tube, was mixed with hexane (10× volume) and mixed vigorously by hand to extract oil and peptides. The hexane extract was filtered through Whatman no. 2 filter paper, 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 h, the MeOH phase (lower layer) containing the cyclolinopeptides and phospholipids was recovered and concentrated under reduced pressure in a rotary evaporator. The methanol-soluble residue was washed twice with diethyl ether (100 mL). After each wash, the solvent was decanted and then concentrated in a Büchi rotary vacuum evaporator at 40 °C. After evaporation of the diethyl ether, the dried solids were resuspended in acetone (10× volume), followed by filtration (Whatman no. 2). Phospholipids have low solubility in acetone and would not dissolve in this solvent. The acetone filtrate was concentrated using a rotary evaporator under reduced pressure. Methanol (50 mL) was added to the dried concentrate to dissolve peptides. After evaporation of the MeOH solution (1 mL), Seg-A solution was added as an internal standard.

Alkali Refining of Flaxseed Oil. Pressed flaxseed oil (var. CDC Bethune) was refined by addition of alkali into flaxseed oil to neutralize the free fatty acids (FFAs) such as oleic acid. In the first experiment, flaxseed oil (50 mL) was heated on a hot plate until the temperature of the oil reached 80 °C, and then 1% (v/v) 4 M NaOH was added. The sample was mixed vigorously with a magnetic stirrer at 600 rpm for 5 min at room tempeerature and then centrifuged (9800*g*) at 4 °C for 30 min, to facilitate separation of the oil and soap

Table 3. Cyclolinopeptide Distribution After Cold Pressing

		concn of cyclolinopeptides[a] (μg/g)							
fraction (n = 3)[b]	wt (dry basis) (%)	1	2	3	4	5	6	7	total
meal	57.5 ± 1.1	24.3 ± 1.9d	ND[c]	21.9 ± 1.6d	17.0 ± 1.9c	21.2 ± 1.4d	6.7 ± 0.8c	7.9 ± 0.6	99.0 ± 7.4d
sediment	3.5 ± 0.1	169.8 ± 10.6b	ND	289.2 ± 16.6b	186.5 ± 14.5b	251.5 ± 16.6b	92.2 ± 3.6a	266.2 ± 13.1a	1255.4 ± 75.0b
crude oil	34.4 ± 0.6	466.7 ± 6.8a	98.0 ± 3.8	368.6 ± 27.2a	227.5 ± 6.8a	462.7 ± 35.9a	86.3 ± 4.3a	262.7 ± 6.8a	1972.5 ± 86.7a
whole seed	100.0 ± 1.0	63.1 ± 1.4c	ND	78.7 ± 3.6c	9.9 ± 0.2c	70.2 ± 1.2c	13.1 ± 0.7b	33.3 ± 1.1b	268.3 ± 7.9c

[a]Values in the same column followed by the same letter are not significantly different at $p < 0.05$. [b]Fraction separation was repeated three times. [c]Not detected by HPLC.

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Table 4. Comparison of Cyclolinopeptide Levels in Retail and Freshly Pressed Crude Flaxseed Oil

flaxseed oil[a] (n = 3)[c]	concn of cyclolinopeptides[b] (μg/g)							
	1	2	3	4	5	6	7	total
I	310.5 ± 17.5b	68.0 ± 2.6b	286.0 ± 12.2b	179.7 ± 11.6b	293.6 ± 16.0b	31.6 ± 1.7b	91.4 ± 4.2b	1260.9 ± 65.9b
II	194.8 ± 14.1c	37.6 ± 4.5b	187.1 ± 13.8bc	31.5 ± 1.7c	211.4 ± 15.5c	35.0 ± 4.1b	101.2 ± 7.4b	798.6 ± 61.2c
III	169.1 ± 16.2c	ND[d]	208.7 ± 16.1bc	22.0 ± 3.4c	206.3 ± 10.7c	17.9 ± 2.9c	32.1 ± 1.6c	656.1 ± 50.9c
IV	300.9 ± 21.6b	122.0 ± 11.8a	112.4 ± 5.3c	21.0 ± 2.9c	173.7 ± 11.0c	14.6 ± 2.3c	52.7 ± 5.1bc	797.2 ± 60.1c
V	273.8 ± 15.4b	ND	182.3 ± 11.1bc	47.8 ± 7.4c	175.0 ± 10.5c	22.5 ± 2.3bc	16.4 ± 1.4c	717.8 ± 48.2c
VI	454.0 ± 16.1a	90.5 ± 7.8a	358.3 ± 15.5a	214.1 ± 4.3a	445.5 ± 14.7a	82.4 ± 4.5a	249.7 ± 3.7a	1894.6 ± 66.6a

[a]Five commercial flaxseed oils (I–V) and crude oil from freshly pressed CDC Bethune flaxseed without treatment (VI). [b]Values in the same column followed by the same letter are not significantly different at $p < 0.05$. [c]Fraction separation was repeated three times. [d]Not detected by HPLC.

Table 5. H_3PO_4 Acid Degumming Effects on Cyclolinopeptides from Flaxseed Oil

method[a] (n = 3)[b]	concn of cyclolinopeptides (μg/g)							
	1	2	3	4	5	6	7	total
a	ND[c]	ND	ND	ND	ND	ND	ND	ND
b	37.1 ± 1.8	ND	ND	ND	24.7 ± 0.1	ND	ND	61.7 ± 2.0
c	193.5 ± 7.2	ND	ND	ND	ND	ND	ND	193.5 ± 7.2
d	454.0 ± 16.1	90.5 ± 7.8	358.3 ± 15.5	214.1 ± 4.3	445.5 ± 14.7	82.4 ± 4.5	249.7 ± 3.7	1894.6 ± 66.6

[a]Methods: a, 1% (v/v) of 75% H_3PO_4; b, acid degumming twice with 0.1% (v/v) of 75% H_3PO_4; c, 1% (v/v) of 50% H_3PO_4; d, crude oil from the same fresh oil (Table 4, VI) without treatment. [b]Fraction separations after refining treatments were repeated three times. [c]Not detected by HPLC.

Table 6. Alkali Refining Effects on Cyclolinopeptides from Flaxseed Oil

method[a] (n = 3)[c]	concn of cyclolinopeptides[b] (μg/g)								
	1	2	3	4	5	6	7	total	recovery (%)
A	135.8 ± 2.6c	ND[d]	49.9 ± 0.5e	ND	85.9 ± 1.1c	ND	ND	271.5 ± 4.2c	14.3 ± 0.2
B	235.7 ± 5.2b	ND	33.6 ± 0.5e	ND	47.4 ± 0.9d	ND	ND	316.6 ± 6.6c	16.7 ± 0.1
C	241.3 ± 3.2b	ND	71.7 ± 0.4de	ND	60.1 ± 1.0cd	ND	ND	373.2 ± 4.6bc	19.7 ± 0.2
D	226.4 ± 2.9b	ND	79.1 ± 1.8de	ND	89.1 ± 3.3 cd	ND	ND	394.7 ± 8.0bc	20.8 ± 0.1
E	239.2 ± 7.8b	ND	112.5 ± 3.0 cd	ND	74.6 ± 2.0 cd	ND	12.2 ± 0.1b	438.5 ± 12.9bc	23.1 ± 0.1
F	277.2 ± 7.0b	ND	153.3 ± 3.2c	ND	102.0 ± 2.9c	ND	19.3 ± 0.2b	551.7 ± 13.3b	29.1 ± 0.2
G	239.1 ± 4.1b	ND	253.6 ± 6.3b	ND	231.8 ± 3.4b	ND	21.8 ± 0.1b	746.4 ± 13.9b	39.4 ± 0.3
H	454.0 ± 16.1a	90.5 ± 7.8	358.3 ± 15.5a	214.1 ± 4.3a	445.5 ± 14.7a	82.4 ± 4.5a	249.7 ± 3.7a	1894.6 ± 66.6a	100.0 ± 0.0

[a]Methods: A, 1% (v/v) of 4 M NaOH; B, 1% (v/v) of 4 M KOH; C, 1% (v/v) of 2 M K_2CO_3; D, 1% (v/v) of 2 M Na_2CO_3; E, 1% (v/v) of saturated $NaHCO_3$; F, 1% (v/v) of 1.3 M K_3PO_4; G, 1% (v/v) of 1.3 M Na_3PO_4; H, crude oil from the same fresh oil (Table 4, VI) without treatment. [b]Values in the same column followed by the same letter are not significantly different at $p < 0.05$. [c]Fraction separations after refining treatments were repeated three times. [d]Not detected by HPLC.

that formed from the neutralization. In subsequent experiments, the conditions of alkali refining were the same as described above except 1% (v/v) of 4 M KOH, 1% (v/v) of 2 M K_2CO_3, 1% (v/v) of 2 M Na_2CO_3, 1% (v/v) of saturated $NaHCO_3$, 1% (v/v) of 1.3 M K_3PO_4, and 1% (v/v) of 1.3 M Na_3PO_4 were used, respectively. Crude oil without alkali treatment was used as a negative control. The concentrations of cyclolinopeptides in base-treated oils were determined, and the results are included in Table 6.

HPLC Analysis. All chromatographic separations were performed on an Agilent 1200 series HPLC system (Agilent Technologies Canada Inc., Mississauga, ON, Canada) equipped with a quaternary pump, an autosampler, a variable-wavelength diode array detector (DAD, 190–300 nm), and a degasser. The samples were separated on a ZORBAX Eclipse XDB-C18 column (150 × 4.6 mm i.d., 5 μm, Agilent Technologies Canada Inc.). Eluting peaks were detected at wavelengths of 214 nm with a 10 nm bandwidth and against a reference signal at 300 nm with a 10 nm bandwidth using Chemstation LC 3D system software (Agilent Technologies Canada Inc.). The injection volume was maintained at 10 μL for all investigations. The mobile phase consisted of a gradient of water–acetonitrile as detailed previously.[20]

Optical Microscopy. The microstructure of selected oil body suspensions was determined using optical microscopy (Nikon microscope Eclipse E400, Nikon Corp., Japan). Oil body suspensions were gently agitated in a glass test tube before measurement to ensure that they were homogeneous. A drop of the oil body suspension was then placed on a glass slide and observed under the microscope at a magnification of 400×. Images of oil bodies were acquired using digital image processing software (Micro Video Instruments Inc., Avon, MA, USA). Optical microscopy measurements were made after the oil body suspensions were stored for 24 h at room temperature.

Statistical Analysis. All statistical analyses were conducted using the Statistical Analysis System (SAS for Windows, release 9.2, SAS Institute Inc., Cary, NC, USA). One-way ANOVA was used to analyze the differences of cyclolinopeptides among different commercial flaxseed oils and among different processing treatments. Post hoc multiple-comparison test was Tukey's test. Differences were considered to be significant at $p < 0.05$. All results were expressed as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Three procedures were used to process flaxseed in this study. The purpose was to compare the impact of the processes on the fate of the peptides. The first method of processing seed was a noncommercial separation of seed into fractions

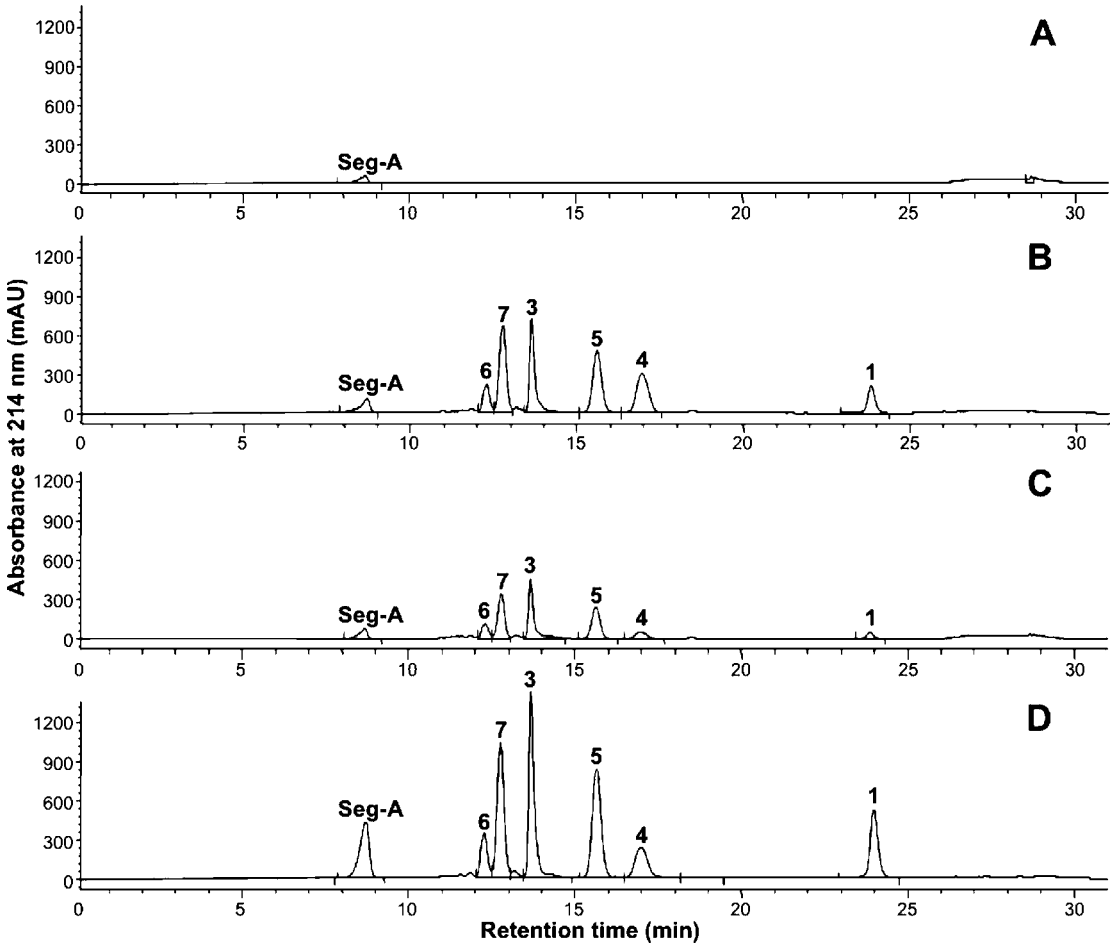

Figure 1. HPLC chromatograms of cyclolinopeptides from flaxseed fractions: (A) mucilage; (B) cotyledon; (C) seed coat; (D) whole seed.

representing gross anatomical structures. In this process seed was first degummed using water, and then gums, seed coats, and seeds were separated, dried, and extracted with acetone. This process provided information on the possible location of the peptides in the plant tissues. In a second process that emulated an existing commercial process,[23] oil bodies were isolated from whole flaxseed following seed hydration. A third process involved a more conventional cold pressing of the seed with a small expeller press. Oil extracted by pressing was subsequently refined, and the fate of the peptides during oil refining was followed.

Levels of Cyclolinopeptides in Flaxseed Fractions. Flaxseed used in this trial had a total cyclolinopeptide concentration of 268.3 μg/g (Table 1), which was similar to 272.9 μg/g reported previously in CDC Bethune cultivars.[20] Mucilage was removed from 10.0 g of flaxseed by soaking the flaxseed in hot distilled water. The solution was freeze-dried, yielding 0.7 g of mucilage. A seed coat fraction and seed coat free seed were separated from the water-degummed flaxseed (Figure S2 of the Supporting Information). The concentration of cyclolinopeptides in each fraction is presented in Table 1. No cyclolinopeptides were detected by HPLC analysis of mucilage extracts (Figure 1A). Cyclolinopeptides are comparatively more hydrophobic than the flaxseed mucilage, which is a hydrophilic mixture of polysaccharides composed of rhamnose, fructose, arabinose, xylose, galactose, and galacturonic acid.[24,25] Cyclolinopeptide B (**2**) was not detected in any samples, probably because of the oxidation of the methionine to methionine sulfoxide (Table 1 and Figure 1). This oxidation is equivalent to the oxidation of reduced cyclolinopeptide **5** to its sulfoxide form noted in bottled flaxseed oil by Brühl et al.[11] The concentrations of cyclolinopeptides **1** (25.2 μg/g), **3** (98.8 μg/g), **4** (13.1 μg/g), **5** (80.2 μg/g), **6** (19.6 μg/g), **7** (55.0 μg/g), and overall cyclolinopeptides (291.9 μg/g) were higher in the cotyledon than in the seed coat fraction, in which the concentrations were **1** (19.3 μg/g), **3** (31.0 μg/g), **4** (15.6 μg/g), **5** (30.7 μg/g), **6** (6.7 μg/g), and **7** (19.8 μg/g), respectively, giving a total cyclolinopeptide concentration of 123.1 μg/g. The prevalence of cyclolinopeptides in the cotyledon fraction could be explained if the peptides are present in oil storage bodies as cotyledons are the major location for oil storage. The cyclolinopeptides observed in the seed coat fraction were probably contributed by the endosperm attached to the seed coats, which also contributed oil. Overall, cyclolinopeptide recovery from seed coats and cotyledons was 75.1% of the whole seed amount. The loss could be explained by incomplete extraction, systematic errors, matrix effects, and loss of material during processing.

The observation that the majority of cyclolinopeptides 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 structures in flaxseed. The diameter of oil bodies isolated from flaxseed was approximately 1.3 μm, and the major components were triacylglycerol (97.7%), protein (1.3%), phospholipid (0.9%), and FFAs (0.1%).[26] The diameter of oil bodies obtained in this study

varied from 0.5 to 2.0 μm, with an average of 1.0 μm, which is consistent with the literature (Figure 2). A brownish crude oil body pad was isolated from flaxseed by homogenization and centrifugation. Repeated homogenization and centrifugation (six times) of the oil body pad substantially reduced contaminants. The resulting fractions were freeze-dried, yielding oil body, supernatant, and pellet fractions. Subsequently, the levels of cyclolinopeptides were measured by HPLC (Figure 3 and Table 2). Peptide containing reduced methionine such as cyclolinopeptide **2** was not found in any of the fractions. After the initial homogenization/centrifugation treatment, higher concentrations of cyclolinopeptides were detected in crude oil bodies (643.8 μg/g) than in dried supernatant (99.6 μg/g) or dried pellet (15.7 μg/g). After five more homogenization/centrifugation treatments, the concentration of cyclolinopeptides was reduced in the crude oil body fraction (153.1 μg/g). The overall recovery of cyclolinopeptides from oil bodies, dried supernatant, and dried pellet was 93.0% after the first homogenization/centrifugation and was reduced to 28.0% after the sixth homogenization/centrifugation. All of the cyclolinopeptides **3**, **5**−**7** and 68% of **1** originally present in isolated oil bodies were removed by homogenization/centrifugation, whereas cyclolinopeptide **4** remained. The lower polarity of cyclolinopeptides **1** and **4** might explain their presence in oil bodies when other cyclolinopeptides with higher polarity were redistributed into the aqueous layer during centrifugation. It is possible that cyclolinopeptides were stored in oil bodies along with triacylglycerols before processing and removed from oil bodies by homogenization/centrifugation, but further confirmation of this is not possible at this time.

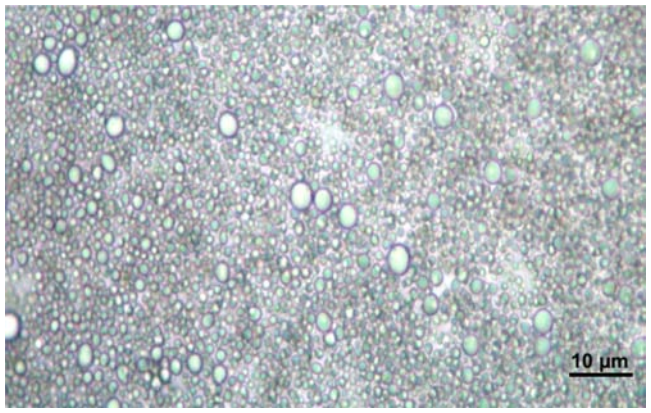

Figure 2. Micrograph of oil bodies after oil body isolation from flaxseed; scale bar represents 25 μm.

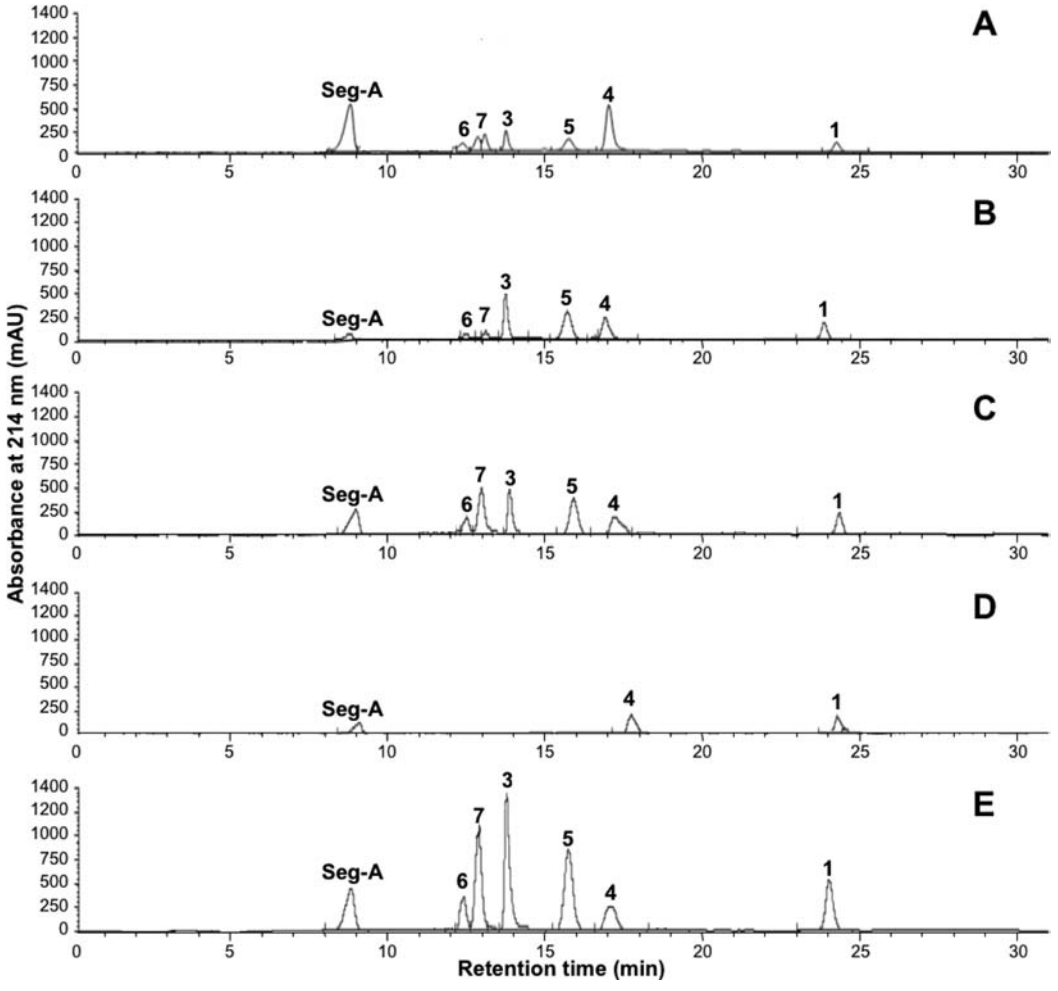

Figure 3. HPLC chromatograms of cyclolinopeptides in fractions derived by aqueous processing of flaxseed: (A) pellet; (B) supernatant; (C) oil body-1 (oil bodies isolated after one homogenization/centrifugation); (D) oil body-2 (oil bodies isolated after six homogenizations/centrifugations); (E) whole seed.

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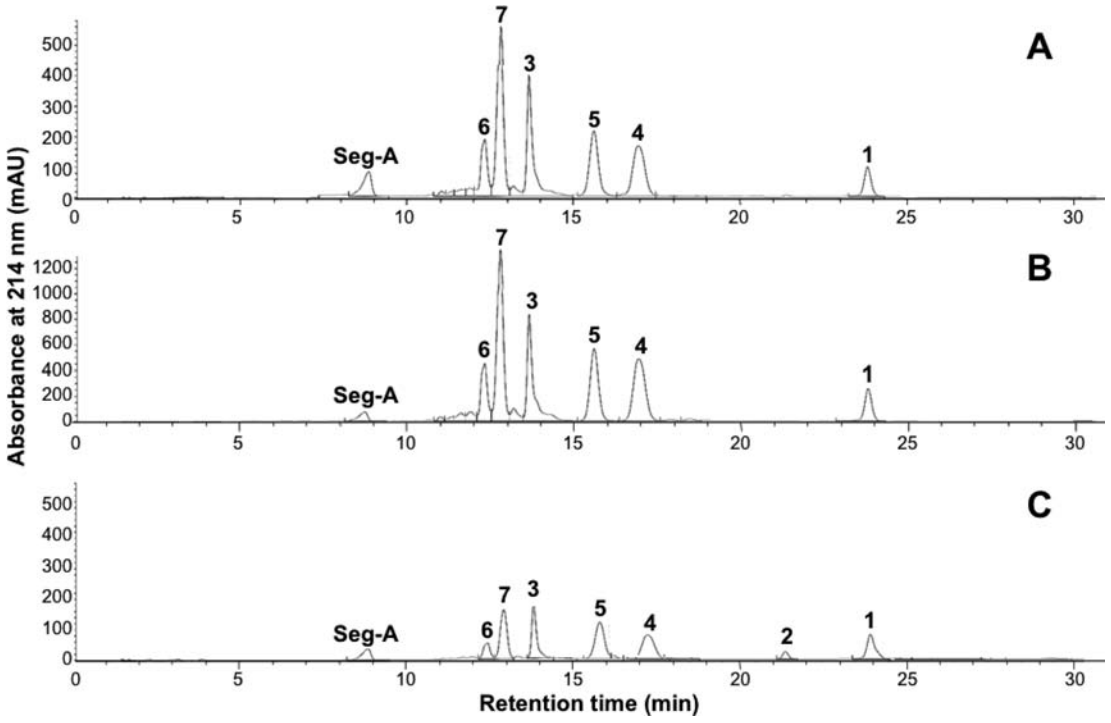

Figure 4. HPLC chromatograms of cyclolinopeptides from flaxseed products after processing: (A) meal; (B) sediment; (C) crude oil.

Cyclolinopeptide Distribution after Expeller Pressing. The extraction of natural products along with vegetable oil occurs during oilseed pressing.[27] Phospholipids, phytosterols, and tocopherols are all found in crude oil, and it is often necessary to remove these compounds from triglyceride oils with further refining to produce vegetable oil of acceptable quality.[28] Crude oil (346.3 g) and flaxseed meal (575.4 g) were obtained after cold pressing of 1.0 kg of flaxseed. The weight loss observed (43.7 g) was likely due to residual material remaining in the expeller after pressing. The oil yield by cold pressing of flaxseed (34.6%) was in agreement with a prior study.[29] The oil with suspended solids was allowed to settle overnight in a 500 mL graduated cylinder to separate suspended solids, commonly found in flaxseed oil after pressing, and clarified oil. Sediment was separated from oil by filtration under vacuum (Figure S3 of the Supporting Information), producing 54.1 g of solids. The filtered oil was combined with clarified oil from the upper layer after settling to obtain oil (343.9 g) that was used for further studies of cyclolinopeptide levels. The concentrations of cyclolinopeptides in crude oil after settling to remove sediment and meal are shown in Figure 4 and Table 3. Cyclolinopeptide **3** was detected in crude oil (99.0 μg/g) but not in other fractions. Cyclolinopeptides **1**, **3**−**5**, which had lower polarity than other peptides on the basis of their later emergence from reverse phase chromatography, were found in greater concentration in crude oil (466.7, 368.6, 227.5, and 462.7 μg/g, respectively), whereas the concentrations of cyclolinopeptides **6** (92.2 μg/g) and **7** (266.2 μg/g), which had relatively higher polarity, were higher in the sediment. The concentration of cyclolinopeptides in meal was relatively low (total = 99.0 μg/g), which was expected after cold pressing as the lipophilic nature of cyclolinopeptides would promote accumulation in the oil during processing. It is worth noting that the total concentration of cyclolinopeptides found in crude oil, meal, and sediment from flaxseed (780.1 μg/g) was higher than the amount of cyclolinopeptides recovered from flaxseed by Goldfisch extraction (371.2 μg/g). This may result from improved extraction of cyclolinopeptides after the applied shear force and heat generated from screw-pressing. Oxidation has been detected in the different stages of vegetable oil processing.[27,30,31] Additionally, storage results in the development of bitter flavor in flaxseed oil. Although the breakdown of α-linolenic acid was suspected as the cause of the unpleasant flavor,[31] Brühl et al.[11] isolated the bitter compound from stored flaxseed oil and proved that oxidation of a cyclolinopeptide to **5** produced the observed bitterness. Expeller pressing efficiently removed peptides from the whole seed as the recovery of peptides was greater in expeller pressed oil (0.3439 × 1972.5 = 678.3 μg/g) than was recovered by solvent extraction of whole seed (268.3 μg/g). The conditions of expeller pressing are more efficient at recovering peptide from seed.

Levels of Cyclolinopeptides in Freshly Prepared Crude Flaxseed Oil and Commercial Flaxseed Oils. The levels of cyclolinopeptides varied among five brands of flaxseed oil obtained from a retail outlet (Table 4). All retail samples had lower peptide levels than flaxseed oil prepared in the laboratory, where **1** (454.0 μg/g), **2** (90.5 μg/g), **3** (358.3 μg/g), **4** (214.1 μg/g), **5** (445.5 μg/g), **6** (82.4 μg/g), and **7** (249.7 μg/g) were found. Brand I had the highest amount of cyclolinopeptide **4** (179.7 μg/g) and the bitter peptide, **5** (293.6 μg/g), among five commercial flaxseed oils. Brand IV had the highest levels of cyclolinopeptide **2** (122.0 μg/g) among all of the flaxseed oils tested, whereas **2** was not detected in brands III and V. Crude oil had the highest concentration of total peptide (1894.6 μg/g) followed by brand I flaxseed oil (1260.9 μg/g), whereas brands II, III, IV, and V had lower overall peptide concentrations (798.6, 656.1, 797.2, and 717.8 μg/g, respectively). 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 these processing operations on cyclolinopeptide removal are described below. Additionally,

it was observed that the reduced cyclolinopeptide **2** was present in crude oil and retail flax oil samples. This result suggests that these products were less oxidized during processing. These samples were not extracted from solids, and perhaps this reflects that oxidation occurred during solid extraction.

Effects of Acid Degumming on Flaxseed Oil Cyclolinopeptides. Crude flaxseed oil contains minor components such as phospholipids, FFAs, and metal-containing compounds.[32] Concentrations of minor constituents of vegetable oils, such as phospholipids, phytosterols, tocopherols, and phytosterol esters, are reduced during refining processes.[33] Degumming of crude soybean oil removed 76.4% of phosphorus, 73.1% of iron, and 51.4% of FFAs.[27] The phosphorus in degummed flaxseed oil was reduced from 325 to 0.5 mg/kg without obvious loss of sterols and tocopherols.[32,34] Other minor compounds from vegetable oil were also partly removed by acid degumming. For instance, 1.1% of oryzanol was removed from degummed rice bran oil.[35] Acid degumming with aqueous H_3PO_4 effectively removed cyclolinopeptides from crude flaxseed oil (Table 5). Degumming by the addition of 1% (v/v) of 75% H_3PO_4 to flaxseed oil removed all of the peptides (method a). Lower concentrations of acid are commonly used in industrial processes for degumming. Sullivan[36] reported the use of 0.13−0.53% of 75% H_3PO_4 in an industrial process. Two-stage degumming was tested using two treatments with just 0.1% (v/v) of 75% H_3PO_4 to mimic an acid-conserving degumming protocol (method b). The two-stage acid degumming treatment removed all of cyclolinopeptides **2**−**7**, leaving trace amounts of **1** (8.2%) and **5** (5.5%) in the crude oil. Acid degumming with a lower concentration of acid [1% (v/v) of 50% H_3PO_4] removed all peptides from the oil except cyclolinopeptide **1** (42.6%) (method c).

Phospholipids found in crude soybean oil are not dissolved but are mostly in micelles that encapsulate sugars and metals.[37] Nonhydratable phospholipids, phosphatidic acid, and part of the phosphatidylethanolamine are present as Ca^{2+} and/or Mg^{2+} salts, which were removed by addition of strong acid, at elevated temperature, to the crude oil.[36,38] Acid degumming using H_3PO_4 proved to be an effective way of removing cyclolinopeptides from crude flaxseed oil. The absence of cyclolinopeptides after degumming may indicate that cyclolinopeptides are trapped in phospholipid micelles that are removed during acid degumming treatment. It is also possible that cyclolinopeptide solubility in oil may require binding to metals that are also removed by acid degumming. These observations may also explain why cyclolinopeptides were primarily found in the dried supernatant of the oil body 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 safe and practical approaches for cyclolinopeptide removal from flaxseed oil as they are commonly used to remove the phospholipids from crude oil in industrial oil refining.

Cyclolinopeptides in Residues after Acid Degumming. The gum/phosphoric acid residues from acid degumming treatments were tested to determine cyclolinopeptide levels (Table S2 of the Supporting Information). Cyclolinopeptide **2** was not found in the gum/phosphoric acid. The yield of cyclolinopeptides from extracted gum/phosphoric acid varied by peptide type. Cyclolinopeptides **1**, **3**, and **5** were recovered with reasonable efficiency (52.5, 69.8, and 52.4%, respectively). The Trp-containing peptides **4**, **6**, and **7** were recovered in low yields of 0, 16.2, and 9.6%, respectively. The partial loss of these peptides and the absence of cyclolinopeptide **4** might be caused by hydrolysis under strongly acidic conditions, the loss of peptides on filter paper, or fractionation of peptides into the hexane and oil layers. The recovery of cyclolinopeptides from gums/phosphoric acid required significant amounts of solvent, and the method was laborious. Recovery procedures could be improved in the future by using liquid−liquid partitioning of cyclolinopeptides in different solvents.

Effect of Alkali Refining on Cyclolinopeptides in Flaxseed Oil. The solubility of nonpolar peptides in oil and organic solvents was reported by Iqbal and Balaram.[39] A nonpolar 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. FFAs are amphiphilic compounds and may have the potential to stabilize cyclolinopeptides in flaxseed oil or even increase their oil solubility. These compounds may be removed from oil by alkali refining.[40,41] Alkali refining reduced FFAs from 0.74 to 0.02% in crude soybean oil.[24] The FFAs of flaxseed oil were neutralized by a number of alkali treatments to evaluate the impact of alkali refining on cyclolinopeptides in oil. HPLC data suggested that the alkalinity and chemistry of the alkali used in alkali refining influenced cyclolinopeptide removal from flaxseed oil (Table 6). All alkaline solutions removed substantial amounts of cyclolinopeptides. However, none of them removed all of the peptides. The stronger alkalis, NaOH, KOH, K_2CO_3, and Na_2CO_3, appeared to be more effective at removing cyclolinopeptides **4**, **6**, and **7**, whereas $NaHCO_3$, K_3PO_4, and Na_3PO_4 removed only cyclolinopeptides **4** and **6**. The total cyclolinopeptides remaining after NaOH, KOH, K_2CO_3, Na_2CO_3, $NaHCO_3$, K_3PO_4, and Na_3PO_4 alkali refining were 13.8, 16.1, 18.9, 20.0, 22.2, 28.0, and 37.8%, respectively. This experiment showed stronger alkalis were more efficient than weaker ones (NaOH = KOH > K_2CO_3 = Na_2CO_3 > $NaHCO_3$ > K_3PO_4 > Na_3PO_4) at cyclolinopeptide removal. The removal of only a portion of the cyclolinopeptides during alkali refining process might be caused by (1) a change in the solubility of cyclolinopeptides in the presence of ions, (2) a change in the solubility of cyclolinopeptides in the presence of soap, and/or (3) release of cyclolinopeptides from FFAs.

Conclusions. The current project investigated (1) the distribution of cyclolinopeptides in different parts of the flaxseed, (2) the concentration of cyclolinopeptides in laboratory-pressed flaxseed crude oil and commercial flaxseed oils, and (3) the effects of acid degumming and alkali refining on the level of cyclolinopeptides in cold-pressed flaxseed oil. A systematic method for cyclolinopeptide extraction, isolation, separation, detection, and identification was developed that increased our knowledge of cyclolinopeptides from flaxseed, including levels in flaxseed and flaxseed fractions, distribution after oil processing, and methods for removing and recovering peptides from flaxseed oil. The presence of cyclolinopeptides in flaxseed ensures that cyclolinopeptides will also be found in flax-related food products (flaxseed oil, flaxseed meal, flaxseed bread). This work also illustrated ways that cyclolinopeptides may be extracted from flaxseed oil, which could lead to large-scale industrial extraction processes. The ability to extract

cyclolinopeptides at a larger scale would allow faster exploration of the potential applications of these molecules and provide the flaxseed industry with potential value-added coproducts.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental figures: structures of cyclopeptides (taken from Gui et al.[20] with permission (Figure S1), flaxseed fractions after water degumming and manual dissection (Figure S2); and products after cold pressing of flaxseed (Figure S3). Supplemental tables: amino acid sequences and chemical formulas of cyclopeptides (Table S1) and cyclolinopeptide recovery from gum/phosphoric acid residues after acid degumming of flaxseed oil (Table S2). Supplemental file: electrospray ionization mass spectrometry spectra of standard cyclopeptides (File S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; MeOH, methanol; Seg-A, segetalin-A; EtOAc, ethyl acetate; FFA, free fatty acids; SD, standard deviation.

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