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Elimination of Saturated Fatty Acids, Toxic Cyclic nonapeptide and Cyanogen Glycoside Components from Flax Seed Oil

Eun-Mi Choi, Jeung-won Kim, Mi-Kyung Pyo, Sung-Jun Jo and Byung Hoon Han*

Life Science Research Institute, E.S. Biotechnology Co. Ltd. Rep. of Korea

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Abstract – Flax seed (*Linseed, Linum usitatissimum L.*) and its oil, a richest source of alpha-linolenic acid (ALA) (ω-3), contain saturated fatty acids, neurotoxic cyanogen glycosides and immuno-suppressive cyclic-nonapeptides. Present paper describes the development of two chemical processes, Process-A and –B, to remove saturated fatty acids and to destroy cyclic nonapeptides and cyanogen glycosides from flax seed oil. Process-A consists of three major steps, i.e., extraction of fatty acid mixture by alkaline saponification, removal of saturated fatty acid by urea-complexation, and triglyceride reconstruction of unsaturated fatty acid via fatty acyl-chloride activation using oxalyl chloride. Process-B consists of preparation of fatty acid ethyl ester by transesterification, elimination of saturated fatty acid ester by urea-complexation, and reconstruction of triglyceride by interesterification with glycerol-triacetate (triacetin). The destruction of lipophilic cyclic nonapeptide during saponification or transesterification processes could be demonstrated indirectly by the disappearance of antibacterial activity of bacitracin, an analogous cyclic-decapeptide. The cyanogen glycosides were found only in the dregs after hexane extraction, but not in the flax seed oil. The reconstructed triglyceride of flax seed oil, obtained by these two different pathways after elimination of saturated fatty acid and toxic components, showed agreeable properties as edible oil in terms of taste, acid value, iodine and peroxide value, glycerine content, and antioxidant activity.

Key words ☐ flax seed oil, urea-fatty acid complex, transesterification, interesterification, cyanogens glycosides, cyclic-nonapeptides

INTRODUCTION

Flax seed oil, known as drying oil in the painting industry, is now noticed as the richest source of ALA (Hettiarachchy et al., 1990; Visjaimohan et al., 2006) which has been reported to reduce effectively the plasma level of cholesterol and triglyceride (Kinsel *et al.*, 1952; Ahrens *et al.,* 1954) and also to improve the blood circulation through the intervention in arachidonate-pathway (Willls, 1981; Hwang, 1980). However, it is also well known that intact fresh flax seed contains such toxic components as cyanogen glycosides (i.e., linamarine) (Irena, 1998; U.S. Department. 1997) and immuno-toxic minor components, cyclic nonapeptides(Terry *et al.*, 1998; Hiroshi Morita *et al.*, 1997). When intact fresh flax seed will be taken by chewing, cyanogen glycosides in the flax seed will release toxic hydrogen cyanide in the gastro-intestinal tract due to the interaction of mixed enzymes linamarinase and nitrile lyase(Harald *et al.*, 1994; Karl, 2004) contained in the fresh flax seed. Hence, taking the fresh flax seed as a food or food supplement has been legally prohibited. Some company in Canada have licenced to produce and supply edible flax seed or flax seed oil after heat treatment(patented) to destroy cyanogen glycosides without giving any harmfull chemical effects to ALA and the company is recommending to take the heat treated whole flax seed by chewing (Canmar Grain). However it was found in our laboratory that the patented heat treatment of flax seed is deactivating only the enzymes linamarinase and nitrile-lyase contained in the intact fresh flax seed but not destroying the cyanogens glycosides. Therefore, it will not give any guaranty for the safety of the edible flax seed or flax seed oil obtained by heat treatment only. In addition, the commercial product of edible flax seed available in market seems to have no consideration for the counter-measure to the immuno-toxic components cyclic nonapeptides. On the other hand, it has been reported that saturated fatty acids, such as palmitic and stearic acids will elevate the plasma level of cholesterol and triglyceride with the twice potency compared to the lowering effect of α-linolenic acid (Hegsted, *et al.*, 1965; Keys, *et al.*, 1957).

*Corresponding author
Tel: +82-41-556-9166, Fax: +82-41-556-916
E-mail: bhhan3312@yahoo.co.kr

Present paper describes experimental details of two chemical pathways developed in our laboratory to remove saturated fatty acids and at the same time to destroy or remove toxic components, cyanogen glycosides and cyclic nonapeptides from the flax seed oil and the reconstruction of triglyceride oil composed of unsaturated fatty acids only. This paper includes also some data for the quality evaluation of the final product obtained by the chemical processes as edible oil.

MATERIALS AND METHODS

Golden species of fresh flax seeds were purchased from Cosmos Co. in Korea, heat treated edible flax seed from Canmar Grain Corp., oxalyl chloride, GLC-standards for fatty acid methyl-ester, bacitracin and ABTS-$(NH_4)_2$ from Sigma-Aldrich Co., linamarine standard from A. G. Scientific Inc. and clinical assay kit for plasma triglyceride from Youngdong Pharm. Co. LTD. in Korea, and M-plate counting-broth from Difco Co. *Micrococcus luteus*(IFO 12708) was donated from Prof. Oh Ki Bong College of Life Science in Agriculture SNU.

1) Extraction of flax seed oil

Two kilograms of fresh flax seed or heat treated edible flax seed were pulverized by mechanical grinding, extracted three times with 5 liter-portions of boiling hexane and distilled hexane to obtain 800 g flax seed oil.

2) Elimination of saturated fatty acids from flax seed oil by Process-A

Process-A is consisting of saponification of flax seed oil, elimination of saturated fatty acids by fractional crystallization of fatty acid-urea complex and reconstruction of flax seed triglyceride oil-A via activation of unsaturated fatty acids to fatty acylchloride.

2-a) Saponification of flax seed oil

Three hundred grams of flax seed oil was mixed with 300 ml of 95% ethanol and 60 g sodium hydroxide in 700 ml water-solution and refluxed in water-bath for 24 hours to ensure complete saponification. The saponified reaction product was acidified by addition of 200 ml Conc. hydrochloric acid and refluxed for 4 hours to destroy cyanogens glycosides contained in the oil as impurity. The ethanol was removed by vacuum-distillation and partitioned with hexane to give 280 g fatty acid mixture.

2-b) Removal of saturated fatty acid by urea-complexation (Christie, 1973)

Two hundred grams of fatty acid mixture and 80 g urea were dissolved in 500 ml ethanol by refluxing in water-bath, and set aside for 24 hours to give crystalline urea complex of saturated fatty acids. The urea-complexes of saturated fatty acids were filtered off to obtain urea complex of unsaturated fatty acid from the filtrate. The filtrate was concentrated to remove ethanol and the resulting oily residue was acidified to decompose the urea-complex. The resulting unsaturated fatty acid was partitioned with hexane to give 160 g unsaturated fatty acid by conventional treatment.

2-c) Reconstruction of triglyceride-A composed of unsaturated fatty acids only

Seventy eight grams (0.62 mole) oxalylchloride was mixed carefully and slowly into a ice-cooled 150 g (0.615 mole) unsaturated fatty acid in the draft and set aside for three days under gentle stirring by magnet stirrer under humidity protection. The reaction starts with vigorous gas evolvement producing oily fatty acyl-chloride. After the cease of gas evolvement, remaining excessive unreacted oxalyl chloride was removed by vacuum distillation in a water-bath (90). Resulting fatty acyl-chloride was reacted with 19.04 g (0.207 mole) anhydrous glycerine under the presence of 48.6 g (0.615 mole) pyridine as acid trap. The reaction product was dissolved in 500 ml hexane and washed successively with d-HCl, sodium-bicarbonate solution and water until the washed water show neutral pH. Hexane layer was dehydrated with 30 g anhydrous sodium sulfate. Finally 145 g of reconstructed triglycerides composed of unsaturated fatty acids including oleic, linoleic and linolenic acid were obtained upon the evaporation of hexane. NMR 300 MHz(TMS, $CDCl_3$, δ) ; 0.96(methyl, 9H, t, J = 7.5Hz), 1.296 (aliphatic methylenes, 36~38H, br. s,), 1.497(carboxyl adjacent methylene, 6H, t, J = 7.5Hz), 2.046(olefine adjacent methylene, 12H, m), 2.302(=CHC$\underline{\mathbf{H}}_{\underline{\mathbf{a}}}$$H_b$CH=, 6H, m), 2.795(=CHC$H_a$$\underline{\mathbf{H}}_{\underline{\mathbf{b}}}$CH=, 6H, m), 4.136(glyceryl C$\underline{\mathbf{H}}_{\underline{\mathbf{a}}}$$H_b$O-,2H, m, J = 6.0Hz,, 16Hz), 4.287(glyceryl C$H_a$$\underline{\mathbf{H}}_{\underline{\mathbf{b}}}$O-,2H, m, J = 6.0Hz,, 16Hz), 5.259(glyceryl CH_2(O-)C$\underline{H}$(O-)CH_2(O-), 2H, m, J = 6.0Hz, 16 Hz), 5.35(olefinic, =CH-, 14~16H, m) The structural assignments of the above proton peaks are well accorded to the compositions(GLC-data) of unsaturated fatty acids of finally reconstructed triglyceride

3) Elimination of saturated fatty acids from flax seed oil by Process-B

Process-B is consisting of transesterification of flax seed oil,

elimination of saturated fatty acid-alkylesters by fractional crystallization of fatty acid alkylester-urea complex and reconstruction of flax seed triglyceride oil-B by interesterification

3-a) Preparation of fatty acid-ethyl ester from flax seed oil by transesterification

Vacuum dried flax seed oil (50 g) was mixed with 500 ml absolute ethanol and 250 μl 20% sodium-ethoxide solution and mixed well for 5 hours by magnetic stirring. Complete reaction of transesterification could be checked by the disappearance of interphase of two liquid phases. At the end of transesterification the catalytic activity of sodium methoxide was destroyed by the addition of equivalent amount (70 μl) of glacial acetic acid.

3-b) Elimination of saturated fatty acid ethyl ester by urea complexation

To the above reaction mixture (transesterification), 80 g urea was dissolved by refluxing in water bath and set aside for one night at room temperature to crystallize urea complex of saturated fatty acid ethyl-ester. Crystalline urea complex was removed by filtration. The absolute ethanol could be recovered for next use by vacuum distillation of the filtrate. Unsaturated fatty acid-ester could be obtained by conventional treatment shown in experiment-3).

3-c) Reconstruction of triglyceride-B composed of unsaturated fatty acids by interesterification

The mixture of 33 g ethyl-ester of unsaturated fatty acids, 8.0 g triacetin (glycerine- triacetate) and 320 μl of 20% sodium ethoxide was heated in 95~110°C silicon-oil bath and ensured the interesterification reaction through the removal of ethyl-acetate by vacuum distillation under reduced pressure <50 mmHg). After the cease of gas evolution of ethyl-acetate, sodium ethoxide in the reaction mixture was neutralized by d-HCl and diluted with water and partitioned to hexane. The hexane layer was washed with d-$NaHCO_3$ and water to give 42.1 g triglyceride oil composed of unsaturated fatty acids. by conventional treatment.

4) GLC-analysis of fatty acid composition

Twenty microliters of flax seed oil or reconstructed triglyceride oil were mixed with 100 μl 28% sodium methoxide in MeOH and mixed well with 100 μl hexane and centrifuged for 10 minutes at 12000 rpm. Five microliters hexane-layer was taken and diluted with 95 μl hexane. Ten microliters portion of diluted hexane layer was injected for the gas-chromatographic analysis. Gas-chromatograpic conditions; Hewlett-Packard 5890-II Series (HP Co., Wilmington, DE, USA), detector; FID, coloumn; DB-23 capillary(60 m, 0.25 mm ID, 0.25 um), temp; oven; initial 130°C, programmed 2.7/min. to final 230°C, injector; 270°C, carrier gas; nitrogen, flow rate; 30 ml/min. The results of GLC-analysis for untreated flax seed oil and reconstructed triglyceride are tabulated in Table I.

5) Indirect evidence for the degradation of cyclic-nonapeptides in a reaction condition of saponification or transesterification: Bioassay for antibacterial activity of bacitracin by using *Micrococcus luteus* culture (Jorgensen, and Sahm, 1995 and Lorian, 1996)

A cyclic-decapeptide antibiotic bacitracin was adopted as the substitute for the cyclic-nonapeptide in flax seed oil, and treated it with the same reaction condition of saponification or transesterification as followings.

5-a) Akaline hydrolysis of bacitracin in saponification mixture

Saponification mixture, prepared by dissolving five mg of bacitracin, 3 g of flax seed oil and 500 mg sodium hydroxide in 8 ml 50% ethanol was sealed in glass ampoule and heated for 4 hours in a boiling water-bath. The reaction product was diluted with 40 ml water, acidified to pH 4.0 by the addition of d-HCl and the resulting fatty acid was removed by exhaustive extraction with hexane. The volume of resulting water layer was adjusted to 50 ml after neutralization to make test solution-A (final Conc. of bacitracin; 0.1 mg/ ml) to check the hydrolytic degradation of bacitracin.

5-b) Negative and positive control samples

Negative control sample was prepared as above a), but omit-

Table I. GLC-anaylysis of fatty acid compositions of fresh flax seed oil and reconstructed triglyceride (%)

	Palmitic Me-ester C16:0	Stearic Me-ester C18:0	Oleic Me-ester C18:1,n9	Linoleic Me-ster C18:2,n6	Linolenic Me-ester C18:3,n3
Fresh flax seed oil	4.8	4.8	21.60	15.30	53.50
Reconstructed triglyceride	0.0	0.0	7.66	16.52	75.81

ting bacitracin only to make test solution-B. To prepare positive control, the saponification mixture was neutralized by the addition of d-HCl in advance to the bacitracin addition and treated same way as above 5-a) to make test solution-C (final bacitracin concentration; 0.1 mg/ ml).

5-c) Assay of IC_{50} value

Test solution-A, -B and –C were diluted with sterile water two-fold sequentially to give final assay sample solutions having final bacitracin concentration of 100, 50, 25, 12.5, 6.25, 3.13, and 1.67 ug/ml respectively. Each 20 μl of assay sample solutions or sterile water were pipetted on 96-well plate and 180 μl of *Micrococcus luteus* , cultured in advance in the M-plate broth for 5 hours, were pipetted on each well and cultured for 24 hours at 37°C. The growth of *Micrococcus luteus* were quantified by turbidity-assay at 630 nm on the ELISA Reader (TECAN, GENios Austria). IC_{50}-values were calculated based on the difference of cell growth between test solution-A and –C, and the results are tabulated in Table II.

6) Assay of cyanogen glycosides

In order to assay cyanogen glycoside contents in flax seed or flax seed oil, the assay samples were treated with mixed enzymes linamarinase and nitrile-lyase to liberate hydrogen cyanide. The resulting hydrogen cyanide was visualized by chloro-succinimide-pyridine-barbiturate procedure for the spectrophotometric assay by the absorbancy at 580 nm (John, 1997; Merina, 1997) as followings.

6-a) Enzyme solution (mixed enzymes of linamarinase and nitrile lyase)

Ten grams of acetone powder prepared from the homogenate of fresh flax seed (Merina, 1997) was suspended in 40 ml of 0.1M-acetate buffer (pH. 7.0) and dialyzed repeatedly against acetate buffer at 4°C to remove completely any cyanogens glycosides contained in the acetone powder and the dialysate was centrifuged to obtain clear supernatant. The supernatant was freeze-dried to obtain crude enzyme powder of mixed enzymes. Fifty milligrams of crude enzymes were dissolved in 5 ml distilled water to use as the enzyme solution.

6-b) Preparation of assay samples

Twenty grams of powdered fresh flax seed was extracted with 100 ml hexane by refluxing 3 hours in a boiling water-bath and evaporated to give 6 g flax seed oil (sample-1). The residue after hexane extraction was extracted with 100 ml portions of methanol by refluxing for 4 hours in a boiling water-bath, to give 1.1 g methanol extracts (sample-2). Heat treated edible flax seed was also treated as same way to give hexane extract (sample-3), and methanol extract (sample-4) respectively. Two hundred milligrams of hexane extract were saponified as previously by semi-micro scale process, and acidified with d-HCl to remove fatty acids by hexane extraction. The resulting water layer was neutralized with d-NaOH and adjusted to 10 ml (Sample-1 and –3). Seventy five milligrams of methanol extracts (sample-2 and –4) were mixed well with 500 μl water, centrifuged for 5 Min. at 12000 rpm to give supernatant and 50 μl of supernatants were taken for cyanide-assay.

6-c) Assay of cyanogens glycoside content

Fifty microliters of each assay sample solutions or standard linamarine solutions for calibration were added in test tube and 150 μl of enzyme solution was mixed and left 2 hours at room temperature for the enzyme reaction. Hydrogen cyanide liberated was oxidized to give cyanide cation by mixing 5 ml 0.05 M chlorosuccinimide solution dissolved in 0.1 M phosphate buffer (pH. 7.0) and 1 ml of pyridine-barbituric acid solution* to develop red color reaction. After 10 minutes of color reaction, the absorbance at 580 nm was determined. Cyanogen-glycoside contents in each fraction are tabulated in Table III.

*Barbituric acid (12 g) was dissolved in pyridine (60 ml) and 3 ml of c-HCl was mixed carefully under stirring. Final volume was adjusted to 250 ml by dilution with water.

Table II. Determination of IC_{50} value of bacitracin-zinc mixed in flax seed oil before and after saponification to *Micrococcus luteus*

Flax seed oil	IC_{50}
Flax seed oil without bacitracin	>10.00 ug/ml
Bacitracin before saponification	1.32 ug/ml
Bacitracin after saponification	>10.00 ug/ml

Table III. Cyanogen glycoside contents in fresh and heat treated flax seed and their extracts; Flax seed was extracted with hexane and followed by methanol extraction

Samples	Cyanogen glycoside content (mg/gm flax seed as Linamarine)
Sample 1 (hexane ex. of fresh flax seed)	Not detected
Sample 2 (MeOH ex. of fresh flax seed)	1.10 mg
Sample 3 (hexane ex. heat treated edible flax seed)	Not detect
Sample 4 (MeOH ex. heat treated edible flax seed)	0.83 mg

7) Assay of glycerine content in triglycerides

Glycerine content in the reconstructed triglyceride was assayed by using clinical enzyme kit. The clinical kit consisting of lipase, glycerol kinase and glycerophosphate oxidase is suitable for the analysis of plasma lipids. In the preliminary experiment using the clinical kit, the lipase activity in the clinical kit was not enough for the synthetically reconstructed triglyceride samples due to the absence of biological surfactant. Therefore the reconstructed triglycerides were saponfied by chemical process instead of using lipase in the kit. The saponified product was acidified and extracted with hexane to remove fatty acid. The resulting water layer was subjected for glycerine assay by using enzymes in the clinical-kit. The glycerine content in fresh flax seed oil and reconstructed triglyceride is tabulated in Table IV.

8) Quality evaluation of reconstructed triglyceride in respect to edible oil

8-a) Acid value, Iodine-value, and peroxide-value of flax seed oil and reconstructed triglyceride were determined by the methods appeared in Korean Food CODEX.

8-b) Total antioxidant activity of flax seed oil and reconstructed triglycerides

Seven millimoles solution of ABTS[2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] in water was oxidized with 2.45 mM potassium persulfate (final concentration) overnight in the dark room temperature to produce ABTS radical cation (ABTS•+). The working radical cation solution was prepared by dilution with ethanol to give absorbance 0.7~0.72 at 734 nm. Ten microliters sample solutions (each 10% solution of untreated flax seed oil or reconstructed triglyceride oil-A, and -B, and fatty acid–ethyl ester in dichloromethane) were added to 1.0 ml of ABTS working solution of radical cation. After six minutes incubation at 30°C, the absorbance decreases were measured at 734 nm as the index for total antioxidant activity.

Table IV. Quality comparison of flax seed oil and reconstructed triglyceride by their acid value, peroxide value and iodine number and glycerine content

	Untreated flax seed oil	Reconstructed-oil-A
Acid value	2.01	2.90
Peroxide value	0.99	2.67
Iodine number	193.80	211.50
Glycerine content	104.30 ug/mg	101.80 ug/mg

RESULTS AND DISCUSSION

1) Fatty acid composition of flax seed oil and reconstructed triglyceride

Saturated and unsaturated fatty acids bound on same triglceride molecules of flax seed oil were segregated as separate molecules by two different ways in process-A by saponification to obtain free fatty acids and in process-B by transesterification to obtain ethylester of fatty acids. Saturated fatty acids of free acid or ethyl ester forms could be efficiently removed by employing urea-complexation. The reconstruction of triglyceride composed of unsaturated fatty acids in the process-A was successfull via oxalyl chloride activation. GLC-data of both the fatty acid compositions of untreated flax seed oil and reconstructed triglyceride oil are tabulated in Table I. As shown in Table I, saturated fatty acids in the flax seed oil was completely eliminated by urea-complexation in the reconstructed triglyceride-oil-A and –B. Reconstruction of triglyceride starting from ethyl-ester of unsaturated fatty acid in the process-B was very successful by employing inter-esterification reaction with triacetin to produce reconstructed triglyceride-oil-B, in respect to its easy manipulation and to its least possibility of side reaction.

2) Fatty acid compositions and the expected effect on plasma lipid profile of untreated and reconstructed flax seed oil

ALA (ω-3) content was highly enriched from 53% to 75% as shown in Table I, due to total elimination of saturated fatty acids together with a part of oleic acid. When we estimate the balance of beneficial effects of PUFA and deleterious effects of saturated fatty acids of flax seed oil on blood lipid profile including cholesterol level and neutral lipid content, normal flax seed oil will give score 49.6 (sum of the total content of PUFA minus twice of saturated fatty acids content; 53.5 + 15.3 - 2×9.6 = 49.6), whereas the balance for reconstructed triglyceride will give score 92.33 (sum of linoleic acid and ALA content minus saturated fatty acid content; 75.81 + 16.52 – 0 = 92.33). This will imply that elimination of saturated fatty acids

Table V. Total antioxidant activity of flax seed oil and reconstructed triglyceride. Absorbancy decrease of ABTS radical cation at 734nm by the antioxidant components in the oil

Untreated flax seed oil	Reconstructed triglyceride-oil-A	Reconstructed triglyceride-oil-B	Fatty acid-ester
0.532	0.573	0.423	0.514

from flax seed oil will enhance the beneficial effect of PUFA in reconstructed triglyceride oil almost twice of the untreated flax seed oil.

3) Degradation of immuno-toxic cyclic-nonapeptides

Immunotoxic cyclic-nonapeptide is reported to be minor components of flax seed ranging from 0.0002% to 0.0058%, hence the deleterious immunosuppressive activity may be negligible. However it may give some problems, when medication of untreated flax seed or flax seed oil is repeated for long period, due to the accumulation of immunosuppressive acitivity. Cyclic peptides are generally lipophilic, hence a part of immunotoxic cyclic nonapeptide must be present dissolved in flax seed oil. Peptide bond is generally highly susceptible to alkaline-hydrolysis therefore, prolonged heating in saponification mixture will ensure the degradation of peptide bonds in the cyclic-nonapeptide molecules. Degradation of cyclic nonapeptide during the saponification process was verified indirectly in a model experiment using bacitracin-antibiotics a structural analogue cyclic-decapeptide by the disappearance of antibacterial activity due to the saponification reaction. As shown in Table II, IC_{50} values of bacitracin was highly elevated from 1.32 μg/ml before saponification to >10 μg/ml after saponfication (as shown in Table II). This is indirect evidence for the destruction of cyclic nonapeptides in flax seed oil due to saponification. Destruction of cyclic nonapeptide in the transesterification reaction was also verified by same indirect method using bacitracin-antibiotics (data is not shown).

4) Cyanogen-glycoside contents in various fractions of flax seed extract

It was found from the cyanide assay that commercial edible flax seed, a patented product of heat treated flax seed contained cyanogen glycosides as much as fresh flax seed. It was also found that cyanogen glycosides are remaining unextracted in flax seed dregs after hexane extraction. Although the cyanogens glycosides are not destroyed in the heat treated edible flax seed, the enzymes linamarinase and nitrilelyase must be denatured due to heat treatment, hence there will be lesser opportunity of cyanide intoxication. However, these facts imply also that chewing the edible flax seed of patented heat treated product will not ensure the safety in special cases when the intestinal microbial flora will be adversely changed. On the contrary, flax seed oil obtained by hexane extraction may be safe, since it doesn't contain any cyanogen glycosides to be eliminated by chemical process as long as the oil is obtained by hexane extraction.

5) Quality of reconstructed triglyceride

Finally the qualities of the reconstructed flax seed oil were assessed by its acid value, peroxide content, iodine number, fatty acid composition by GLC and peak assignment of proton-NMR-spectra of reconstructed triglyceride. As shown in Table IV, iodine value was highly elevated due to elimination of saturated fatty acids. Peroxide-value was not changed significantly when all the chemical processes were carried out under nitrogen atmosphere and under the protection from light. Acid-value of reconstructed triglyceride was slightly elevated compared to fresh flax seed oil. This slight elevation of acid value of the reconstructed flax seed oil could not be reduced to the level of untreated flax seed oil by repeated washing with water and sodium bicarbonate solution. Glycerine contents of untreated flax seed oil and reconstructed flax seed oil showed almost same value with experimental error level deviation.

6) Total antioxidant activity of fresh and reconstructed flax seed oils

ABTS radical cation quenching activities were assayed respectively for the fresh flax seed oil, reconstructed triglyceride-oil-A and-B and ethyl ester of unsaturated fatty acid.

As shown in Table V unsaturated fatty acid ester and reconstructed triglyceride-oil-A give elevated anti-oxidant activity compared to untreated flax seed oil probably due to selective elimination of saturated fatty acids by urea complexation. Triglyceride-oil-B showed slightly reduced antioxidant activity probably due to the combined effects of elevated temperaturere and exposure to light and air during interesterification reaction. Reaction temperature of interesterification could be decreased to lower than 90°C when the amount of sodium ethoxide catalysts was slightly increased. In other experiments on process-B under sufficient protection from light and air the peroxide value and antioxidant activity of reconstructed triglyceride-oil-B could be highly improved almost to the normal level of untreated flax seed oil as appeared in the Table V.

7) Industrial applicability of Process-A for edible oil production

Process-A consists of three steps, i.e., a) saponification, b) elimination of saturated fatty acids by urea complexation and c) glyceride formation via acid chloride formation by using very reactive oxalyl-chloride. Oxalyl-chloride is so reactive that it is

not included in the GRAS-list (U.S.-FDA-list of Generally Recognized As Safe for Food), hence process-A will not be recognized as a suitable method for the industrial production of edible oil. Actually oxalylchloride is so reactive that it was classified as toxic substance, however there will be no remaining toxic residues after the reaction with fatty acid as exemplified by the following reaction equation.

$$RCOOH + (COCl)_2\ RCOCl + CO + CO_2$$

Sometimes excessive amount of oxalylchloride may be used to finish up reaction. However the excessive oxalylchloride can be completely removed by vacuum distillation. A minor part of oxalylchloride may react with phenolic antioxidant components in the fatty acid fraction destroying the antioxidant activity. However the decrease of antioxidant activity was not observed in the reconstructed triglyceride oil produced by process-A as shown in Table V. In some special case, some unsaponifiable substance in the fatty acid fraction may produce oxalyl-ester. Considering the above special case, a small amount of reconstructed triglyceride-oil-A was saponified completely, and oxalic acid fraction could be obtained by acidification followed by hexane extraction. Oxalic acid in water layer was titrated with 0.1N-potassium permanganate solution, and finally we could have a conclusion that the reconstructed triglyceride-oil-A containing a negligible amount of oxalyl-ester as minor component. There may be also some possibility of oxalylester formation from the phenolic antioxidant contained in the fatty acid fraction as an minor components. If this is true, the antioxidant activity of reconstructed triglyceride oil-A produced by process-A should give decreased value compared to that of untreated flax seed oil. As shown in Table V, the antioxidant activity of reconstructed triglyceride oil-A showed rather elevated value. This fact may be another additional evidence that formation of oxalyl ester of phenolic antioxidant may not be occurred. However it will be necessary to have some safety tests by using animal model for the reconstructed triglyceride-oil-A.

7) Industrial applicability of process-B for edible oil production

Process-B is consisting of three steps, i.e., a) transesterification, b) elimination of ethylester of saturated fatty acids by urea complexation and c) glyceride formation through interesterification. This process does not use any harmful reagents, since both urea and triacetin are found in the GRAS-list. Furthermore all three reactions including transesterification, urea complexation and interesterification are customarily employed in the food-chemical industry in order to modify the physical natures of edible vegetable oils.

CONCLUSION

A new type of flax seed oil devoid of deleterious components as saturated fatty acid, immunotoxic cyclic-nonapeptides and neurotoxic cyanogen glycosides was created by a few step of chemical process starting from fresh flax seed oil. These chemical reactions will not alter the chemical nature of the flax seed oil except that saturated fatty acid and two toxic substances were removed. The ALA (ω-3) content of reconstructed triglyceride oil of flax seed was highly enriched from 53% to 75% compared to untreated flax seed oil. The improving effect of reconstructed triglyceride of flax seed oil on plasma lipid profile may be enhanced twice compared to that of untreated flax seed oil due to elimination of saturated fatty acids. This reconstructed flax seed oil must be highly recommendable source of ALA (ω-3) compared to other source as fish oil, since this new product doesn't contain any deleterious components as saturated fatty acid and cholesterol and doesn't have disagreeable persisting smell as fish oil. Combination of three already known chemical processes in the process-A; 1) saponification, 2) elimination of saturated fatty acid by urea complexation and 3) conventional glyceride synthesis and in process-B; 1) transesterification, 2) elimination of saturated fatty acid by urea complexation and 3) interesterification with triacetin are all of necessary chemical processes for the elimination of saturated fatty acids and toxic components.

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