PROTEIN PRODUCTS AND SPROUTS FROM FLAXSEED (Linum usitatissimum L.)

JANITHA PRIYAKANTHI DEEPANI WANASUNDARA

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PROTEIN PRODUCTS AND SPROUTS FROM FLAXSEED (*Linum usitatissimum* L.)

BY

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ABSTRACT

Polysaccharides of flaxseed coat (flax mucilage) were removed by soaking of whole seeds in water or sodium bicarbonate solution or treatment with commercially-available carbohydrases. The extent of polysaccharide removal was determined by monitoring the concentration of total sugars and pentoses as well as viscosity values of the aqueous polysaccharide extracts prepared from treated flaxseed. A direct relationship existed between the amount of seeds dispersed in the extraction medium and the total contents of sugars and pentoses or viscosity values (correlation coefficients of 0.957, 0.974 and 0.921, respectively). These indicators served well in quantitating the content of polysaccharides in aqueous extracts. Soaking in a 0.10 M sodium bicarbonate solution for 12 h removed most of the mucilage from seed coats. Treatment of seeds with Viscozyme® L (22.5 mg protein/100 g seeds) for 12 h removed more polysaccharides from seed coat than Pectinex™ Ultra SP or Celluclast® 1.5L under similar conditions. Scanning electron microscopy provided evidence that polysaccharides were removed from seed coats by pre-treatments employed. Nitrogen solubility and protein recovery from meals of mucilage-reduced seeds was improved since there was less interference from seed coat polysaccharides which were co-extracted with proteins. Sodium hexametaphosphate-assisted protein isolation from mucilage-reduced seeds was optimized under laboratory conditions for pH, meal-to-solvent ratio and concentration of sodium hexametaphosphate using response surface methodology with a central composite rotatable design. Maximum nitrogen solubility and protein recovery were obtained at pH 8.89 and 9.04, meal-to-solvent ratio of 1:33.5

and 1:33 and sodium hexametaphosphate concentration of 2.75 and 2.85%, respectively. The prepared flax protein isolate contained 78% protein. Lysine was the first limiting amino acid of the protein isolate. The computed protein efficiency ratio and biological value indicated a reasonably good quality of protein in the isolate. The isolate contained elevated levels of phytic acid and total phosphorus. Electrophoretic results showed that the isolate was composed of total proteins of flaxseed.

The isolated flax proteins were acylated with acetic or succinic anhydride. Approximately 84.5 and 56.9% of the free amino groups of protein isolates were acylated when 0.2 g anhydride/g protein was used. The colour and solubility of the protein isolate were improved due to acylation. Emulsifying activity and stability were enhanced by succinylation, but acetylation had a negative influence. Furthermore, foaming ability and fat binding capacity of succinylated products were less than those of their acetylated counterparts. Acylation reduced the *in vitro* digestibility of flax proteins.

In another study flaxseeds were germinated for 8 days. Germinated seeds had lowered content of dry matter due to the use of their reserve components as an energy source for the developing seedlings. The crude protein content did not show a significant ($p<0.05$) change but the content of non-protein nitrogen compounds including free amino acids as well as agmatine and spermidine increased during the germination period. The *in vitro* digestibility was lowered but the calculated values for protein efficiency ratio and biological value of seeds did not change upon germination.

Lipid content of seeds was reduced by 58% during germination but their fatty acid composition did not change. The content of neutral lipids, accounting for 96% of total flax lipids, was significantly ($p<0.05$) decreased while those of glyco and phospholipids increased. There was also a decrease in the content of triacylglycerols and an increase in the diacylglycerols, monoacylglycerols and free fatty acids of the neutral lipid fraction. The content of phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine in the phospholipid fraction was decreased while that of phosphatidic acid and lysophosphatidylethanolamine increased significantly ($p<0.05$) due to germination. The quantities of raffinose and sucrose in the seeds were reduced while those of fructose and glucose increased after germination. The mineral composition of the seeds was not affected by germination. The content of known antinutrients of seeds namely, cyanogenic glycosides, phytic acid and trypsin inhibitors were lower in the germinated seeds as compared to those in their ungerminated counterparts.

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LIST OF ABBREVIATIONS

AA	- Acetic Anhydride
ACS	- American Chemical Society
AEP	- Aqueous Extraction Process
ALA	- Alpha Linolenic Acid
ANOVA	- Analysis Of Variance
ANS	- 1-Anilinonaphthalene-8-sulphonate
AOAC	- Association of Analytical Chemists'
ASTM	- American Standards for Testing Materials
BSA	- Bovine Serum Albumin
BV	- Biological Value
CCRD	- Central Composite Rotatable Design
CRD	- Complete Randomized Design
C-PER	- Computed Protein Efficiency Ratio
DAG	- Di-Acylglycerol
DHA	- Docosahexaenoic Acid
EA	- Emulsifying Activity
EPA	- Eicosapentaenoic Acid
ES	- Emulsion Stability
FAME	- Fatty Acid Methyl Ester
FAO/WHO	- Food and Agriculture Organization/World Health Organization

FFA	- Free Fatty Acid
FID	- Flame Ionization Detector
GL	- Glyco-Lipids
GRAS	- Generally Recognized As Safe
HPLC	- High Performance Liquid Chromatography
HLB	- Hydrophilic-Lypophilic Balance
IMP	- Industrial Membrane Process
IR	- Infra-Red
LA	- Linoleic Acid
LDL	- Low Density Lipoprotein
LPC	- Lysophophatidylcholine
LPE	- Lysophospatidylethanolamine
MAG	- Mono-Acylglycerol
NL	- Neutral Lipids
NMR	- Nuclear Magnetic Resonance
NPAGE	- Non-Denaturating Polyacrylamide Gel Electrophoresis
NPN	- Non-Protein Nitrogen
NSI	- Nitrogen Solubility Index
OSA	- Octanesulphonic Acid
PA	- Phosphatidic acid
PC	- Phosphatidylcholine

PCA - Perchloric Acid

PE - Phosphotidylehanolamine

PER - Protein Efficiency Ratio

PI - Phosphatidylinositol (PI)

PS - Phosphatidylserine

SA - Succinic Anhydride

SAS - Statisical Analytical System

SD - Secoisolariciresinol Diglucoside

SDS-PAGE - Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis

SHBG - Sex Hormone Binding Globulin

SHMP - Sodium Hexametaphosphate

TAG - Tri-Acylglycerol

TCA - Trichloro Acetic Acid

TI - Trypsin Inhibitor

TLC - Thin Layer Chromatography

UV - Ultra-Violet

THIS WORK IS DEDICATED TO

MY LOVING PARENTS, HUSBAND & SON

CHAPTER 1

INTRODUCTION

Canada is the largest producer of flax (*Linum usitatissimum* L.) seed in the world by contributing 29% to its total world production; however, only 1 to 4% of flaxseed oil is produced in Canada (Anonymous, 1995). The oil which is traditionally the most important product of flaxseed, is largely used in industrial products due to its high content (45 to 63%) of α-linolenic acid (Easterman, 1968). The whole seed and oil of flax have been used for edible purposes in some European and Asian countries. Flaxseed meal contains 36 to 38% protein (Oomah and Mazza, 1993) and is widely used as an animal feed ingredient. In North America, whole seeds of flax are used in different health foods while the oil is used in oil-based industrial products and the meal is used as an animal feed ingredient or fertilizer.

In recent years potential use of flaxseed for value-added products has been investigated. Presence of biologically-active phytochemicals such as α-linolenic acid, lignans and soluble fibre has generated new and increased interest about the nutritional and pharmaceutical value of flaxseed. Due to the presence of a wide spectrum of biologically active phytochemicals in flaxseed, this oilseed has been identified as an item for the "Designer Foods" project in the United States (Caragay, 1992). Development of low-linolenic acid flaxseed varieties (LINOLA™ and SOLIN™) has been achieved mainly for their oil for edible purposes and these are already available in the Canadian market.

The major components of flaxseed meal are proteins and non-starch polysaccharides (or mucilage). Cyanogenic glycosides, phytic acid, phenolic compounds, lignans, linatine, minerals and possibly protease inhibitors comprise the minor components of flaxseed meal. Flaxseed mucilage which is located mainly in the seed coat accounts for 70% of total carbohydrates of the seed. The mucilage is soluble in water and has good thickening and emulsifying properties, suitable for use as a stabilizer and thickening agent in food products, as a lubricant in the chemical industry and as an additive in the cosmetic and pharmaceutical industries. In addition, flaxseed mucilage has special nutritional value as a source of dietary fibre which appears to play a role in reducing diabetes and risk of coronary heart disease, preventing colon and rectal cancer and reducing the incidence of obesity (Mazza and Oomah, 1995; Wolever, 1995).

Vassel and Nesbitt (1945) have reported the isolation of major flax protein, named "linin". Madhusudhan and Singh (1983) reported that globulins constitute 70 to 85% of flaxseed proteins, two third of which had a molecular weight of 25 kDa and the remainder was low molecular weight in nature. Albumins and globulins, the total proteins of the seeds, may be extracted into mild salt solutions. Flax proteins have a balanced amino acid composition, comparable to those of soy and canola, and contain high levels of arginine, aspartic acid, glutamic acid and leucine, and limiting only in tryptophan compared to FAO/WHO (1973) standard values (Oomah and Mazza, 1993). However, flaxseed proteins have not been commercially exploited due to lack of scientific and technical data on their quality attributes. There are few reports available on preparation of protein products from flaxseed or meal and their possible utilisation. Proteins from flax meal have previously been prepared by isoelectric precipitation of their aqueous extracts (Smith *et al.*, 1946; Sosulski and Bakal, 1969; Madhusudhan and Singh, 1983). Isolation of flaxseed proteins has inherent problems similar to other oilseeds. Co-extraction and co-precipitation of seed coat polysaccharides is the major practical impediment in separating and isolating flaxseed proteins by conventional alkali solubilisation and isoelectric precipitation process. Therefore, presence of polysaccharides in the seed coat may hinder separation of proteins due to their swelling in the aqueous medium (Smith *et al.*, 1946; Sosulski and Bakal, 1969). Use of dehulled or mucilage-free seeds may improve isolation and yield of protein from flaxseed. Dehulling of flax is tedious due to the shape of the seed and the nature of seed coats. The liquid cyclone process developed for canola seeds by Sosulski and Zadernowski (1981) may be successfully applied to flaxseed. Soaking in water may solubilize seed coat polysaccharides and provides a means of reducing mucilage content of flaxseeds.

The functional properties are the major determining factors for potential use of a new protein ingredient in food systems. The major functional properties required by the food industry for use of protein ingredients are solubility, water and fat binding capacities, foaming, emulsifying and viscoelastic characteristics. Alteration of certain functional properties of a native protein may be necessary to meet the desirable requirements for foods. Biological, chemical and physical treatments which modify protein structure and conformation at primary, secondary, tertiary and quaternary levels are employed to confer the functional properties as desired for the particular food system. These modifications of proteins may be carried out for the purpose of changing functional properties, removing off-flavours, toxic or inhibitory substances, retarding deteriorative reactions and to include other chemicals and nutrients (Feeney and Whitaker, 1985).

1.1 Objectives of the present study

Development and production of low-linolenic acid flax varieties may have a positive impact on domestic crushing and oil processing of flaxseed in Canada. However, possible food utilisation of flaxseed meal, the by-product of oil processing has not yet been adequately addressed. The major objectives of this study were to investigate the possibility of preparing a food-grade protein product from flaxseed meal. Development of a cost-effective and simple method to reduce the content of seed coat mucilage to obtain a higher protein recovery from the seeds/meals was first studied. Possibility of preparation of a protein isolate with sodium hexametaphosphate was evaluated and process parameters for the recovery proteins were optimized. The resultant flaxseed proteins were subjected to chemical modification by acylation in order to improve their functional properties. Germination of whole flaxseed and its effect on nutritional value of the sprouts was also investigated.

CHAPTER 2

LITERATURE REVIEW

2.1 Flax: Botanic, economic and historic value

Flax or linseed (*Linum usitatissimum* L.) is one of the oldest arable crops of the world. The origin of the plant goes back to the high Asia or Caucasus with subsequent spreading to China and India. Historical evidence has proven that flax was first cultivated for oil and fibre (linen) in Egypt along the Nile Delta (Van Sumere, 1992).

The botanical classification of the flax plant has been summarised by Haywood (1978) as given below:

Class : *Angiospermae*
Subclass : Dicotyledon
Super order : *Rosidae*
Order : *Ferangiales*
Family : *Linaceae*
Genus : *Linum*
Species : *Linum usitatissimum* L.

The domesticated species of *Linum*, *L. usitatissimum* L. is believed to be a species developed during cultivation throughout civilization. During cultivation two ecotypes of *L. usitatissimum* have been developed; the fibre type, usually known as flax, and the oilseed type referred to as linseed (Keijzer and Metz, 1992). However, recent literature has also referred to linseed as flax.

The flax plant has a single stem nearly 1.0 to 1.2 m in height. Several branches are developed at the apex of the stem which bear flowers. The perfect, regular, hypogeneous and tetracyclic flowers are developed on a cymose inflorescence or a panicle. Each flower consists of five sections (5 sepals, 5 petals and 5 stamens). The flower has green coloured sepals and white, purple or blue coloured petals. Five stamens are alternate with the petals and are fused at their base. Pollination of flowers is mainly by insects.

Historically, flax plants may have been used mainly for their fibre for making linen. Nowadays, there are more uses for flax fibre in industries such as manufacturing of rugs, fine writing papers, currency paper, fine carbon papers, cigarette papers, etc. (Easterman, 1968). Fibres obtained from flax stems consist of elongated, thick walled sclerenchyma cells from the phloem regions of vascular bundles (Easu, 1977; Dybing and Lay, 1981).

Traditionally flaxseed oil has been used as a valuable ingredient for the paint and varnish industry. The oil has also been used in topical applications for eczema, acne and dry skin (Kolodziejczyk and Fedec, 1995) and as a cooking oil among certain cultures (Pan, 1992). The whole flaxseed has been used as an ingredient in cereal mixes and baked goods. Possible uses of flaxseed for human consumption are summarised in Figure 2.1. Important new applications relevant to human consumption are developing, in which meat (Romans, 1995), eggs (Jiang *et al.*, 1991), milk (Kennelly and Khorasani, 1992) and fish (Dick and Yang, 1995) may be enriched with Omega-3 fatty acids by feeding flaxseed to farm animals and fish. Flaxseed has a long tradition of use in folk medicine for gastric disorders, stomach and duodenal ulcers and

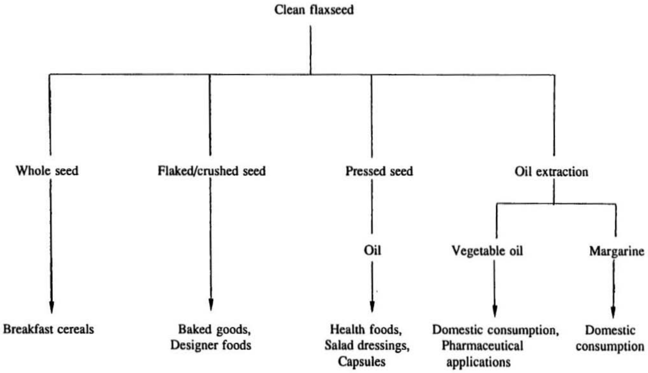


Figure 2.1 Applications of flaxseed for human food use (adapted from Kolodziejczyk and Fedec, 1995)

also as a laxative. The Food and Drug Administration of the United States of America allows inclusion of 12% flaxseed in foods but flaxseed and cold-pressed flaxseed oils have not attained "generally recognized as safe" (GRAS) status (Carter, 1993).

Flaxseed is the third major oilseed crop grown in Canada after canola and soybean. Canada is the largest producer of flaxseed in the world by contributing 29% to the total global production of the seed in the year 1994-95 (Anonymous, 1995). Flax plants are grown mainly in Western Canada, from latitude 49° to 57° north, from longitude 96° to 121° west, and 225 to 1000 m above sea level (Dorrel, 1975). Flax cultivars grown for oil in Canada are registered by Agriculture and Agri-Food Canada after approval by the Prairie Registration Recommending Committee for Grains. The major cultivars grown in Canada in the 1993 crop year were Flanders, McGregor, NorLin, NorMan, Somme and Vimy (Oomah and Kenaschuk, 1995). In agronomic terms, flax is preferred as a rotation crop because it is not usually affected by the diseases that attack cereals and other oilseed crops. Furthermore, the clean straws can be utilized, thus fibrous matter is not incorporated into the soil (Dorrel, 1975).

2.1.1 Flaxseed development

Flaxseeds are developed inside the five-loculed ovary of terminally produced flax flowers. The five-loculed ovary is divided by false septa resulting ten-loculed structure with two seeds produced in each segment. The fruit is a septicidal capsule or boll, 8 to 15 mm high and 10 to 12 mm in diameter. The boll changes in colour from green to yellow to brown during seed maturation. The mature flaxseed is flat, oblong and one end has a curved nib. The size of the seed varies from 3 to 6 mm in length and 2 to 3.5 mm in diameter and weighs 6 to 12 g/1000 seeds. The seed surface is smooth, highly polished and may be dark brown to yellow in colour (Gray, 1981; Sultana, 1992; Freeman, 1995).

2.1.2 Flaxseed structure

Flaxseed contains a seed coat (testa, true hull or spermoderm), an embryo or the germ, a thin endosperm and two cotyledons. Two large flattened cotyledons constitute the bulk of the seed (57%) while the seed coat and endosperm accounts for 38% and the embryo comprises 5% of the total seed weight (Dorrel, 1970).

The seed coat or testa consists of five distinct cell layers all of which originate from the integument (Boeswinkel, 1980). The five cell layers from outside to inside are the mucilage cells (epidermis), round cells, fibre cells, cross cells and pigment cells. The outermost epidermal cell layer is externally covered with a thick cuticule giving the seed a shiny appearance (Freeman, 1992).

The epidermal cell layer is developed from the external cell layer of the outer integument (Boesewinkel, 1984). The shape of these cells is elongated along the edge of the seed and polygonal in other areas. The epidermal cell layer contains mucilage as secondary cell wall material. In dry seeds the striated mucilage appears to completely fill the cells. Upon hydration the mucilage expands rapidly to many times its original dimensions, lifting the cuticle and outer epidermal wall. Folds in the walls of the mucilage cells allow for considerable stretching but ultimately break under the pressure of the expanding mucilage. Suberized inner walls of the mucilage cells resist internal distortion as well as internal migration of the expanding mucilage into underlying layers of the testa (Boesewinkel, 1980).

One to several layers of round cells are located immediately below the mucilage cells. These parenchymatous cells originate from the inner layer of the outer integument. The fibres comprise the third cell layer and have their origin from the outer layer of the inner integument. The secondary walls of these cells are lignified, porous and may be thick compared to the diameter of cells. Immediately internal to the fibres are the cross cells and comprise the fourth anatomical cell layer of the seed coat. These cells have been recognized as the compressed and partly reabsorbed remnants of the middle layers of the inner integument. The walls of these cells are colourless and oriented at right angles to the fibres. The fifth structural cell layer of the testa consists of pigment cells. The pigment cells originate from the innermost layer of the inner integument and comprise the innermost layer of the testa (Boesewinkel, 1984). Polyphenols contained in these cells determine the colour of the seed. In brown seeds, pigment cells contain comparatively larger amounts of polyphenols than those of the yellow coloured seeds (Freeman, 1995). The pigment cells are connected to the oleaginous endosperm tissues which are not a part of seed coat. The structural variations of seed coat anatomy as well as mucilage and polyphenol contents in different varieties of flaxseed have been reviewed by Freeman (1992).

The endosperm tissues vary in thickness from three to many layers along the seed axis. Cotyledons are the major oil storage tissues containing 75% of seed oil. The seed coat and endosperm account for 22% of the seed oil while the embryo contains only 3% (Dorrel, 1970). The intracellular organelles or oil bodies contain lipids and have an average diameter of about 1.3 µm. Neutral lipids are the major constituent of these oil bodies and comprise about 98% of total lipids. The other constituents present are protein bodies called oleosins (1.3%), phospholipids (0.9%) and free fatty acids (0.1%). Phosphatidylcholine and phosphatidylserine are the major components (about 90%) of the phospholipids (Tzen *et al.*, 1992).

During germination the primary root is the first structure to emerge from the seed. As the hypocotyl hook elongates, the seed coat and cotyledons are pulled above the surface (generally soil surface). The cotyledons break free of seed coat, expand and develop chlorophyll and function as the first photosynthetic organ for the growing seedling (Freeman, 1995).

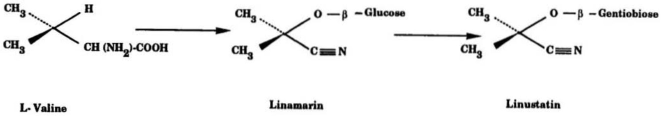
2.2 Composition of flaxseed

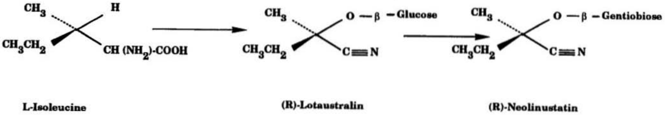
The Canadian grown flaxseed contains, on the average 41% oil (on a moisture-free basis), 26% protein (%N × 6.25), 4% ash, 5% acid detergent fibre and 24% nitrogen-free extract (Oomah and Mazza, 1993). Proximate composition of the whole flaxseed varies considerably depending on the cultivar, growing conditions, seed processing and also analytical methods employed for analyses. There are other minor constituents of flaxseed such as cyanogenic glycosides, phenolic compounds and lignans which are as follows.

2.2.1 Cyanogenic glycosides

Both the vegetative parts and seeds of flax contain cyanogenic glycosides which are nitrogenous secondary plant metabolites capable of liberating HCN upon hydrolysis. Linamarin (2-[(6-O-β-D-glucopyranosyl)-oxy]-2-methylpropanenitrile) and lotaustralin ([(2R)-[(6-O-β-D-glucopyranosyl)-oxy]-2-methylbutanenitrile]) are the monosaccharide cyanogenic glycosides which may be present in flaxseed (Figure 2.2; Butler, 1965; Conn, 1981). Smith *et al.* (1980) have reported that two disaccharide cyanogenic glycosides (Figure 2.2) may also be isolated from flaxseed meal, namely linustatin (2-[(6-O-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy]-2-methylpropanenitrile) and neolinustatin ([(2R)-[(6-O-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy]-2-methylbutanenitrile]). Recent studies by Oomah *et al.* (1992) and Wanasundara *et al.* (1993) have shown the presence of linamarin, linustatin and neolinustatin in the seeds. The content of these three glycosides depends on the cultivar, location and year of production, with cultivar having the most important effect. The predominant cyanogenic glycoside of the Canadian cultivars are linustatin (213 to 352 mg/100 g seed) which accounts for 54 to 76% of the total content of cyanogenic glycosides. The content of neolinustatin

Figure 2.2 Chemical structures of cyanogenic glycosides of flax and their precursors (Poulton, 1989).


CH3
CH3
H
CH (NH2)-COOH
L- Valine
CH3
CH3
O — β — Glucose
C≡N
Linamarin
CH3
CH3
O — β — Gentiobiose
C≡N
Linustatin


CH3
CH3CH2
H
CH (NH2)-COOH
L-Isoleucine
CH3
CH3CH2
O — β — Glucose
C≡N
(R)-Lotaustralin
CH3
CH3CH2
O — β — Gentiobiose
C≡N
(R)-Neolinustatin

ranges from 91 to 203 mg/100 g seed and linamarin is present in less than 32 mg/100 g seeds (Oomah *et al.*, 1992).

2.2.1.1 Biosynthesis

Cyanogenic glycosides are β-glycosidic derivatives of α-hydroxynitriles. They are mainly derived by multi-step biosynthetic sequences from the amino acids namely L-phenylalanine, L-tyrosine, L-leucine, L-isoleucine and L-valine. It has been shown that synthesis of linamarin and lotaustralin (Butler, 1965) and linustatin and neolinustatin (Conn, 1981) in flaxseed is closely associated with the metabolism of valine and isoleucine, respectively (Figure 2.2). Initially, amine nitrogen of the precursor amino acid is oxygenated to form the corresponding aldoxime. Dehydration of aldoxime yields nitrile and stereospecific oxygenation of it produces cyanohydrin. These reactions are catalysed by a membrane-bound enzyme system (Cutler *et al.*, 1985) that exhibits many of the properties of a multienzyme complex. The last step of the biosynthetic sequence is the glycosylation of the cyanohydrin catalysed by a uridine diphosphate-glucose-ketone cyanohydrin β-glucosyl transferase (Hahlbrock and Conn, 1970). It has also been confirmed that the same enzyme is responsible for the formation of both linamarin and lotaustralin (Hahlbrock and Conn, 1971). Linamarin and lotaustralin are cyanohydrins of acetone and 2-butanone, respectively. The unstable cyanohydrin moiety is stabilized by a glycosidic linkage to a single sugar residue (D-glucose) in linamarin and lotaustralin while a disaccharide (D-gentiobiose) stabilizes linustatin and neolinustatin.

The biochemically-related cyanogenic glycosides from valine, linamarin and linustatin, dominate the isoleucine series lotaustralin and neolinustatin (Figure 2.2) during the growth of whole flax fruits from anthesis to maturity (Conn, 1981). The ratio of monoglycosides to total cyanogenic glycosides shifts from 100% to 0% at maturity. Young flax fruits (0 to 18 days after flowering) contain mainly the monoglycosides linamarin and lotaustralin (>90% of total cyanogenic glycosides), whereas about 30% of the cyanogens in older fruits (25 to 40 days after flowering) are the diglycosides linustatin and neolinustatin (Frehner *et al.*, 1990).

2.2.1.2 Chemistry and analyses

A close structural relationship exists among linamarin, linustatin, lotaustralin and neolinustatin. Linustatin and neolinustatin have been isolated as levorotatory crystalline solids. The IR spectra of these compounds show strong hydroxyl absorption (3400 cm^{-1}) and a weak absorption due to C≡N (2240 cm^{-1}) with no carbonyl absorption. The ^{13}C NMR spectra indicate that these compounds are cyclic polyols and their structures have been elucidated as their aglycon moieties (Smith *et al.*, 1980). Glucose is the only monosaccharide produced when linustatin and neolinustatin are completely hydrolysed with mineral acids. Linustatin cleaves regiospecifically into D-glucose and linamarin upon enzymatic hydrolysis with a β-glucosidase.

Extraction of cyanogenic glycosides from defatted flaxseed may be achieved using boiling 80% (v/v) ethanol (Wanasundara *et al.*, 1993) or 70% (v/v) methanol at room temperature (Oomah *et al.*, 1992). Various chemical procedures have been described and used for quantification of total cyanogenic glycosides of plants. Most methods use acid hydrolysis to break down the glycoside to release HCN which is then steam distilled into dilute alkali. The colorimetric assay of the complex formed between HCN with barbituric acid-pyridine is used for quantification of cyanogenic glycosides (Nambisan and Sundaresan, 1984). The most commonly used picrate method (Gilchrist *et al.*, 1967; Cooke *et al.*, 1978) has been improved to allow densitometric quantification of HCN formed upon enzymic hydrolysis on a thin layer chromatographic (TLC) plate (Brimer *et al.*, 1983).

Chromatographic separation of cyanogenic glycosides of flaxseed by TLC (Amarowicz *et al.*, 1993a) and high pressure liquid chromatography (HPLC, Smith *et al.*, 1980; Oomah *et al.*, 1992; Wanasundara *et al.*, 1993) has been established. Unhydrolysed cyanogenic glycosides of flaxseed may be determined by reverse phase-HPLC equipped with a refractive index detector and a C_{18} (bonded silica) column and 15% (v/v) methanol (Wanasundara *et al.*, 1993) or water/methanol/phosphoric acid (94.95:5.00:0.05, v/v/v; Schilcher and Wilkens-Sauter, 1986 and Oomah *et al.*, 1992). Authentic standards of cyanogenic glycosides are used as standards. Since linustatin and neolinustatin are not commercially available, they have to be isolated and purified from readily available sources (Smith *et al.*, 1980; Amarowicz *et al.*, 1993a).

2.2.1.3 Catabolism, toxicity and removal

The aliphatic cyanogenic glycosides of flaxseed are referred to as bound cyanide. The molecular HCN is regarded as free non-glycosidic cyanide. Under normal physiological conditions, tissues of cyanophoric plants contain little or no detectable HCN. When plant tissues are disrupted, HCN may be released rapidly from cyanogenic glycosides upon enzymic hydrolysis. The catabolism of cyanogenic glycosides is initiated by cleavage of their carbohydrate moiety by one or more β-glucosidases, thus yielding the corresponding α-hydroxynitrile. This intermediate may decompose either spontaneously or enzymatically in the presence of α-hydroxynitrile lyase to yield HCN and an aldehyde or a ketone (Paulton, 1989).

Crude flaxseed extracts contain two distinct β-glucosidases which cooperate in stepwise removal of glucose residues. Linustatinase catalyses the hydrolysis of β-(*bis*-1,6)- and β-(*bis*-1,3)-glucosides but is inactive toward linamarin and cyanogenic disaccharides having terminal xylose or arabinose moieties. Thus, linustatin and neolinustatin are hydrolysed to linamarin and lotaustralin, respectively by linustatinase. These monosaccharides are further degraded to their corresponding α-hydroxynitriles by linamarase (Fan and Conn, 1985). Flaxseed linamarase shows only a moderate degree of substrate specificity with respect to the aglycone moiety. It catalyses the hydrolysis of both aliphatic and aromatic cyanogenic monosaccharides but is virtually inactive towards cyanogenic disaccharides (Butler *et al.*, 1965)

Potential toxic levels of cyanogenic glycosides for animals consuming plant materials containing cyanogenic glycosides depend on factors such as species and size of animal, the level of β-glucosidases in the plant, the length of time between tissue disruption and ingestion, the presence and nature of other components in the meal and the rate of detoxification of HCN by the animal (Poulton, 1989). For acute toxicity to occur, enough plant materials must be ingested in a sufficiently short period by the animal. In humans, the minimum lethal dose of HCN taken orally is approximately 0.5 - 3.5 mg/kg body weight or 35 - 245 mg for a 75 kg person (Poulton, 1989). Cyanide exerts an acute toxic effect by combining with metalloporphyrin-containing enzyme systems, most importantly cytochrome oxidase. Cyanide content of approximately 33 μmole can completely inhibit the mitochondrial electron transport system, thus swiftly preventing utilization of oxygen by the cells (Conn, 1981).

Several attempts have been made to remove the cyanogenic compounds from flaxseed meal. These treatments include boiling in water, dry and wet autoclaving and acid treatment followed by autoclaving, among others (Madhusudahan and Singh, 1985b,c). A dry milling process to remove flaxseed toxic compounds has been described by Dev and Quensel (1988). Furthermore, a simultaneous extraction procedure for the removal of cyanogenic glycosides from flaxseed and oil has been reported (Wanasundara *et al.*, 1993). A two-phase solvent system composed of methanol-ammonia-water (95:10:5, v/v/w)/hexane was employed in these studies. The solvent system examined was able to reduce over 50% of both linustatin and neolinustatin in a single extraction step. The solvent-to-seed ratio and duration of extraction period were the main factors affecting the degree of detoxification. A multistage extraction procedure employing the same solvent system was able to remove over 90% of cyanogenic glycosides originally present in flaxseed (Wanasundara *et al.*, 1993).

2.2.1.4 Cyanogenic glycosides and selenium toxicity

The high selenium levels of soil in certain geographical locations of Canada and United States has been identified as a cause of selenium toxicity in farm animals grazing vegetation grown in these areas. Reduced weight gain and lowered reproductive rates are the major economic losses associated with selenium toxicity in farm animal although it is not fatal. Flaxseed meal at a dietary level of 25% (w/w) protected rats against adverse effects from 10 ppm Se (Palmer, 1995). The complete isolation and identification of chemical compounds of flaxseed responsible for protection against Se toxicity in rats was achieved by Smith and co-workers (1980). These authors were able to show that linustatin and neolinustatin were involved. At 2 mg/g level both linustatin and neolinustatin were able to overcome the adverse effects of Se and showed a significant ($p<0.05$) growth increase in rats (Palmer, 1995). The monoglucoside linamarin present in flaxseed was not as effective as the two diglucosides in preventing selenium toxicity.

Free cyanide has been shown to block Se stimulation of the thiol-induced swelling of liver mitochondria from Se deficient rats, possibly through the formation of $SeCN^-$ (Levander *et al.*, 1973a,b). It has also been reported that cyanide can inhibit the Se-containing enzyme glutathione peroxidase and release Se from the enzyme in the form of $SeCN^-$ (Prohaska *et al.*, 1977). These observations suggest that CN^- derived from the cyanogenic glycosides must be responsible for the protective action of purified glycosides and/or flaxseed meal against Se toxicity (Palmer, 1995).

2.2.2 Dietary fibre

Flaxseed meal is high in total dietary fibre, crude, acid detergent (cellulose and lignin) and neutral detergent fibre (cellulose, lignin and hemicellulose). The total dietary fibre content of flaxseed meal is 39 to 45% (Bhatty and Cherdkiatgumchai, 1990) which is somewhat more than the amount present in barley or oat bran (Bhatty, 1993a). Meal from yellow-seeded flax contains less crude (8.7 versus 11.7%) and neutral detergent fibre (24 versus 29%) than meal from brown-seeded flax (Bell and Keith, 1993). Most of the total dietary fibre in flaxseed meal is in the insoluble form and the ratio of soluble fibre to total dietary fibre is 1:7 while it is 1:13 in ground full-fat seeds (Ranhotra *et al.*, 1993)..

2.2.2.1 Structure of soluble fibre and its functional characteristics

Flaxseed polysaccharides (gum or mucilage), accounting for 5 to 8% of the seeed weight, may be isolated via aqueous extraction of the whole seed or meal (BeMiller, 1973, Mazza and Biliaderis, 1989; Oomah *et al.*, 1995). Soluble fibre fraction of flaxseed is mainly polysaccharides. Flaxseed gum is a mixture of acidic and neutral polysaccharides (Erskine and Jones, 1957; Hunt and Jones, 1962). The neutral fraction is composed of a backbone of a β- (1→4) linked D-xylose to which arabinose and galactose side chains are attached at position 2 and/or 3 (Muralakrishna *et al.*, 1987; Cui *et al.*, 1994). The acidic fraction contains mainly (1→2) linked D-galactopyranosyluronic acid backbone with fucose and galactose side chains (Muralakrishna *et al.*, 1987).

Polysaccharides of flaxseed can be extracted in the crude form by adding boiling water and allowing the mixture to cool for about 3 h with constant stirring. Flaxseed polysaccharides may be obtained in the dried form by ethanol (80%, v/v) precipitation and lyophilization (Mazza and Biliaderis, 1989). The viscosity of flaxseed polysaccharides is influenced by concentration, pH and temperature of their solutions and the concentration of the electrolyte in the gum (BeMiller, 1973; Mazza and Biliaderis, 1989). Flax polysaccharides at concentrations of 0.05 to 0.20% (w/v) showed a Newtonian behaviour (pH 6.5, 25 °C), while at concentrations above 0.20% (w/v), a shear thinning behaviour was observed with increasing shear rate, which is typical for polymeric solutions. When compared with other carbohydrate hydrocolloids at equal solid levels (0.3%, w/v) the viscosity of flaxseed polysaccharides at low shear rates is between those of gum arabic and locust bean or guar gum at optimal pH range of 6.0 to 8.0 (Mazza and Biliaderis, 1989). Presence of electrolytes reduces viscosity of flaxseed polysaccharide solutions due to increased association of counter-ions with the polymer molecules that diminishes the electrostatic repulsion of charged groups on the polymer chains (Susheelamma *et al.*, 1991; Mazza and Biliaderis, 1989). According to Fedeniuk and Biliaderis (1994) the neutral fraction of flax mucilage has higher intrinsic viscosity, exhibits more pronounced shear thinning and viscoelastic responses in solution than its acidic counterparts.

2.2.2.2 Significance and uses of flaxseed polysaccharides

A number of applications of flaxseed polysaccharide have been described in food, medicine, pharmaceutical and cosmetic preparations (BeMiller, 1973) as well as other industrial applications (Lummus and Randall, 1967; Lummus, 1967). Recently, flaxseed gum has been patented as a saliva substitute and also as a carrier for oral pharmaceuticals. Flaxseed polysaccharide is low in cost, bland in taste, and has high lubricating and retention properties which are advantageous for the treatment of hyposalivation (reduced secretion of saliva) that causes burning of tongue, mouth, pharynx and esophagus and sensitivity to beverages and spicy foods. Antibiotics and corticosteroids are the suggested pharmaceuticals in which flaxseed polysaccharide-based saliva substitute may be incorporated. Flaxseed polysaccharides can also be used as a laxative and a cough emollient (Mazza and Oomah, 1995).

Flaxseed fibre has potential beneficial effects on glucose metabolism (Cunnane *et al.*, 1993) by slowing carbohydrate digestion and absorption due to a combination of factors, including soluble fibre, and the presumed effects of a starch-protein interaction and antinutrients such as phytic acid (Wolever, 1995). Slowing down of carbohydrate digestion by other methods has been shown to reduce mean blood glucose, insulin and lipid concentrations and thereby reduce the risk for coronary heart disease and possibly diabetes. According to Ratnayake *et al.* (1992) 60 to 70% of total dietary fibres of flaxseed are fermentable by intestinal microflora mostly owing to their soluble fibre fraction. This is a high value compared to wheat bran and the fermentation products, notably short chain fatty acids have beneficial effects including lowering pH in the large intestine lumina and regulation of lipid metabolism in the liver by propionate. Lowering of pH in the lumen is a key step in modification of bile acid degradation by inhibiting the 7-α-dehydroxylation of primary bile acids that has a direct relationship to hypocholesterolemic effect of foods with high fermentable fibre content (Chen *et al.*, 1984).

Novel uses of flaxseed polysaccharides in food and beverages have been reported. Nikkila (1966) has patented the use of flaxseed polysaccharides as an egg white substitute for use in bakery products and ice creams. Successful incorporation of flaxseed polysaccharides into fruit drinks and wheat flour for bread making (Garden-Robinson, 1993) has also been reported. Utilisation of flaxseed mucilage as an ingredient in traditional foods (*idli*) was reported by Susheelamma (1989). Susheelamma and co-workers (1991) have also studied isolated flaxseed polysaccharides as clarifying agents in sugar processing due to their ability to form a gel-like material with sucrose which then floats on top and is easy to remove during the concentration of sugar cane juice.

2.2.3 Lignans

The potential value of flaxseed as a source of plant lignans was recently recognized. Studies carried out in the past few years have shown that flaxseed has anticarcinogenic effects attributed to the antiestrogenic activity of its lignans.

2.2.3.1 Flaxseed lignans and their relationship to mammalian lignans

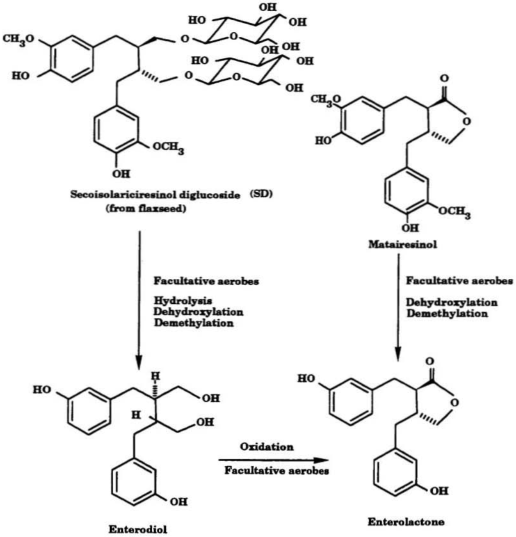
Lignans are phenolic compounds formed by the union of two cinnamic acid residues. The basic structure of these compounds is 2,3-dibenzylbutane (Haworth, 1942). Lignans are related to lignin in that their synthesis involves a similar pathway, however, it is probable that lignans are byproducts of the lignin synthesis pathway (Setchell, 1995). These compounds are ubiquitous within the plant kingdom (Ayers and Loike, 1990) and also possess multiple oxygenated substituents in the aromatic ring in the *para-* position (Rao, 1978), thus differing in their structure from mammalian lignans. Although lignans are found in all plant products their biological role is not clearly defined. The antifungal and insecticidal activities of these compounds indicate a possible role in plant-host defence mechanism and also in some plants lignans as growth regulators (Ayers and Loike, 1990).

Mammalian lignans are formed by the action of intestinal bacteria on plant lignan precursors in the diet. The most important mammalian lignans by quantity are enterolactone (*trans*-2,3-*bis*[(3-hydroxyphenyl)methyl]-γ-butyrolactone; molecular weight 298) and enterodiol (2,3-*bis*[(3-hydroxyphenyl)methyl]-butane-1,4-diol; molecular weight 302) which are formed by the removal of two methyl groups and two hydroxyl groups from plant precursors matairesinol (3R-*trans*-dihydro-3,4-*bis*[(4-hydroxy-3-methoxyphenyl)-methyl]-2(3H)-furanone; molecular weight 358) and secoisolariciresinol [(2,3-*bis*[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediol; molecular weight 362], respectively (Figure 2.3; Ayers and Loike, 1990).

Flaxseed contains the lignan precursor secoisolariciresinol as its diglucoside (Brekke and Klosterman, 1956; Axelson *et al.*, 1982). Secoisolariciresinol diglucoside (SD) is metabolized by intestinal bacteria to the mammalian lignan enterodiol, which is then oxidized to enterolactone. Enterolactone may also arise from metabolism of the plant lignan, matairesinol (Figure 2.3).

Following synthesis by intestinal bacteria, lignans are absorbed from the intestine, transported to the liver and secreted in the bile (Axelson and Setchell, 1981). This enterohephatic circulation of enterolactone and enterodiol is common with steroid hormones (Adlercreutz and Martin, 1980). Concentrations of lignans in plasma are relatively high compared with those of steroid hormones (Axelson and Setchell, 1981; Adlercreutz *et al.*, 1993) which are eliminated from the body via renal clearance in urine. Enterolactone and enterodiol are found in urine at concentrations which correlate directly with the amount of plant lignans or lignan precursors present in the diet (Thompson, 1995). A lower incidence of cancer among vegetarians and populations

Figure 2.3 Chemical structures of flaxseed lignan and matairesinol and their relationship to mammalian lignans (Borriello *et al.*, 1985).


Secoisolariciresinol diglucoside (SD)
(from flaxseed)
Matairesinol
Facultative aerobes
Hydrolysis
Dehydroxylation
Demethylation
Facultative aerobes
Dehydroxylation
Demethylation
Oxidation
Facultative aerobes
Enterodiol
Enterolactone

who consume plant-based diet as well as relatively high urinary levels of enterolactone and enterodiol in them has been well documented (Setchell, 1995; Thompson, 1995).

In vitro studies have shown clear effects of lignans on the synthesis of female and male sex hormones as well as on their availability and receptor binding activity. The urinary excretion of enterolactone in both pre- and post-menopausal women has been shown to correlate positively with plasma sex hormone binding globulin (SHBG) and negatively with plasma percentage of free estradiol and free testosterone (Mousavi and Adlercreutz, 1993). The SHBG is known to bind estrogen and thereby to decrease the concentration of free estrogen. Also low levels of SHBG are associated with high urinary excretion of estradiol and 16-OH estrone (Setchell, 1995) which are strong risk factors for breast cancer. These observations suggest that lignans may protect against breast cancer and other hormone-related cancers by stimulating SHBG synthesis in the liver. The biological properties of plant and mammalian lignans also support their anticancer effects. *In vitro* studies have demonstrated the growth inhibitory and antiproliferative effects of lignans on cells of selected estrogen-sensitive breast cancer cell lines, human neuro blastoma cells and other tumour cell lines (Hartwell, 1976; Hirano *et al.*, 1990).

Flaxseed extracts as well as purified lignans exhibited antioxidant effects *in vitro* and *in vivo* (Fukuda *et al.*, 1985; 1986; Amarowicz *et al.*, 1993b). Some lignans are capable of inhibiting the activity of lipoxygenase and cytochrome P-450 dependent monooxygenase. These activities indicate that the activation of promutagens and procarcinogens may be inhibited by lignans (Agrawal *et al.*, 1991). Lignans also influence cholesterol homeostasis and inhibit the activity of cholesterol-7-α-hydroxylase, the rate limiting enzyme in the formation of primary bile acids from cholesterol (Hirose *et al.*, 1991). Colonic bacteria convert primary bile acids to secondary bile acids such as deoxycholic acid. The increase in secondary bile acid formation has a positive correlation with the risk of colon cancer (Thompson, 1995).

Several animal model studies have shown a protective effect of flaxseed on both mammary and colon carcinogenesis when flaxseed was included in the diet. Purified flaxseed lignans were able to show similar effects as ground or defatted flaxseed suggesting that the anticarcinogenic effect of flaxseed is primarily related to the secoisolariciresinol diglucoside (SD) content not the α-linolenic acid. A significant relationship between urinary lignan excretion and cancer risk markers or tumour characteristics provides further support for the cancer-protective role of mammalian lignans derived from SD (Shultz *et al.*, 1991; Thompson, 1995).

Harris and Haggerty (1993) were able to quantify SD from a methanolic extract of flaxseed meal by HPLC at levels of 0.7 to 0.9 μg/g. Thompson *et al.* (1991) screened various plant foods for their mammalian lignan production by simulated colonic fermentation *in vitro*. Flaxseed flour and its defatted meal were able to produce the highest amount of mammalian lignans ranging from 52.7 to 67.5 mg/100 g sample.

2.2.4 Oil

2.2.4.1 Composition

Oil is the major constituent of flaxseed varying from 35 to 45% of the seed weight, depending on genetic and environmental conditions at growth. Triacylglycerols compose approximately 95% of flaxseed oil; however, accurate quantification of lipid classes of flax oil is not available in the literature. Flaxseed or linseed oil is unique among the major vegetable oils (Table 2.1) in that it contains a very high level (45-63%) of α-linolenic acid (C18:3 ω3), very low levels of saturated fatty acids and oleic acid (C18:1 ω9). The susceptibility of the highly polyunsaturated fatty acids to oxidation and polymerization imparts drying properties to flax oil which is traditionally used as an industrial oil in the production of paints, varnishes, ink and linoleum. However, flavour reversion associated with the oxidative instability of flax oil is a major constraint for its use as an edible oil, as such, in the Western market.

Generally, cold-pressed flaxseed oil is regarded as unsuitable for frying at high temperatures, but it has been used as a cooking oil in rural China (Carter, 1993) where low-temperature stir-frying is practised during which the oil is not oxidized rapidly (Pan, 1992). Traditionally, flaxseed oil has been extracted by cold pressing followed by solvent extraction of press cake for additional oil recovery. Kolodziejczyk and Fedec (1995) have suggested that refining steps similar to other edible vegetable oil processing are required for flaxseed oil intended for health-food markets.

The two major polyunsaturated fatty acids that occur in economically important

Table 2.1 Fatty acid (%) composition of some economically important oilseeds.

Fatty acid	Canola[a]	Cottonseed[b]	Corn[b]	Flax		Soybean[b]	Sunflower[b]
				Traditional[c]	Low-linolenic[d]		
14:0	-	0.5-2.0	<1.0	-	-	<0.5	<0.5
16:0	4.0	16.5-29.5	6.0-28.0	3.8-9.2	5.6-5.8	7.0-12.0	4.5-9.0
18:0	1.4	1.0-4.0	0.5-1.5	1.3-6.2	3.4-4.0	2.0-5.5	1.0-6.5
20:0	1.1	<0.5	<1.0	-	-	<1.0	<1.0
22:0	0.3	-	-	-	-	<0.5	<1.0
16:1	0.5	<1.5	<1.0	-	-	<0.5	-
18:1 ω9	56.9	13.5-14.0	14.0-64.5	13.3-25.2	14.5-15.9	20.0-50.0	14.0-70.0
20:1	1.5	<0.5	<0.5	-	-	-	<0.5
18:2 ω6	23.8	33.5-58.0	10.0-71.0	10.4-20.9	71.9-73.9	35.0-60.0	20.5-75.0
18:3 ω3	10.1	<0.5	<2.0	45.5-63.1	1.8-2.0	2.0-13.5	<0.5
Oil content, %	22-44	15-24	3-16	35-45	35-45	12-30	22-36

[a] Adapted from White (1992) and Hammond (1992)
[b] Adapted from Ackman (1990)
[c] Adapted from Green and Marshall (1981)
[d] Adapted from Green and Dribnenki (1994); Kolodziejczyk and Fedec (1995)

oilseeds are linoleic acid (C18:2 ω6, LA) and α-linolenic acid (C18:3 ω3, ALA). Linoleic acid is the principal component of polyunsaturated edible oils such as canola, corn, soybean and sunflower. Flaxseed oil has an ω6 to ω3 fatty acid ratio of about 0.3:1 while those for canola, corn and soybean are 2:1, 35:1 and 7:1, respectively. Linolenic acid is formed by enzymatic desaturation of linoleic acid and it occurs in relatively smaller amounts in canola and soybean oils. Recently, plant breeders have been able to increase linoleic acid content of flaxseed by genetically blocking the conversion of linoleic to linolenic acid that occurs during lipid synthesis via chemically-induced mutation. With combined efforts of Australian (Green and Marshall, 1981;1984, Green 1986;1988) and Canadian (Dorrell, 1972; Rowland and Bhatty, 1990, Rowland, 1991) scientists, conversion of industrial flaxseed oil into an edible cooking or salad oil has been achieved in recent years. The new low-linolenic flaxseed varieties, available as Linola™ or Solin™ since 1994, contain elevated levels of linoleic acid ranging from 65 to 76%, depending on the variety and growing conditions (Green and Dribnenki, 1994).

2.2.4.2 Nutritional significance

The traditional flaxseed oil is one of the richest plant sources of α-linolenic acid (ALA). Therefore, flaxseed oil has beneficial effects, with respect to its polyunsaturated fatty acids, on the nutrition of animals and humans. However, most of the past studies on flaxseed oil were performed on laboratory animals while anticipating that extrapolation of the results to humans would be valid.

ALA is considered as an essential dietary fatty acid for mammals due to the fact that only plants can synthesize it. Plants can convert oleic acid to linoleic acid (LA) via δ-12 desaturase and convert LA to ALA via a δ-15 desaturase (Tinoco, 1982). Once consumed, ALA is digested and absorbed by pathways common to all other long-chain fatty acids. ALA is important as a precursor of eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) which are essential for synthesis and metabolism of eicosanoids. The eicosanoids are biologically-active compounds that regulate several physiological functions required for normal health. It has been clear that ALA has a protective effect in cancer, degenerative and coronary heart diseases. Short term studies on the effect of flaxseed and flaxseed oil consumption on the eicosanoid formation and platelet composition have shown that ALA in flax oil actually promotes eicosanoid activity (Cunnane, 1995).

Epidemiological studies have shown low incidence of inflammatory and autoimmune diseases among human populations with high dietary intake of fish oils that contain long chain ω3 fatty acids. Enhanced immune status indices were also observed in healthy humans fed flaxseed oil as a source of ω3 fatty acids. Such effects of flaxseed oil may be useful in management of diseases like arthritis, lupus and allergies using diets containing flaxseed/oil (Kelly, 1995).

Cunnane *et al.* (1993) have reported that flaxseed-supplemented diets significantly reduce total serum cholesterol as well as low density lipoprotein (LDL)-

cholesterol of human subjects. Raised ALA and EPA levels in plasma triacylglycerols were also observed after including flaxseed oil in the diets, but no significant effect was noticed on the cholesterol levels (Dyerberg *et al.,* 1978; Budowski *et al.,* 1984; Adam *et al.,* 1986). Therefore, the effect of lowering blood cholesterol level due to flaxseed consumption is hypothesized to be a combination effect of high fibre and ALA contents.

There is evidence that dietary ALA retards tumour growth in rodent mammary-tumour models (Carrol, 1990). Cameron *et al.* (1989) reported that both fish oil and flaxseed oil feeding reduced chemically-induced mammary tumorigenesis in mice and the effect was dependent on both the type and level of fat. The hypothesized mechanism is the intervention of EPA on the production of prostaglandins and leukotrienes which are related to the inhibition of tumour growth. EPA may be produced *in vivo* from ALA of flaxseed as well as flaxseed/soybean oil mixes (Kelly, 1995).

A study by Levander and co-workers (1992) showed that ethyl ester of ALA, the only ω3 fatty acid of flaxseed oil, was capable of providing good protection against infection by malarial parasites in vitamin E-deficient mice. The same research group was also able to show that dietary sources rich in ω3 fatty acids (various fish oils) may provide a similar protection. This activity of polyunsaturated fatty acid containing oils was explained as being due to the prooxidant activity in the vitamin E-deficient diet. A sufficient oxidative stress could be exerted by these oils to inhibit the growth of the parasite that causes malaria (Levander and Ager, 1995).

2.2.5 Protein

Plant proteins are divided into two groups, namely reserve proteins of the seed and functional proteins of the vegetative part of plant. The storage proteins are located in distinct organelles of the seed, which are called as protein bodies/vacuole/granule or aleurone grains. It has also been reported that besides storage proteins, protein bodies contain other proteins such as enzymes, lectins, etc. (Prakash and Narasinga Rao, 1986). Albumins, globulins and prolamines are the three classes of proteins of the seed storage proteins. Albumins are soluble in water, globulins are soluble in dilute salt solutions but are relatively insoluble in water and prolamines are soluble in aqueous ethanol. Globulins and prolamines function primarily as carbon and nitrogen sources for the germinating seed. Albumins are generally considered as enzymic or metabolic proteins (Dieckert and Dieckert, 1985). According to the available literature, oilseed storage proteins consist of one group of high molecular weight proteins and the other of low molecular weight proteins. The high molecular weight proteins of oilseeds serve as a nitrogen source and hydrolysed to amino acids during seed germination. Globular shape and high molecular weight of these proteins are believed to facilitate a dense packing in the cellular structures (Prakash and Narasinga Rao, 1986).

The total protein content of Canadian grown flaxseed cultivars is 36 to 38% on an oil-free, moisture-free basis, with differences attributed to both environmental and genetic factors. As in other oilseeds, a negative correlation was observed between the contents of oil and protein of the seeds (Bajpai *et al.*, 1985; Naqvi *et al.*, 1987). A higher seed protein content may be obtained by increasing the application of nitrogen fertilizer. Nitrogen deficiency lowers the content of total and non-protein nitrogens (NPN) in flaxseed. Sulphur deficiency increases the content of protein nitrogen (Anderson and Spencer, 1950).

2.2.5.1 Physico-chemical properties of flaxseed protein

Solubility of flaxseed nitrogen is dependent on pH, composition of the solvent, ionic strength, meal-to-solvent ratio and temperature (Madhusudhan and Singh, 1983; Dev and Quensel, 1986). The broad pattern of nitrogen extractability of flaxseed at varying pH and ionic strength is comparable with other oilseed meals. The minimum extractability of nitrogen of oil-free flaxseed meal is between pH 3.5 and 3.8 (Smith *et al.*, 1946; Painter and Nesbitt, 1946; Sosulski and Bakal, 1969; Wanasundara and Shahidi, 1994b); however, a broader range of pH 3.0 to 6.0 was reported for demucilaged, defatted and dehulled flaxseed meal (Madhusudhan and Singh, 1983). About 20 to 24% of total nitrogen of flaxseed meal is soluble at the minimum solubility pH due to the presence of high levels of non-protein nitrogen compounds (Bhatty *et al.*, 1973; Sosulski and Bakal, 1969). According to the solubility of flax proteins in different solvent systems, approximately 25% of them are water-soluble, 34 to 47% soluble in 5% (w/v) NaCl, 1 to 2% soluble in 70% (v/v) ethanol and 3 to 3.5% soluble in 0.2% (w/v) NaOH (Sosulski and Bakal, 1969). According to Madhusudhan and Singh (1983) 70 to 85% of flaxseed proteins are globulins, of which two thirds are high molecular weight and the remainder are low molecular weight proteins.

There are not many studies published on characterization of flaxseed proteins, but considerable detail is available on their physicochemical properties as summarized in Table 2.2. As in other oilseeds, albumins and globulins are the major groups of proteins of flaxseed and are classified on the basis of their solubility.

Vassel and Nesbitt (1945) described isolation of linin, a major protein from flaxseed. The purified linin was shown to be homogeneous with an isoelectric pH of 4.75, containing 17% nitrogen, 0.6% sulphur and 0.5% carbohydrate. Madhusudhan and Singh (1985a) isolated and studied the physico-chemical properties of the major protein of flaxseed (Table 2.2). The protein has a sedimentation coefficient ($S_{20,w}$) of 12 in the presence of 1 M NaCl while Dev and Sienkiewicz (1987) have reported a sedimentation coefficient of 11.5 for flaxseed globulins. The molecular weight of purified flax globulins varied between 252 and 294 kDa depending on the method of determination. The 12S globulin had a largely nonhelical secondary structure. Sodium dedocylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the protein contained at least five nonidentical subunits with molecular weights ranging from 11 to 61 kDa. Urea-polyacrylamide gel electrophoresis at acidic and alkaline pHs showed six bands. From the mobility of the bands the protein was assumed to contain

Table 2.2 Physico-chemical properties of flaxseed albumin and globulin proteins[1].

Property	Albumin[2]	Globulin[3]
Total protein (%)	20	66
Sedimentation coefficient ($S_{20,w}$)	1.6	12
Diffusion coefficient ($D_{20,w} \times 10^{-7}$, cm^2/s)	10.7	3.7
Molecular weight (kDa)		
Archibald method	17.0	294.0
Sedimentation diffusion	16.0	298.0
From viscosity data	-	252.0
Gel filtration	15.0	-
Secondary structure (%)		
α-Helix	26	3
β-Structure	32	17
Aperiodic	42	80
Subunit composition		
SDS-PAGE	1	5
Urea-PAGE	-	6

[1] Bhatty (1995)

[2] Madhusudhan and Singh (1985d)

[3] Madhusudhan and Singh (1985a)

one acidic, two neutral and three basic subunits (Madhusudhan and Singh, 1985a).

Vassel and Nesbitt (1945) first reported separation of a low molecular weight protein of flaxseed, referred to as conlinin. The low molecular weight protein (albumin) may constitute up to 42% of the total seed protein (Youle and Huang, 1981). Madhusudhan and Singh (1985d) isolated the major low molecular weight protein using carboxymethylcellulose-sephadex C-50 chromatography; its sedimentation coefficient was 1.6 and it had a molecular weight of 16 kDa. The N-terminal amino acid was alanine and the C-terminal amino acid was lysine. The low molecular weight fraction exhibited β-structure and helical conformation of 32% and 26%, respectively (Table 2.2). The protein consisted of a single polypeptide chain and the amino acid composition showed the presence of a large amount of lysine, cysteine, glutamic acid and glycine. About 93% of the isolated protein was soluble in water and 99% was soluble in 0.05 M NaCl (Youle and Huang, 1981).

2.2.5.2 Uses of flaxseed protein

Smith *et al.* (1946) reported isolation of protein from defatted flaxseed meal by alkali extraction, acid precipitation, separation and drying of the curd. This method which is suitable for isolating soybean protein is not effective for flaxseed, due to the interference of flaxseed hull polysaccharides with the isolation and settling of proteins (Oomah and Mazza, 1993).

Flaxseed protein products containing different levels of polysaccharides were prepared by alkali extraction followed by isoelectric precipitation and subsequently tested in food systems (Dev and Quensel, 1986; 1988 and 1989). The protein product containing high levels of polysaccharides exhibited better water absorption and emulsifying properties and foaming capacity but had lower nitrogen solubility, oil absorption and foam stability (Dev and Quensel, 1988). A good emulsion stabilizing effect was observed in canned fish sauce containing flax protein concentrate with high polysaccharide content. It gave a creamy and smooth fish sauce devoid of any undesirable flavour and a marked reduction of red colour of the sauce. When these protein products were used as meat extenders a reduced fat loss during cooking as well as loss of firmness of the meat emulsion and meaty flavour owing to the poor gelling ability was observed for both low- and high-polysaccharide containing flax protein products. In ice cream, both products gave a stabilizing effect similar to gelatin with increased product viscosity, specific gravity and overrun but a reduced melt-down time (Dev and Quensel, 1989).

2.2.6 Minor compounds of flaxseed

2.2.6.1 Anti-pyridoxin factor

Incorporation of high levels of flaxseed meal in chick diets has shown typical symptoms of a B vitamin deficiency which was alleviated by addition of pyridoxin to the ration (Kratzer *et al.*, 1946) and resulted in improved growth rate of the birds (Klostermann *et al.*, 1960). It was suggested that flaxseed meal contains a vitamin B_6

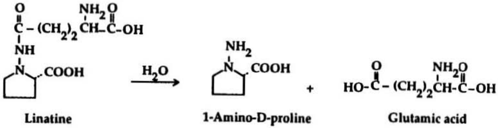
antagonist counteracting the effect of naturally-occuring vitamin B_6 in the feed. Klostermann *et al.* (1967) were able to isolate and characterise this vitamin B_6 antagonist as γ-glutamyl derivative of 1-amino-D-proline (Figure 2.4). This compound was independently synthesized and named as "linatine" because of its association with plant *Linum usitatissimum* (Klostermann *et al.*, 1967). Although linatine was first identified in seeds, its presence in immature flax plants was recognized later (Nugent, 1971).

Linatine was found to be very soluble in water but insoluble in anhydrous organic solvents (Klostermann *et al.*, 1967). Hydrolysis of linatine produced 1-amino-D-proline, an asymmetrically substituted secondary hydrazine which readily condenses with the carbonyl groups of pyridoxal and pyridoxal phosphate to form a stable hydrazone (Klostermann, 1974). While 1-amino-D-proline is the reactive component of linatine, it has not been found in the free state in flaxseed. Hydrolysis of linatine by dilute acid or by enzymes can occur in the digestive tract or liver of poultry or mammals. Linatine, 1-amino-D-proline, and other α-hydrazino acids has also been shown to possess bactericidal properties (Klostermann, 1974).

2.2.6.2 Enzyme inhibitors

Unlike other oilseeds, enzyme inhibitors of flaxseed have not been much reported, probably due to their low content or absence in the seeds. Trypsin inhibitor activity of flaxseed meal was very low compared to that of soybean meal (Madhusud-

Figure 2.4 Chemical structures of linatine and its hydrolytic products (Klosterman, 1974).


Linatine
H_2O
1-Amino-D-proline
+
Glutamic acid

han and Singh, 1983; Bhatty, 1993). Madhusudhan and Singh (1983) reported that amylase inhibitors and hemagglutins were absent from flaxseed meal.

2.2.6.3 Phenolic acids and tannins

Flaxseed as compared to other oilseeds contains low amounts of phenolic acids (Kozlowska *et al.*, 1983; Dabrowski and Sosulski, 1984; Shahidi and Naczk, 1989; Wanasundara and Shahidi, 1993a). Kozlowska *et al.* (1983) reported the presence of free phenolic acids (22.4 mg/100 g) in the methanolic extracts of defatted flour, but Dabrowski and Sosulski were unable to detect any free phenolic acids in the meal, perhaps due to the choice of a different solvent (tetrahydrofuran) extraction system. Phenolic acids released from soluble esters were the largest fraction (55-89% of total phenolic acids). According to Dabrowski and Sosulski (1984), ferulic acid was the predominant phenolic acid in the soluble esters and the insoluble residue.

Amarowicz *et al.* (1994) studied the phenolic compounds of flaxseed meal extracted into 80% ethanol (v/v) by chromatographic techniques. Both hydrophilic and hydrophobic phenolic compounds were present. The separated hydrophilic fractions had a UV maximum absorption between 270 and 290 nm which was different than that observed for phenolic acids. Tannin content of flaxseed meal was very low (125 to 137 mg/100 g of defatted meal) compared to that of canola and high glucosinolate rapeseed (Shahidi and Naczk 1988; Shahidi *et al.*, 1988; Wanasundara and Shahidi, 1994a).

Phenolic compounds contribute to the dark colour, bitter taste and astringency of oilseed meals. In addition to the sensory effects, phenolic acids of oilseed meals interact with other seed constituents. Oxidation of phenolics of oilseeds, catalysed by atmospheric oxygen or enzymes, results in production of quinoidal compounds and hydrogen peroxide. Both of these products are destructive to labile amino acids, denature proteins and inhibit enzyme activity (Shahidi *et al.*, 1988). In oilseeds, cinnamic acid and its esters are the preferred substrates for phenolases and polyphenol oxidases. The *o*-diphenols, especially caffeic and chlorogenic acids are oxidized to form *o*-quinones by the action of enzyme phenol oxidase. The *o*-quinones so produced polymerize or bind covalently to amino, thiol and methylene groups. The ε-amino group of lysine and thiol group of methionine are usually attacked and are thus rendered nutritionally unavailable to the digestive systems of monogastrics (Clandinin and Robblee, 1981).

2.2.6.4 Phytic acid

Phytic acid or *myo*-inositol hexaphosphate (1,2,3,4,5,6-hexakis-dihydrogen phosphate) is the major storage form of phosphorus (60 to 90% of total phosphorus) in seeds and is produced as a secondary product of carbohydrate metabolism (Loewus and Loewus, 1980). Phytic acid exists typically as salts of calcium, magnesium or potassium (Mills and Chong, 1977; Yiu *et al.*, 1983). In cottonseed, peanuts (Saio *et al.*, 1977), rapeseed (Yiu *et al.*, 1983) and soybean (Tombs 1967; Maga, 1982) phytic acid is found in globoids inside the protein bodies of cotyledon cells. However, phytic acid in cereals is largely found in the protein bodies of the aleurone layer (Thompson, 1990). Therefore, it is difficult to remove the phytic acid of oilseeds by traditional processing due to their strong association with proteins. Therefore, phytic acid is concentrated with proteins during preparation of protein concentrates and isolates from oilseed meals (Thompson, 1989).

The phytic acid molecule has 12 replaceable protons and is negatively charged at pH conditions generally encountered in food and feedstuff. Therefore, it is highly reactive towards positively charged groups such as metal ions and proteins (Erdman, 1979; Thompson, 1990). In general, one or two phosphate groups of phytic acid may bind with cations (Gosselin and Coughlan, 1953). The mixed salt of phytic acid is formed when several cations complex within the same phytic acid molecule. The binding of phytic acid with minerals is pH dependent, and complexes of varying solubilities are formed (Cheryan, 1980). Most polyvalent metal ions, especially calcium (Reinhold *et al.*, 1973), magnesium (Nolan *et al.*, 1987), zinc (Erdman, 1979; Nosworthy and Cladwell, 1988; Champagne and Phillipy, 1989) and iron (Davis and Nightingale, 1975) bind to phytic acid and form insoluble complexes which makes them unavailable for metabolism.

As mentioned earlier phytic acid can react with proteins, depending on the pH of the medium. At pH values below the isoelectric point of proteins, phytic acid binds directly with the positively charged proteins as a result of electrostatic attraction. At intermediate pH values, above the isoelectric point of the protein both phytic acid and protein molecules are negatively charged and phytic acid binds primarily with proteins mediated by polyvalent cations such as those of calcium or magnesium. However, direct binding of proteins with phytic acid does not take place to any considerable extent (Cheryan, 1980). Complexing of proteins with phytic acid, directly or through mediation by mineral ions, may alter the protein structure and consequently decrease solubility or functionality and digestibility of proteins (Cosgrove, 1980).

Phytic acid appears to be structurally capable of binding with starch through phosphate linkages or indirectly through its association with proteins. The nutrient digestibility may also be affected by binding of phytic acid with digestive enzymes. These, in turn, may be responsible for both the adverse and beneficial health effects of phytic acid in foods passing through the digestive system (Thompson, 1986, 1989).

2.3 Oilseed protein products

Oilseeds are good sources of oil and protein. After oil extraction, the residual protein-rich meal may be used as protein supplements in animal rations. However, there is a continuous desire to use these sources of proteins in human foods to alleviate increasing world demand for proteins and to address religious concerns about certain animal sources of proteins.

Plant proteins from canola/rapeseed, cottonseed, mustard, peanut, soybean and sunflower meals have been used in a variety of applications. However, the success of including these ingredients in traditional foods depends on reformulating the food in such a manner that the traditional quality of the product is maintained. Oilseed proteins may be used in processed meat products (sausages, luncheon loaves, poultry rolls and related items), textured meat-like analogs/spun fibre (bacon like-bits, simulated beef, ham and chicken breast), dairy-type foods (coffee whiteners, cheese, frozen deserts, whipped toppings, etc.), bakery products (doughnuts, layer type cakes, pancakes, cookies), beverages (fruit-flavoured), special dietary items (infant formula, geriatric foods, weight loss foods and specialized medical and nutritional products) and pasta products (Meyer, 1970; Kinsella, 1979; Kolar *et al.*, 1985). Recent research interests on oilseed proteins have been focused on their industrial application as substitutes for adhesives as well as edible and biodegradable films for packaging purposes.

2.4 Methods of preparation of oilseed protein products

Since oilseed meals are by-products of the oil processing industry they have the advantage of being low-cost starting materials for recovery of proteins. The sensory, functional and nutritional qualities of proteins are very important, but these qualities may be altered in the separated protein afterwards.

First the cleaned (may be dehulled) seeds or kernels are extracted with hexane under mild thermal conditions after initial processing of seeds following their flaking of press cake. After countercurrent extraction, hexane is drained and the meal is desolventised and toasted by moist heat. Defatted meals are converted to flours by grinding to pass through a 100 mesh screen. An air classification process may also be used to separate a protein-rich fraction from flour (Cheftel *et al.*, 1985; Pomeranz, 1985).

Oilseed meals or flours may be used to prepare protein concentrates or isolates. Protein concentrates are prepared by extracting soluble carbohydrates and minerals. Concentrates are more bland than defatted flour but still contain fibre components. The dried protein concentrates contain at least 65% protein (Lusas and Rhee, 1986).

Protein isolates are prepared by solubilizing proteins in alkali solutions and eliminating the insoluble constituents. Proteins are recovered by their precipitation at isoelectric pH and dehydration after washing; the pH of the acid precipitate may be adjusted to neutrality. Protein isolates contain a minimum of 90% protein (Wolf, 1970; Pomeranz, 1985).

Aqueous extraction process (AEP) is a modification of the earliest methods of oil extraction in which finely ground dehulled seeds are mixed with hot water and then centrifuged into a three phase separation. The oil is removed as an emulsion which can be broken by various means to recover oil. The bulk of protein remains in the solution and may be recovered as concentrate or isolate depending on the pH conditions selected (Lawhon *et al.*, 1981; Lusas and Rhee, 1986). This method has been under continuous investigation due to lack of involvement of organic solvents.

A variety of processing options are possible through industrial membrane processing (IMP). Ultrafiltration membranes of 20 kDa molecular weight cut off keep proteins in the retentate, while sugars and water-soluble components pass through as permeate. The permeate can be processed by reverse osmosis to obtain pure water and the concentrated soluble compounds. Various combinations of AEP and IMP techniques have also been studied to prepare vegetable protein concentrates and isolates (Lawhon *et al.*, 1981; Lusas and Rhee, 1986). Soybean has been used mostly for its protein in several countries and has been granted GRAS status and used for human consumption.

2.5 Functional properties of food proteins

Characteristics of oilseed proteins that determine their food utilisation are collectively known as functional properties. Functional properties denote the physico-chemical properties of proteins that determine their behaviour in foods during consumption, processing, preparation, and storage. These physico-chemical properties and the manner in which proteins interact with other food components affect processing applications, quality and ultimately acceptance of food, both directly and indirectly. The type of functional property required for a protein or a protein mix varies with the particular food system in which it is present (Kinsella, 1982). Tables 2.3 and 2.4 list typical functional properties of oilseed proteins and their importance in food applications along with examples. It is also important to note that there are no standardized tests or guidelines to evaluate each functional property of food proteins.

Functional properties of a protein are fundamentally related to its physical, chemical and structural/conformational characteristics. These include, size, shape,

Table 2.3 Functional properties of oilseed proteins important in food applications[1]

General property	Specific functional attribute
Hydration	Wettability, water absorption, water-holding capacity, swelling, solubility, thickening, gelling, syneresis
Kinesthetic	Texture, mouthfeel, smoothness, grittiness, turbidity, chewiness
Sensory	Colour, flavour, odour
Structural and rheological	Viscosity, elasticity, adhesiveness, cohesiveness, stickiness, dough formation, aggregation, gelation, network formation, extrudability, texturizability, fibre formation
Surface	Emulsification, foaming/aeration/whipping, protein-lipid film formation, lipid binding, flavour binding
Other	Compatibility with other food components, enzymatic activity, antioxidant properties

[1] Kinsella (1982)

Table 2.4 Typical functional properties conferred to foods by oilseed proteins[1]

Functional property	Mode of action	Food system
Cohesion-adhesion	Protein acts as an adhesive material	Meats, sausages, baked goods, cheese, pasta products
Elasticity	Hydrophobic bonding in gluten, disulphide links in gels	Meats, bakery products
Emulsification	Formation and stabilization of fat emulsions	Sausages, bologna, soup, cakes
Fat absorption	Binding of free fat	Meats, sausages, doughnuts
Flavour binding	Adsorption, entrapment, release	Simulated meats, bakery goods
Foaming	Formation of stable films to entrap gas	Whipped toppings, chiffon desserts, angel food cakes
Gelation	Protein matrix formation and setting	Meats, curds, cheese
Solubility	Protein solvation	Beverages
Viscosity	Thickening, water binding	Soups, gravies
Water absorption and binding	Hydrogen bonding of water, entrapment of water without dripping	Meats, sausages, bread, cakes

[1] Kinsella (1979 and 1982)

amino acid composition and sequence, charge and charge distribution, hydrophilicity/hydrophobicity ratio, secondary structures and their distribution (eg. α-helix, β-sheet and aperiodic structure) tertiary and quaternary arrangement of polypeptide segments, inter- and intra-subunit cross links (e.g. disulphide bonds) and the rigidity/flexibility of the protein in response to external conditions (Kinsella, 1979; 1982). Functional properties of food proteins may be regarded as manifestations of hydrodynamic and surface related molecular properties of proteins (Damodaran, 1989). Viscosity and gelation are manifestations of hydrodynamic properties which are affected largely by the shape and size of the macromolecule and are independent of amino acid composition and distribution. Emulsifying and foaming properties, fat and flavour binding and solubility are surface-related properties that are largely affected by amino acid compostion/distribution and molecular flexibility rather than size and shape of the macromolecule. Factors such as processing conditions, the method of isolation, environmental factors (temperature, pH and ionic strength, etc.) and interactions with other food components (carbohydrates, flavours, ions, lipids, proteins and water, etc.) alter the functional properties of a protein (Kinsella, 1979).

The fundamental relationships between conformational properties of food proteins and their functional behaviour in food systems is poorly understood. There has been a continuous interest among food scientists to investigate the molecular basis for the expression of functional properties of food proteins which helps to increase utilisation of novel food proteins in conventional foods. Therefore, it is necessary to develop better processing techniques to retain or enhance protein functionality and also to develop genetic engineering strategies by altering the conformational characteristics of underutilised food proteins in order to improve their functionality.

2.5.1 Colour

The first encounter with any food product is visual; thus colour is the major immediate criterion used to evaluate quality and past experience plays a role in this evaluation. Off-colour is associated with poor flavour and quality. The colour that plant protein products impart to food products must be considered as an important factor in consumer acceptability. Undesirable colours in raw and processed foods have often been minimized by removing causative pigments during processing, adding antioxidants and employing bleaching agents to reduce colour change or masking unwanted colours. Proteins contribute to the colour of foods by participating in Maillard browning reactions (Kinsella, 1979; Cheftel *et al.*, 1985).

Colour is a sensory property that depends on both physical and psychological factors related to the object, the conditions of testing and personal bias. Objective methods of colour evaluation use light reflecting or light transmitting properties of the coloured product. The colour of an object is quantitatively defined as i) hue or spectral colour, which identifies the object as red, green, blue or an intermediate colour between these, ii) saturation or purity which is the strength or intensity of the hue and iii) lightness or luminance which is the amount of light reflected or transmitted from the object (Blouin *et al.*, 1981). Numerous visual and instrumental methods and colour scales have been devised to measure and express these three qualities.

2.5.2 Emulsifying properties

The ability of a protein to aid in the formation and stabilization of emulsions is important for preparation of chopped or comminuted meats, cake batters, coffee whiteners, frozen desserts, homogenized milk, mayonnaise and salad dressings. Emulsions are thermodynamically unstable mixtures of immiscible liquids such as vegetable oil and water. If energy is applied, the systems may be dispersed but increased surface energy causes the phases to coalesce rapidly unless an energy barrier is established. Emulsified droplets can be stabilized against coalescence by the addition of molecules that are partly soluble in both phases. In foods, a number of substances may serve this function. Proteins are capable of unfolding at the interphase and may also function as emulsifiers and emulsion stabilizers. The much larger and complex protein molecules as compared with small-size emulsifiers coat lipid droplets and provide an energy barrier to both particle association and to phase separation. The emulsifier protein molecules must first reach the water/lipid interface and then unfold so that their hydrophobic groups can contact the lipid phase (Mangino, 1989). To migrate towards the interface, a protein molecule should be mobile. Since good mobility cannot be expected from coagulated or insoluble proteins, good solubility of proteins is also essential for the formation of a stable emulsion (Nakai and Li-Chan, 1988).

Once a protein molecule reaches the interface it must be able to unfold enough to expose hydrophobic groups to function as an emulsifier. Molecules that contain cross links such as disulphide bonds are more rigid and less able to unfold, thus are less effective in emulsion formation. Reduction of disulphide bonds enhances emulsifying ability of some proteins as long as the molecules do not unfold to the point where there is a large increase in viscosity. Highly cross-linked, small protein molecules tend to perform poorly as emulsifiers (Haque and Kinsella, 1988; Mangino, 1989).

When protein molecules begin to unfold they must possess hydrophobic groups to interact with the nonpolar phase. According to Nakai and Li-Chan (1988), hydrophobic sites on the surface of protein molecules could be a critical factor; hydrophobic groups deeply buried inside proteins do not unfold to be able to participate in emulsion formation. Therefore, surface hydrophobicity as measured by hydrophobic probes has been well related to the ability of various proteins to form stable emulsions. Surface hydrophobicity also correlates well with surface tension, interfacial tension and emulsifying activity of soy, canola and sunflower proteins and their derivatives obtained by treating with surfactants or proteinase (Nakai *et al.,* 1980).

Three main tests have been devised to assess the efficiency of proteins to serve as emulsifiers in food systems; a number of which measure emulsifying capacity (Swift *et al.,* 1961; Carpenter and Saffle, 1964; Inklaar and Fortuin, 1969). These methods generally involve adding lipids to an aqueous solution of the protein to be tested until phase inversion occurs. Thus, the test measures the capacity of the protein to emulsify fat at a very high lipid to protein ratio and the emulsifying capacity is expressed as millilitres of oil emulsified per gram of protein.

The other means is to form an emulsion under conditions that resemble those in the actual food product. The emulsion is then allowed to separate, either under the influence of gravity or after exposure to centrifugal force. In the centrifugal field fat globules are compacted into a cream layer, while an aqueous layer devoid of fat is formed. The ratio of the cream layer formed to the volume of the initial emulsion is used as an indicator of the emulsifying activity. The change in lipid distribution throughout the sample with time can be measured and the phase separation with time is taken to account as emulsion stability (Inklaar and Fortuin, 1969). The size distribution of particles in an emulsion can also be used as an indicator of the effectiveness of the emulsifier but determination of size distribution is tedious (Mangino, 1989). Many investigators have recently used a method based on turbidity of the emulsion formed as first described by Pearce and Kinsella (1978). This method has the advantage of using a very small amount of sample. The emulsifying activity index (EAI m^2/g) and emulsion stability index (ESI, min) measured according to this method correlate well with surface hydrophobicity of food proteins (Nakai and Li-Chan, 1988).

2.5.3 Fat and water binding ability

Fat binding ability of a protein is important as it enhances flavour retention and improves mouthfeel. The mechanism of fat absorption by proteins is not well understood. Fat absorption of proteins is usually measured by adding excess liquid fat (oil) to the protein powder, thoroughly mixing and holding, centrifuging and determining the amount of adsorbed or bound oil as the difference between the total amount of added oil minus free oil (Lin *et al.*, 1974; Wang and Kinsella, 1976). The fat absorption (capacity), assessed according this method, is attributed to the physical entrapment of oil. A correlation coefficient of 0.95 was found between the bulk density and fat absorption of alfalfa leaf protein (Wang and Kinsella, 1976). The turbidimetric method described by Voutsinas and Nakai (1983) for measuring fat binding capacity minimizes physical entrapping effects and reflects the true fat binding capacity.

The surface-related properties of protein molecules contribute to their fat binding ability (Damodaran, 1989). Factors affecting protein-lipid interaction include protein conformation, protein-protein interaction and the spatial arrangement of the lipid phase resulting from lipid-lipid interactions (Hutton and Campbell, 1981). Non-covalent interactions (hydrophobic, electrostatic and hydrogen bonding) are also involved in protein-lipid interactions. Hydrogen bonding is of secondary importance in lipid-protein complexes though it is indirectly involved in hydrophobic bonding (Karel, 1973) as water-water interactions by hydrogen bonding in aqueous media are much stronger than interaction between water and non-polar groups. Nakai and Li-Chan (1988) have suggested that electrostatic attraction can occur between negatively charged phosphate groups of phospholipids and positively charged protein groups (lysyl and guanidyl) or between a positively charged group in the phospholipid (eg. choline) and a negatively charged amino acid side chain (glutamyl, aspartyl). According to Ryan (1977), hydrophobic interactions are most important in stabilizing interactions of both polar and non-polar lipids with proteins. Voutsinas and Nakai (1983) found a strong relationship between fat binding capacity and surface hydrophobicity of food proteins.

Water absorption, water binding and water holding are terms that are used interchangeably in the literature. Water absorption provides critical information about functional properties of proteins such as those in meat processing which affect, tenderness, juiciness, colour, taste, cook loss and drip on freezing and thawing. Proteins have been reported to be primarily responsible for water absorption, although other food constituents may have an effect (Hutton and Campbell, 1981, Nakai and Li-Chan, 1988). Water binding and water holding usually refer to the water that is retained by the sample following filter paper press, centrifugation or gravitational filtration (Nakai and Li-Chan, 1988). Porteous and Wood (1983) introduced absorbed moisture and retained moisture to differentiate between the water holding parameters determined in uncooked and cooked samples, respectively. Hermansson and Åkesson (1975) have suggested that swelling properties may be used to measure water absorption. The property, water adsorption is referred to water taken up spontaneously by a dry powder after equilibration against water vapour of a known relative humidity

(Kinsella, 1976).

Primary water-protein interactions occur at polar amino acid sites of the protein molecules such as amino, carbonyl, hydroxyl and sulphydryl groups. Water binding can vary with the number and type of polar groups, protein conformation and environmental factors that affect protein charge and/or conformation. Conformational changes in the protein molecules can affect the nature and availability of hydration sites. Transition from globular to random coil conformation may expose previously buried amino acid side chains, thereby making them available to interact with aqueous medium; the unfolded proteins bind more water than the globular form of proteins (Hutton and Campbell, 1981).

2.5.4 Foam and film forming properties

Protein foams are important in preparation of several food products including chiffon desserts, bakery products, fudges, ice creams, icing, meringues, soufflés and whipped toppings. Foams are thermodynamically unstable colloidal systems in which gas is maintained as a distinct dispersed phase in a liquid matrix. In food foams the kinetic barrier to bubble coalescence and rupture is typically provided by a protein film surrounding the bubble. In foam formation, soluble proteins are subjected to an interfacial exposure/adsorption that alters their structure and allows for their subsequent association with other proteins in the interface (German *et al.*, 1985). Protein solubility makes an important contribution to the foaming behaviour of proteins (Wang and

Kinsella, 1976).

Various molecular forces are believed to be responsible for the film and foam formation, the nature of which is not entirely understood. German and Phillips (1989) have described film formation as a four step process. The generation of new interface is the initial event which needs high energy inputs. Then the soluble proteins maintained in a particular conformation (primarily by entropically favourable solvent interactions such as hydrophobic) should arrive at this interface by diffusion. The proteins unfold and pursue favourable associations such as intramolecular ones. Then as more proteins arrive and unfold at the interface, intermolecular associations are increased. Forces and structures which favour intermolecular associations at this point improve foaming ability, while forces and structures which prevent intermolecular associations decrease foaming ability (Phillips, 1981; German *et al.*, 1985). Films provide stability to foams by forming a continuous kinetic barrier to coalescence.

The structure of a protein in solution and forces which maintain it are clearly important to its ability to interact at an interface. Proteins which owe their solution structure mostly to the solvent entropy unfold readily after leaving the solvent and adsorbing to a surface. The formation of new intermolecular associations becomes more favourable and desorption from the interface occurs, thus helping to form a good film. In contrast, proteins that are stabilized predominantly by intramolecular interactions in solutions (ion pair, disulphide bonds, etc.) may not easily alter their native structure and form new intermolecular associations. Such molecules even if they adsorb to a clean interface, have a higher desorption tendency and may not form a good film (German and Phillips, 1989).

Several methods may be used to measure foaming properties of proteins. Most of these methods use injection, shaking, sparging or whipping to incorporate air into the protein solution. The most widely reported method is the whipping method that uses whipping of a highly concentrated protein solution in a standard mixer. Although this method is closer to the practical method of foam formation, it requires a large quantity of protein. Therefore, this method is not suitable for purified proteins. Once the solution is completely incorporated into the foam, the volume of the overrun (ratio of volume of foam to the initial liquid volume as a percentage; Halling, 1981) is measured and the maximum overrun developed is used as a measure of foaming ability. The sparging method is suitable when sample size is small. The gas bubbles are forced through a rather dilute protein solution and a column of foam is allowed to form above the solution. Waniska and Kinsella (1979) have improved the sparging or bubbling method regulating temperature by using water jacketed columns and less volume of protein solution. Finally, the shaking method is rarely used. Foam formation by shaking tends to be slower than bubbling or whipping under similar conditions (Halling, 1981). Stability of the foams produced by these methods are determined by the drainage/leakage or syneresis rates (Halling, 1981; German *et al.*, 1985).

2.5.5 Gel formation

The process of gelation is defined as aggregation of denatured protein molecules to form an ordered network. Gelation is a very important functional property and plays a major role in preparation of various dairy products, coagulated egg white, gelatin gels, soybean protein gels, vegetable proteins texturized by extrusion or spinning, bread dough and heated comminuted fish and meat products. Protein gelation is utilised for the formation of solid viscoelastic gels as well as for improved water absorption, thickening, particle binding (adhesion) and emulsion or foam stabilizing effects (Cheftel *et al.,* 1985).

The mechanism and interactions related to the formation of three dimensional protein networks and characteristics of gels are not very clear. All studies have shown the necessity of protein denaturation and unfolding prior to the step of ordered protein-protein interaction and aggregation. The formation of a protein network results from the balance between protein-protein and protein-solvent (water) interactions as well as attractive and repulsive forces of adjacent polypeptide chains. The attractive forces are hydrophobic interactions, electrostatic interactions, hydrogen bonding and disulphide cross links. Their relative contribution may vary with the nature of the protein, environmental conditions and the various steps in the gelation process. Electrostatic repulsions and protein-water interactions tend to keep polypeptide chains apart. Intermolecular protein attraction (and gelation) takes place more readily at high protein concentrations because of the greater probability of intermolecular contacts. At high concentrations gelation may take place even in environmental conditions that are not specially favourable for aggregation (eg. without heat, pH values away from pI). The establishment of covalent disulphide cross links usually leads to the formation of heat irreversible gels (eg. ovalbumin and β-lactoglobulin gels). However, gelatin gels which are mainly stabilized by hydrogen bonding melt upon heating and setting-melting cycle can be repeated many times. Different proteins can form gels when heated together (cogelation) or through interaction with polysaccharide gelling agents. Many gels exist as highly expanded (open) and hydrated structures with water and other food constituents entrapped in the protein network. The gel-forming ability of soybean proteins have been extensively studied (Kinsella, 1979; Cheftel *et al.*, 1985; Foegeding, 1989).

2.5.6 Solubility

Solubility is an important functional property that determines the use of proteins in various food products. Solubility behaviour under various conditions also provides a good criterion for the potential application of proteins. Solubility is the most important attribute of proteins selected for use in beverages. The degree of insolubility is probably the most practical measure of protein denaturation and aggregation, because proteins that initially exist in a denatured, partially aggregated state, often exhibit impaired ability to participate effectively in gelation, emulsification and foaming (Kinsella, 1976).

Protein solubility at neutral or isoelectric pH is the first functional property measured at each stage of preparation and processing of a protein ingredient. The nitrogen solubility index (NSI) and solubility profile as a function of pH, ionic strength or temperature are the most frequently monitored (Kinsella, 1979; Cheftel *et al.*, 1985).

The solubility of most proteins is markedly and irreversibly reduced with heat treatments. A protein with a high initial solubility permits rapid and extensive dispersion of its molecules or particles, thus leading to a finely dispersed colloidal system. Also initial solubility facilitates protein diffusion to air/water and oil/water interface thus improve their surface activity (Cheftel *et al.*, 1985).

Proteins interact with water through their peptide bonds (dipole-dipole or hydrogen bonding) or through their amino acid side chains (interactions with ionized, polar and even non-polar). From a thermodynamic standpoint solubilization corresponds to separating the molecules of solvent and/or protein and dispersing the protein molecules in the solvent with maximum interaction between the protein and the solvent. The protein must interact (dipole-dipole, hydrogen, ionic) as much as possible with the solvent in order to become soluble (Shen, 1981).

The solubility of a protein under a given set of conditions can be expressed as the manifestation of the equilibrium between the protein-solvent (hydrophilic) and the protein-protein (hydrophobic) interactions. Many of the molecular and functional properties of food proteins are related to the content of hydrophobic and hydrophilic amino acids and their distribution in their primary structure. The average hydrophobicity ($H\Phi_{ave}$) and the charge distribution are the most important molecular features that influence physical properties such as solubility of proteins (Bigelow, 1967, Nakai and Li-Chan, 1988). However, it has become clear that physical and chemical characteristics of the protein surface and thermodynamics of protein interaction with the surrounding solvent are also critically important. The extent of exposure of hydrophobic surfaces at the exterior of protein molecules determines the solubility as well as other solution properties related to physico-chemical characteristics of the protein. Polypeptide chains of proteins are folded in such a way that the majority of the non-polar residues are inside and most of the polar residues are at the surface where they are exposed to the solvent (Damodaran, 1989). Nakai and Li-Chan (1988) have explained the relationship of protein solubility to hydrophobicity and charge of the protein molecule. The aromatic hydrophobicity, measured using 1-anilino 8-napthosulphonic acid (ANS), has shown a significant ($p<0.001$) relationship with the insolubility of the proteins.

Proteins at pH values higher or lower than their isoelectric point, carry a net negative or positive charge. Water molecules interact with these charges and contribute to the solubilisation of proteins and dissociation of aggregates and/or unfolding of their native structure. The solubility of a given protein as a function of pH is V- or U-shape reaching a minimum in the vicinity of the isoelectric pH (pI). At this point, protein molecules show minimum interactions with water and their net charges are sufficiently minimal to prevent aggregate formation and protein precipitation (Shen, 1981; Cheftel *et al.*, 1985).

The ions of neutral salts at 0.5-1.0 M may increase the solubility of proteins due to salting-in effect. The ions interact with the charges of proteins and decrease the electrostatic attraction between opposite charges of the neighbouring molecules. Moreover, the solvation connected with these ions serves to increase protein solubility. At concentrations of greater than 1 M, neutral salts reduce protein solubility and may cause their precipitation due to salting-out effect. At high salt concentrations water molecules are occupied by solvating ions and there are not enough water molecules available for protein solvation. Thus, protein-protein interactions become stronger than protein-water interactions which lead to aggregation followed by precipitation of protein molecules (Cheftel *et al.*, 1985).

2.6 Modification of functional properties of oilseed proteins

Oilseed proteins in the native state do not always meet the desirable functional properties for particular food systems. Therefore, altering the functional properties by using biological, chemical or .physical modification of proteins is necessary. Modification of proteins involves changes in protein structure or conformation at all the primary, secondary, tertiary and quaternary levels. Besides, modification of functional properties, retarding deteriorative reactions (eg. Maillard reaction), removing of toxic or inhibitory ingredients (eg. phytic acid, phenolic acids and tannins) and attachment of nutrients and additives by formation of new covalent bonds (eg. amino acids, carbohydrates, flavour compounds and lipids) can also be achieved by biological or chemical modification of proteins (Feeney and Whitaker, 1985; Nakai and Li-Chan, 1988).

2.6.1 Biological modification

Enzymatic modification of food proteins may be regarded as biological modification. Endogenous enzymes are responsible for hydrolytic reactions which may affect quality of foods such as tenderization of meat, cheese ripening as well as development of off-flavours and spoilage of foods. In addition, *in vivo*, enzymes catalyse many post-translational modifications of proteins, including cross linking of polypeptide chains, phosphorylation, glycosylation, hydroxylation and methylation (Nakai and Li-Chan, 1988).

Proteolytic enzymes that participate in the hydrolysis and resynthesis of peptide bonds have been widely used to improve functionality of a variety of foods. The hydrolysis of peptide bonds results in an increase in the number of charged groups (extra amino and carboxyl termini) and hydrophilicity, decrease in molecular weight and alterations in molecular configuration (Phillips and Beuchart, 1981). Thus, breakage of peptide bonds may be followed by a complex series of changes which can alter functionality through dissociation of subunits or unfolding of a compact structure to expose hydrophobic regions. So as hydrolysis proceeds, an increase in solubility and a decrease in viscosity of proteins is observed. In addition, proteins may undergo altered gelation properties, enhanced thermal stability, increased emulsifying and foaming abilities and decreased emulsion and foam stabilities (Nakai and Li-Chan, 1988).

Extensive proteolysis often results in the formation of bitter-tasting hydrophobic peptides containing leucine or phenylalanine terminal residues. However, controlled hydrolysis of vegetable proteins may produce functional hydrolysates with desired flavour and aroma. Such products serve as important ingredients for incorporation into the diets of patients with impaired digestive function or those allergic to milk or gluten. The resynthesis that may occur upon incubation of highly concentrated solutions of protein and peptide hydrolysates under the influence of enzymes is known as the plastein reaction. Controlled plastein reaction is useful for the removal of unwanted odourants, debittering of protein hydrolysates, enrichment of proteins with desired essential amino acids and also in preparation of speciality foods (eg. low-phenylalanine high-tyrosine peptide type foods for patients with phenylketonuria; Fujimaki *et al.*, 1977; Cheftel *et al.*, 1985; Adler-Nissen, 1986; Nakai and Li-Chan, 1988).

2.6.2 Chemical modification

Chemical modification of proteins has been widely used in studies of structure-activity relationships. This technique has also been applied to study the structure of food proteins as well as to improve their nutritional and functional properties. Some of the most commonly used methods of chemical modification of amino acid residues are listed in Table 2.5. Most studies on food protein modification involve derivatization of ε-amino group of lysine residues. This type of derivatization may directly affect the net charge and charge-density of protein molecules. Conformational changes and alterations in intra- and intermolecular interactions may also occur that modify effective hydrophobicity of proteins (Means and Feeney, 1971; Feeney *et al.*, 1982; Nakai and Li-Chan, 1988). Since reactive side chains of proteins are nucleophilic (e.g. amino, thiol and phenolic hydroxyl) or may be easily oxidized or reduced, reactions involving modification of these groups are commonly carried out.

2.6.2.1 Acylation

The most commonly used chemical modification of food/plant proteins is the formation of an amide (isopeptide) bond, particularly with lysine residues using an acid anhydride (Feeney and Whitaker, 1985). Reaction with acetic anhydride (acetylation) replaces the positively charged ε-amino group of the lysine residue by a neutral acetyl group (Figure 2.5). Acylation with cyclic anhydrides such as succinic anhydride results in a two-charge change, from the positive charge of the ε-amino group to the negative charge of succinyl anionic groups (Figure 2.5). The double-charge change of amino group to the carboxyl group due to succinylation increases the net negative charge and has a marked effect on protein conformation. (Means and Feeney, 1971; Hirs and Timasheff, 1972).

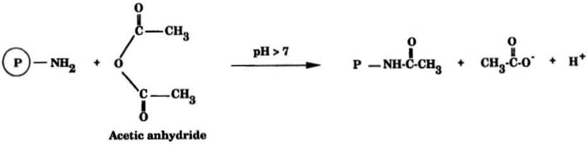
Acylation reactions are known to follow the carbonyl addition pathway. While

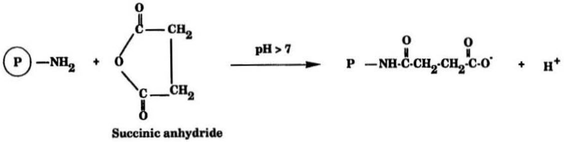
Table 2.5 Chemical modification of amino acid side chains[1].

Side chain	Amino acid residue	Commonly used modification
Amino	Lysine	Alkylation, acylation
Carboxyl	Aspartic and glutamic acid	Esterification, amide formation
Disulphide	Cystine	Reduction, oxidation
Sulphydryl	Cysteine	Alkylation, oxidation
Thioether	Methionine	Alkylation, oxidation
Imidazole	Histidine	Alkylation, oxidation
Indole	Tryptophan	Alkylation, oxidation
Phenolic	Tyrosine	Acylation, electrophilic substitution
Guanidino	Arginine	Condensation with dicarbonyls

[1] Adapted from Nakai and Li-Chan (1988)

Figure 2.5 Acylation of amino groups of proteins with acetic or succinic anhydrides (Means and Feeney, 1971).


P
NH2
O
C
CH3
Acetic anhydride
pH > 7
P —NH-C-CH3
CH3-C-O-
H+


P
NH2
O
C
CH2
Succinic anhydride
pH > 7
P —NH-C-CH2-CH2-C-O-
H+

amino- and tyrosyl-groups are easy to acylate, histidine and cystine residues are only rarely observed to undergo acylation. The hydroxyl groups of serine and threonine are weak nucleophiles and never acylate in the aqueous medium. Acylation reactions are generally influenced by the pH of the medium. Table 2.6 summarises the effect of acylation on functional properties of some oilseed proteins.

2.6.2.2 Other chemical modifications

Alkylation of the amino groups of a protein with an aldehyde or a ketone in the presence of a reducing hydride donor reagent marginally alters the basicity of the amino groups (Nakai and Li-Chan, 1988). This reaction can be used to attach a sugar residue to the protein which would change some of its functional properties (Lee *et al.*, 1979). Incorporation of a hydrophobic group into the protein molecule via esterification, amidation, acylation or reductive alkylation is referred to as lipophilization. Attachment of palmitoyl residues to soy protein glycinin resulted in its improved emulsification activity, foaming ability and foam stability (Haque *et al.*, 1982). Modification of ε-amino group of proteins by dimethylation or ethylation and reduction is particularly suitable for preventing Maillard browning reactions from occuring (Feeney, 1977; Galembeck *et al.*, 1977). Covalent attachment of various L-amino acids such as methionine and tryptophan through peptide or isopeptide linkage may change proteins of low nutritive value to proteins fortified with essential amino acids (Shukla, 1982).

Table 2.6 Acylation of oilseed proteins and the effect on functional properties.

Protein source[1]	Modification reaction	Changes in chemical and physical properties	Reference
Cottonseed	Acetylation	Improved water holding, oil binding, emulsifying and foaming capacities.	Child and Parks, 1976
	Succinylation	Improved water solubility, heat stability, emulsion and oil binding capacities, gel strength, viscosity, water hydration and retention, decreased bulk density.	Child and Parks, 1976; Choi *et al.*, 1981
Canola	Succinylation	Improved solubility, emulsifying activity and stability, thermally induced gelation, hydrophobicity and net negative charge.	Paulson and Tung, 1987;1988;1989a,b
Rapeseed	Acetylation	Improved nitrogen solubility, emulsifying properties, specific viscosity.	Ponnampalam *et al.*, 1990
	Succinylation	Improved emulsifying properties, foam capacity and stability, heat stable gel formation, dissociation into subunits and unfolding.	Gueguen *et al.*, 1990; Schwenke *et al.*, 1993

[1] Isolates, unless otherwise specified

Continued.....

Table 2.6 continued......

Protein source[1]	Modification reaction	Changes in chemical and physical properties	Reference
Rapeseed flour	Acetylation	Decreased phenolic acid extractability and peptic and tryptic hydrolysis, increased nitrogen extractability, decreased phytic acid and mineral extractability.	Thompson and Cho, 1984; Ponnampalam *et al.*, 1987
	Succinylation	Increased nitrogen extractability, decreased phytic acid and mineral extractability.	Thompson and Cho, 1984
Peanut	Succinylation	Enhanced solubility at low pH, water absorption and viscosity, dissociation into subunits and swelling.	Beuchart, 1977
Soybean	Acetylation	Decreased water binding and gel strength, increased solubility.	Barman *et al.*, 1977; Franzen and Kinsella, 1976a
	Succinylation	Increased emulsifying activity and stability, foam capacity, decreased isoelectric pH.	Franzen and Kinsella, 1976a

[1] Isolates, unless otherwise specified

2.6.2.3 Nutritive value and toxicity of chemically modified food proteins

Modified protein products must have acceptable flavour, improved nutritive value and functional properties as well as being non-toxic. Creamer *et al.* (1971) have reported that acetylated casein or whey proteins gave a moderate protein efficiency ratio (PER) and showed no degeneration of organs (liver, lung, adrenal and kidney) in three generations of rats. However, succinylated casein exhibited a lower PER value than acetylated casein indicating that rats were able to deacylate acetylated proteins only. Groninger and Miller (1979) fed acetylated and succinylated myofibrillar fish proteins to rats and reported a reduction in protein efficiency ratio, partial utilization of protein-bound acetyllysine and metabolic unavailability of both succinyl- and acetyllysine. These authors also reported that hydrolysis of succinylated proteins was dependent on the type of protein, extent of modification and the type of protease employed.

2.6.3 Physical modification

Physical modification of proteins includes use of mechanical or thermal energy or pressure to forge desired alterations. Common examples of physical processes which alter food proteins are emulsification, extreme dilution, extrusion, fibre spinning, freezing, heating, radiation, sonication and whipping. These processes usually result in the denaturation of proteins, therefore, alter the conformation of the polypeptide chains without breaking their primary covalent bonds (Nakai and Li-Chan, 1988). Improved protein solubility, foaming properties and fat and water absorption were observed in dry milled products as compared with their unmilled counterparts. Mechanical forces also play an important role in texturization process of proteins such as those brought about by dough or fibre formation and extrusion cooking. Molecular alignment, disulphide bond interchange and formation of protein networks are some of the alterations that may occur in proteins due to the shear forces applied (Cheftel *et al.*, 1985). Thermal treatment of protein results in structural changes, hydrolysis of peptide bonds, modification of amino acid side chains and condensation with other molecules. These treatments bring about changes in solubility, surface properties, flavour and colour of products (Kinsella, 1982; Cheftel *et al.*, 1985).

2.7 Nutritional value and utilisation of sprouted cereals, legumes and oilseeds

The process technologies of using the whole seeds of cereals or legumes include milling, cooking, fermentation, and malting or sprouting/germination. In the Orient and Far East Asia, fermentation and sprouting of legumes are widely and routinely practised. This method of seed alteration has recently become popular in Western countries and non-traditional seeds such as alfalfa, onion and sunflower sprouts are available in the Canadian market besides the traditional bean sprouts.

The terminology, sprouting, malting and germination are often used interchangeably in the literature to describe the process of soaking or steeping the dry seeds in water until they are saturated followed by germination under controlled conditions for a specified period. However, the term malting is more commonly used when the grains, particularly barley, are soaked and germinated for brewing purposes. A similar treatment of seeds such as those of cereals and legumes for food applications, other than brewing, is referred to as sprouting and germination by many investigators (Salunkhe *et al.*, 1987).

To achieve maximum yield of sprouts and nutritional benefits, the starting seeds should be clean, free from broken or infected individuals, untreated and viable. Several factors including seed viability, water availability, temperature and conditions of air and light influence the germination of seeds and the development of sprouts.

The ability of seeds to germinate under favourable conditions is known as viability. The healthy, untreated, dry seeds can retain their viability for several years under storage conditions favouring low metabolic activity such as low temperature and moisture and high carbon dioxide concentration. The physical condition, inheritance, microflora and insects and use of fungicides or insecticides also influence seed viability (Roberts, 1979; Chavan and Kadam, 1989).

A viable dry seed requires water for the germinative process. Water is imbibed by the seed during soaking. The amount of water imbibed is influenced by seed size, seed coat permeability, quantity of water available, composition of seed and concentration of solutes in the soaking medium. The water imbibition results in the development of pressure within the seed. The magnitude of this imbibition pressure indicates the water-retaining power of the seed and the amount of water available for hydration of the seed during germination and subsequent sprouting. Therefore, maximum water imbibition during soaking is advantageous for good sprouting. However, controlling water uptake during soaking and the supply of water during subsequent sprouting is recommended because excessive water uptake during soaking may break the seed coat and affect the vigour of the sprouts (Chavan and Kadam, 1989).

The temperature is a crucial factor for producing a higher percentage of germination in a short time. Most seeds require 20 to 30 °C as the optimum temperature for maximum germination at a faster rate with differences owing to genetic factors and age of seeds. Therefore, it is important to establish and maintain the optimum temperature of germination for a specific seed to produce maximum sprouts (Roberts, 1979, Chavan and Kadam, 1989).

Most seeds germinate at the normal level of oxygen (20%) and carbon dioxide (0.03%) in the air. Seeds germinate equally well under light or dark; however, dark conditions may be more advantageous to avoid photosynthetic activity in developing sprouts. The stored seeds are often treated with insecticides and fungicides which can retard or inhibit germination. Therefore, untreated seeds are recommended for sprouting purposes (Chavan and Kadam, 1989).

2.7.1 Nutritional value

The metabolic activity of resting dry seeds increases as soon as they are hydrated during soaking. Complex biochemical changes occur during hydration and subsequent sprouting in various parts of the seed. The major reserve chemical constituents namely carbohydrates, proteins and lipids are enzymatically broken down into simple molecules to synthesize new compounds or transported to the parts of the growing seedling. Since no external nutrients are added, only water and oxygen are consumed by the sprouting seeds from external sources. The desirable nutritional changes that occur during sprouting are mainly due to the breakdown of complex compounds into simple molecules, and essential constituents as well as transformation of nutritionally undesirable constituents. There are increases in the levels of B vitamins in germinated cereals (Lemar and Swanson, 1976); however, depending on the type of seed, seed quality and sprouting conditions contradictory results may be observed (Chavan and Kadam, 1989). The sprouting of seeds resulted in a decrease levels of phytic acid, polyphenols and tannins in cereals (Chavan *et al.*, 1981; Glennie, 1983) and flatulence-causing sugars (Balasubramanian and Sadasivam, 1989; Chavan and Kadam, 1989) and protease inhibitors (Mulimani and Vadiraj, 1993; Savelkoul *et al.*, 1994) in legumes and cereals.

2.7.2 Food uses

Improvements in the nutritional quality and desirable changes in the functional properties of sprouts and their dried meals have been very important in using sprouted cereals in traditional as well as non-traditional foods (Chavan and Kadam, 1989). Fresh sprouts can be used as vegetables in various dishes. The dried sprouts milled into flour can be used in preparation of various foods including bakery products, pasta and soups. Drinks prepared from malted grains are very popular among certain cultures in the world (Balasubramaniam and Sadasivam, 1989). Sprouted, dried and milled legumes have been used as partial substitutes for wheat flour in various types of foods in developing countries (Leelavathi and Haridas Rao, 1988; Chavan and Kadam, 1989). Development of low-cost weaning foods using local ingredients with high caloric density has been a focus in major nutritional programmes in developing countries. Germinated cereals and legumes have been well studied as weaning food ingredients (Marero *et al.,* 1988a,b). Such ingredients have the advantage of producing low paste viscosity, reducing dietary bulk and increasing energy density. Also the essential amino acid composition, protein quality with respect to protein efficiency ratio, net protein utilization, biological value and true digestibility of germinated products in rat-feeding studies was found to be comparable with other weaning foods available in the market. However, some of the heat-labile vitamins and lysine may be destroyed during the roller drying, but this can be rectified by supplementation or avoided by using low heat drying processes (Chavan and Kadam, 1989).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Samples

Flaxseed (*Linum usitatissimum* L., variety-Somme) was obtained from Omega Nutrition Company (Vancouver, BC). Seeds were stored in air-tight containers at ambient temperatures in the dark. Defatted meals were prepared by blending of seeds with hexane (1:5 w/v, five times) in a Commercial Waring blender (Waring Products, New Hartford, CT) at ambient temperatures. Flaxseed meals so obtained were air-dried for 12 h and stored in air-tight containers at ambient temperatures and in the dark prior to experimentation. A portion of seeds was processed using liquid cyclone (Sosulski and Zadernowski, 1981) to obtain hull-free flour. Lyophilization of samples was carried out using a freeze dryer (Labconco 5, Labconco Co., Kansas City, MO) at -95 °C, under 0.01 torr pressure. Lyophilized products were stored at 4±1 °C in air-tight containers.

3.1.2 Chemicals

All chemicals used in the present studies were of American Chemical Society (ACS) grade or better. Electrophoretic, spectroscopic and high performance liquid chromatographic (HPLC)-grade chemicals were used for analyses and preparation of reagents as required. Glass-distilled water was used for preparation of reagents. Water was demineralized and its organic matter removed using Ultrapure Bärnstead Reverse Osmosis system (Bärnstead, Boston, MA) coupled with organic removal, demineralization and submicron filtration connected to the Nanopure II system for elemental and HPLC analyses. Weight of samples and chemicals was measured using a Mettler AE100 or AE200 (Mettler Instrumente AG, Greifensee, Switzerland) balance, while all volumes were measured using appropriate measuring devices.

3.2 Methods

3.2.1 Extraction of flaxseed coat mucilage

Mucilage present in the seed coat of flax was extracted into water or aqueous solutions without disintegrating the seeds. Whole seeds (3 to 30 g) were added to 300 mL of boiled distilled water and extracted for 3 h in a wrist-action shaker (Burrel Scientific, Pittsburgh, PA) at room temperature (22 °C). Mazza and Biliaderis (1989) have shown that these conditions allow extraction of over 90% of the mucilage from flaxseed. The weight of seeds changed but the volume of water added was kept constant to make a 1 to 10% (w/v) dispersion. The extract was recovered by filtration through a layer of glass wool and the volume was made to 300 mL with distilled water.

3.2.2 Analyses of mucilage extract

3.2.2.1 Determination of pentose content of mucilage extracts

The mucilage extract (1 mL) was hydrolysed with 1 mL of 4 M HCl in a tightly capped glass vial at 100 °C for 2 h. After cooling, 0.1 to 0.2 mL of the hydrolysed sample was diluted to 3 mL with distilled water to which 3 mL of 0.1% (w/v) $FeCl_3$ in concentrated HCl and 0.3 mL of a 1.0% (w/v) solution of orcinol in absolute ethanol were added. Reagents were well mixed with the sample and allowed to develop colour at 100 °C over a 30-min period. Absorbance of the reaction mixture was read at 670 nm (Hashimoto *et al.*, 1987) using a diode-array spectrophotometer (Hewlett Packard 8452A, Hewlett Packard Co., Palo Alto, CA) and pentose content was expressed as mg D-xylose equivalents per mL of extract. The typical standard curve used for determination of D-xylose content in the reaction mixture is given in Figure A1 of Appendix 1. Reagent blanks were used for all colorimetric determinations employed.

3.2.2.2 Determination of total sugar content of mucilage extracts

A 0.05 to 0.10 mL of the mucilage extract was diluted to 1 mL and 10 mL of ice-cold anthrone reagent (0.05%, w/v, anthrone in 3.25 M H_2SO_4 containing 1.00%, w/v thiourea) were added to it followed by mixing. The reaction mixture was held at 100 °C for 10 min and the absorbance of the reaction mixture was read at 620 nm (Carrol *et al.*, 1955). The total sugar content of the mucilage extracts was calculated as mg D-glucose equivalents per mL of extract. A typical standard curve used for calculation of D-glucose content of the samples is given in Figure A2 of Appendix 1.

3.2.2.3 Determination of viscosity of mucilage extracts

Viscosity measurements were carried out using a Brookfield Synchro-Lectric viscometer (Model LVT, Brookfield Engineering Inc., Stoughton, MA). Readings were taken at the speed of 60 rpm using spindle No.1 at 22±1 °C. The viscosity values were calculated in centipoise units by multiplying the dial readings by the corresponding conversion factors provided by the manufacturer.

3.2.3 Preparation of low-mucilage flaxseed

3.2.3.1 Soaking treatment

Whole seeds were soaked in water, in 0.05, or 0.10 M $NaHCO_3$ solution at a seed-to-solvent ratio of 1:10 (w/v) for 3, 6 or 12 h in glass beakers in a water bath shaker (Model G76, New Brunswick Scientific, New Brunswick, NJ) at 200 rpm and 22±1 °C. After soaking, excess water was drained and seeds were dispersed in 0.10 M HCl (half the volume of liquid used for soaking) and allowed to stand for a few minutes before washing. The resultant slimy suspension of seeds was drained through a wire mesh (mesh size, 2 mm) to recover seeds. Seeds were rubbed against the mesh to remove more mucilage and then washed 5 times with water (1:5 w/v) at 30±1 °C. After washing and draining, seeds were dried in a forced-air oven (Fisher Isotemp 300, Fisher Scientific, Unionville, ON) at 45 °C for about 6 h. Dried seeds were then stored in "Nasco" whirl pack plastic bags (Polycello, Amherst, NS) at room temperature and in the dark. Figure 3.1 provides the flow chart for soaking treatment.

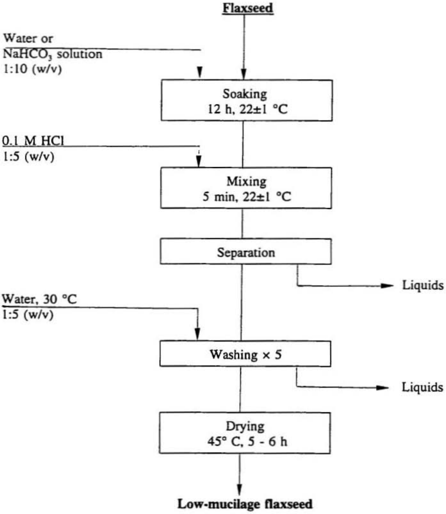


Figure 3.1 Flow sheet for preparation of low-mucilage flaxseed by soaking.

3.2.3.2 Enzyme treatment

Commercially-available carbohydrases used in this study were, Celluclast® 1.5L, Pectinex™ Ultra SP and Viscozyme® L, all of which were kindly provided by Novo Nordisk A/S (Bagsvaerd, Denmark). Details related to these enzymes are given in Table 3.1. Protein content of the enzymes were determined according to the method given in Section 3.2.11.19. Seeds were added to a 0.01 M acetate buffer of appropriate pH at a seed-to-solvent ratio of 1:5 (w/v) and incubated with different concentrations of each enzyme (mg enzyme protein/100 g seed) for 1, 3 or 6 h in a water bath shaker. The reaction was stopped by adding 50 mL of 0.10 M NaOH to the mixture. Seeds were then treated essentially in the same manner as that described for the soaking treatments. Table 3.2 provides a summary of conditions used for enzyme and soaking treatments as well as treatment combinations. Figure 3.2 provides the flow chart for enzyme treatment of flaxseed.

Mucilage remaining in the seed coat, after soaking or enzyme treatment, was extracted as previously described for untreated seeds (see Section 3.2.1). The content of pentoses and total sugars as well as viscosity of mucilage extracts were determined as described in Sections 3.2.2.1, 3.2.2.2 and 3.2.2.3, respectively.

Table 3.1 Sources, activities and optimum conditions of enzymes used.

Enzyme	Source	Mode of activity	Declared activity[a]	Optimum pH and temperature	Protein content[c] (mg/ml)
Celluclast® 1.5L Batch CCN 3041/94-10	*Trichoderma reesei*	Cellulase	1500 NCU/g[b]	4.5 - 6.0 50 - 60°C	54.0 ± 2.0
Pectinex™ Ultra SP Batch T012/94-04	*Aspergillus niger*	Polygalactouronase, hemicellulase	8800 PSU/mL	3.5 - 4.5 30 - 45°C	76.3 ± 2.5
Viscozyme® L Batch KRN 01108 /94-10	*Aspergillus* spp.	Arabanase, cellulase, β-glucanase, hemicellulase, xylanase	100 FBG/g[b]	3.3 - 5.5 40 - 50°C	90.1 ± 3.0

[a] FBG = Fungal beta-glucanase
NCU = Novo cellulose units
PSU = Pectinase S. units

[b] Specific gravity = 1.2 g/ml

[c] Determined according to Lowry's method (Section 3.2.11.19)

Table 3.2 A summary of treatments and conditions used for preparation of low-mucilage flaxseeds.

Treatment	Seed-to-solvent ratio (w/v)	Time (h)	pH	Temperature (°C)
Soaking				
0.00 M $NaHCO_3$	1:10	12	6.8	23.0
0.05 M $NaHCO_3$	1:10	12	8.2	23.0
0.10 M $NaHCO_3$	1:10	12	8.8	23.0
Enzymes (mg enzyme protein/100 g seed)				
0.0	1:5	1,3,6	-	-
22.5	1:5	1,3,6	-	-
45.0	1:5	1,3,6	-	-
Celluclast® 1.5L	1:5	1,3,6	4.8	40.0
Pectinex™ Ultra SP-L	1:5	1,3,6	4.0	25.0
Viscozyme® L	1:5	1,3,6	4.0	40.0

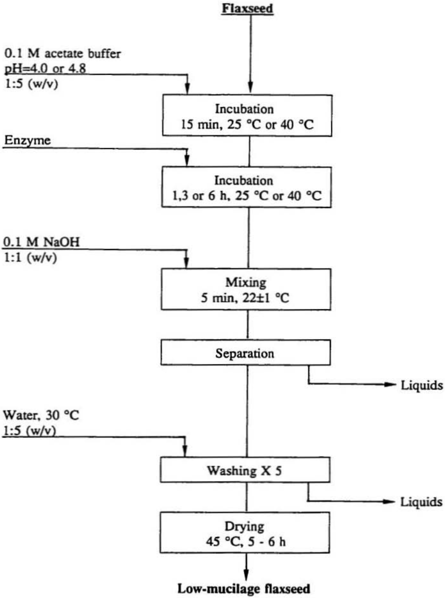


Figure 3.2 Flow sheet for preparation of low-mucilage flaxseed by enzyme treatment.

3.2.4 Determination of protein and soluble nitrogens

Defatted meals of untreated seeds, seeds soaked in 0.10 M $NaHCO_3$ solution for 12 h, and seeds treated with Viscozyme® L (22.5 mg protein/100 g for 3 h) were prepared as described earlier. About 0.3 g of meal were dispersed in distilled water (1:100, w/v) and pH of the dispersion was adjusted between 2.0 to 11.0 using 1 M solutions of NaOH or HCl. The dispersions were shaken at 200 rpm in an orbital shaker (Model G76, New Brunswick Scientific, New Brunswick, NJ) for 30 min and pH values were recorded after mixing. Samples were centrifuged at 1500 × g for 10 min and nitrogen content of the supernatant was determined by Kjeldahl analysis (AOAC, 1990; see Section 3.2.11.15). The content of soluble nitrogen was expressed as the percent ratio of nitrogen in the supernatant to that of the meal.

The pH of the remaining supernatant was adjusted to 3.5±0.1. It was then centrifuged at 4 000 × g for 10 min and nitrogen content of an aliquot of it was determined by Kjeldahl nitrogen analysis (Section 3.2.11.15). The difference in the soluble nitrogen content of supernatants before and after pH adjustment to 3.5±0.1 was considered as protein nitrogen content and expressed as percentage of protein nitrogen recovered from total nitrogen of the meal. Volume of aliquots removed for nitrogen determination was considered in calculations. Percentages of soluble nitrogen and protein nitrogen were calculated using the formula given below.

$$\text{Soluble nitrogen, \%} = \frac{\text{mg of nitrogen}_{\text{extract}}}{\text{mg of nitrogen}_{\text{sample}}} \times 100$$

$$\text{Protein nitrogen, \%} = \frac{\text{mg of nitrogen}_{\text{extract}} - \text{mg of nitrogen}_{\text{supernatant}}}{\text{mg of nitrogen}_{\text{sample}}} \times 100$$

Flour obtained by liquid cyclone process was also studied for its protein and soluble nitrogen contents.

3.2.5 Scanning electron microscopy of flaxseed coats

The untreated as well as water (12 h), $NaHCO_3$ (0.10 M, 12 h) and Viscozyme® L (22.5 g protein/100 g, 3 h)-treated seeds were manually dissected and seed coats were separated from cotyledons. Seed coat fragments were placed on aluminium stubs covered with double sided tapes to face their outermost side up and then sputter coated with gold under vacuum and observed under a Hitachi S570 scanning electron microscope operated at 20kV. The photographs were taken using a Polaroid type 665 positive/negative film.

3.2.6 Preparation of flaxseed protein isolates

3.2.6.1 Extraction of protein and preparation of isolates

Different concentrations (0.00 to 4.52%, w/v) of food-grade sodium hexametaphosphate [$(NaPO_3)_n$, SHMP], obtained from Albright and Wilson Americas (Toronto, ON), under different pH conditions (4.98 to 10.02) and meal-to-solvent ratio (R;1:13.2 to 1:46.5, w/v) were used. Flaxseed meal (1 to 2 g) was added to a solution of SHMP (0 to 4.52%, w/v) and mixed in a water bath shaker (200 rpm) for 5 min at room temperature (22±1 °C). The pH of the dispersion was then adjusted to a desired value using a 3 M HCl or 3 M NaOH solution and the extraction of the meal was continued over a 30-min period under the same conditions. After extraction, the dispersion was centrifuged at 4 000 × g for 15 min. The volume of the supernatant was recorded and its nitrogen content was determined using the Kjeldahl method (Section 3.2.11.15). The content of extracted nitrogen was calculated as the percentage of the total nitrogen in the original meal. The pH of the remaining supernatant was adjusted to 3.5±0.1 using 1 M HCl and the mixture was then centrifuged at 4 000 × g for 30 min. Nitrogen content of the supernatant was determined. Protein nitrogen was then calculated as the difference between the soluble nitrogen content before and after pH adjustment with consideration of the total volume of the extract. Results were expressed as percentage of protein nitrogen with respect to the total nitrogen in the meal that could be extracted at a particular combination of three independent variables described in Table 3.3. Sufficient quantities of flaxseed protein isolate were prepared as outlined in the flow chart (Figure 3.3) using optimum conditions decided from data analysis.

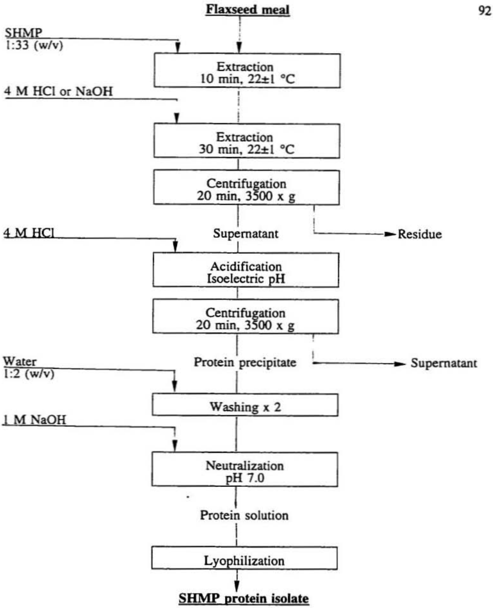


Figure 3.3 Flow sheet for preparation of SHMP protein isolate.

3.2.6.2 Experimental design and data analysis for optimization of protein extraction

A three-factor central composite rotatable design (CCRD, Figure 3.4, Box, 1954; Cornell, 1992) was employed to study two responses; percentage soluble nitrogen extracted (Y_1) and percentage protein nitrogen recovered (Y_2) from low-mucilage flaxseed (0.1 M $NaHCO_3$, 12 h soaked) meal. The pH of the extraction medium (X_1), meal-to-solvent ratio (X_2) and concentration of SHMP (X_3) were the independent variables selected to optimize Y_1 and Y_2. Table 3.3 shows the independent variables and their levels used. Duplicate extractions were carried out at all design points except at the centre point (0,0,0). Experiments were carried out in a randomized order.

A quadratic polynomial regression model was assumed for predicting individual Y variables. The model proposed for each response of **Y** was:

$$\mathbf{Y} = \beta_o + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii} x_i^2 + \sum\sum_{i<j=1}^{3}\beta_{ij} x_i x_j$$

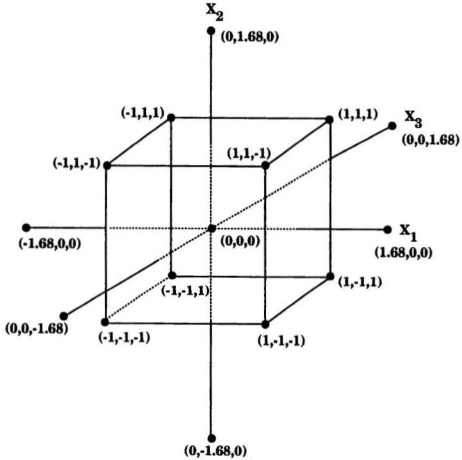
where β_o, β_i, β_{ii} and β_{ij} are intercept, linear, quadratic and interaction regression coefficient terms, respectively, and x_i and x_j are independent variables. Statistical Analytical System (SAS, 1990) was used for multiple regression analysis, analysis of variance (ANOVA), canonical analysis and analysis of ridge maximum of data in the RSREG procedure. Stepwise technique was employed for model selection using STEPWISE procedure. Response surfaces and contour plots were developed using the fitted quadratic polynomial equations obtained from RSREG analysis and holding the

Table 3.3 Independent variables and their levels used for CCRD.

Variable	Symbol	Coded-variable levels[a]				
		- 1.68(-α)	-1	0	1	1.68 (α)
pH of extraction medium	X_1	4.98	6.00	7.50	9.00	10.02
Meal-to-solvent ratio (w/v)	X_2	1:13.2	1:20	1:30	1:40	1:46.8
Sodium hexametaphosphate concentration (%, w/v)	X_3	0.0	0.50	2.00	3.50	4.50

[a] Transformation of coded variable (X_i) levels to uncoded variable (x_i) levels could be obtained from: $x_1 = 1.5X_1 + 7.5$, $1:x_2 = 1:(10X_2 + 30)$ and $x_3 = 1.5X_3 + 2$

Figure 3.4 Central composite rotatable design for three variables (Points are given as coded variable levels).


X_2
(0,1.68,0)
(-1,1,1)
(1,1,1)
X_3
(0,0,1.68)
(-1,1,-1)
(1,1,-1)
(-1.68,0,0)
(0,0,0)
X_1
(1.68,0,0)
(-1,-1,1)
(1,-1,1)
(0,0,-1.68)
(-1,-1,-1)
(1,-1,-1)
(0,-1.68,0)

independent variable with the least effect on the response at a constant value and changing the levels of the other two variables. Confirmatory experiments were carried out to validate the equation, using combinations of independent variables that were not part of the original experimental design but within the experimental region. Regression analyses of experimental and predicted responses were carried out using the GLM procedure of SAS (1990).

3.2.7 Polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (NPAGE) was performed on 7.5% (w/v) vertical polyacrylamide rod gels (5.0 × 0.5 cm, length × diameter) at pH 8.3 using a 25 mM Tris-glycine buffer. Protein samples (100 μg) were loaded onto each rod and electrophoresed (Electrophoresis Apparatus GE-4 II, Pharmacia Fine Chemicals, Uppsala, Sweden) at a constant current at 10 mA per rod supplied by Pharmacia electrophoresis constant power supply (ECPS 2000/300, Pharmacia Fine Chemicals, Uppsala, Sweden).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on rod gels composed of stacking gel (4%, w/v, 1 cm) and resolving gel (10%, w/v, 6 cm) using 0.38 M Tris-glycine buffer at pH 6.8. Protein samples were dissolved in 65 mM Tris-HCl (pH 6.8) containing 2% (w/v) sodium dodecyl sulphate (SDS) and a drop of bromophenol blue. Reduction of disulphide bridges was performed by β-mercaptoethanol (5%, v/v) at 100 °C for 3 min. Protein samples of

100 μg were loaded on to each rod and electrophoresis was conducted at a constant current of 10 mA for stacking gel and 15 mA for resolving gel until the dye travelled the same distance for all rods. The molecular weight markers used were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa). The relationship between the molecular weight of the standards and their mobility on the gel are provided in Figure 4.9 of the Results and Discussion section.

Following electrophoresis, gels were stained with Coomassie Briliant Blue R-250 in methanol/water/acetic acid (5:4:1, v/v/v) and destained in a mixture of acetic acid/water/methanol (1.5:17.5:1, v/v/v) until a desired background colour was obtained. The gels were fixed and stored in a 7% (v/v) acetic acid solution. For comparison total protein of flaxseed was prepared according to Madhusudan and Singh (1983) by extracting the meal with 1 M NaCl and extensively dialysing the extract against the same solution followed by lyophilization.

3.2.8 Preparation of acylated derivatives of flax protein isolate

3.2.8.1 Acetylation and succinylation

Flaxseed protein isolates, prepared according to the procedure described in Figure 3.3, were used for chemical modification by acetylation or succinylation. Protein isolates were acylated at room temperature with three levels of acetic or succinic anhydride (0.05, 0.10 and 0.20 g/g protein equivalents) at pH 8.5±0.1

according to the method described by Thompson and Reyes (1980) for canola proteins. A 3% (w/v) dispersion of protein isolate in distilled water was prepared and its pH was adjusted to 8.5±0.1 with 3 M NaOH. Acetic or succinic anhydride was then added slowly to the reaction mixture. The mixture was stirred for 60 min while maintaining its pH at 8.5±0.1 by addition of 1 M NaOH over a 60 min period. The reaction was terminated by lowering the pH of the mixture to 3.5±0.1 with 3 M HCl. The reaction mixture was then centrifuged for 25 min at 1 000 × g to recover the precipitated proteins. The precipitate was dispersed in distilled water, neutralized and then dialysed against distilled water over a 24-h period at 4±1 °C. Dialysed protein solutions were lyophilized and stored at 4±1 °C.

3.2.8.2 Determination of the degree of acylation

The extent of acylation (acetylation and succinylation) of free amino groups was determined according to the ninhydrin reaction (Moore and Stein, 1943). To 1 mL of protein solution 1 mL of ninhydrin reagent was added and the mixture was incubated in a 100 °C water bath for 20 min. After cooling for 5 min at room temperature, 5 mL distilled water were added to the mixture and the absorbance of the solution was read at 580 nm using water/n-propanol (1:1, v/v) as a blank. The free amino groups of unmodified and modified isolates were determined as lysine equivalents (μg/ml protein) using the standard curve given in Figure A3 in Appendix 1. The ratio of the difference of amino groups of the modified isolate to that of the unmodified isolate was calculated as percentage of amino groups of unmodified isolate and expressed as the degree of acylation as given below.

$$\text{Degree of acylation, \%} = \frac{\text{N}^{\text{o}}\text{ of amino groups}_{\text{unmodified protein}} - \text{N}^{\text{o}}\text{ of amino groups}_{\text{modified protein}}}{\text{N}^{\text{o}}\text{ of amino groups}_{\text{unmodified protein}}} \times 100$$

3.2.9 Evaluation of functional properties of modified and unmodified flax protein isolates

3.2.9.1 Colour

Lyophilized, modified or unmodified, proteins were packed evenly into glass petri dishes (60 mm diameter, 15 mm height). The Hunter **L** (110, white; 0, black), **a** (+, red; -, green) and **b** (+, yellow; -, blue) values of samples were determined by surface reflectance measurement using a Colormet colourimeter (Instrumar Engineering Limited, St. John's, NF). The unit was standardized with a B-143 white calibration tile with Hunter values of **L**, 94.5±0.2; **a**, -1.0±0.1; and **b**, 0.0±0.2.

3.2.9.2 Emulsifying activity

Emulsifying activity of protein isolates were determined according to the modified method of Pearce and Kinsella (1978) as described by Paulson and Tung (1988) for succinylated canola protein isolates. To protein dispersions (0.5%, w/v, 4 mL) in a Britton-Robinson Universal buffer (Britton, 1956) in the pH range of 3.1 to

11.3 and NaCl concentration of 0, 0.35 and 0.70 M were added 4 mL of pure corn oil. The mixture was then homogenized for 1 min at 2 000 rpm using a Polytron homogenizer. A 50 µL volume of the emulsion formed was immediately taken from the bottom of the container and diluted in 10 mL of the same buffer containing 0.10% (w/v) of SDS. Absorbances of the diluted samples were read at 500 nm using a diode array UV/VIS spectrophotometer and recorded as emulsifying activity values.

3.2.9.3 Emulsion stability

Samples for determination of emulsion stability were prepared in a similar manner to those for emulsifying activity determinations. The absorbance at 500 nm was read as soon as the emulsion was formed, i.e. zero time. Subsequently, aliquots were removed at appropriate time intervals and absorbance values were read at 500 nm. Emulsion stability was determined as the time, in min, required for absorbance at 500 nm to reach one half of that for the emulsion at zero time (i.e. half-life; Paulson and Tung, 1988).

3.2.9.4 Fat binding capacity

Fat binding capacity of the unmodified and modified protein isolates was determined by a turbidimetric method as described by Voutsinas and Nakai (1983). To lyophilized sample (40 mg) in a centrifuge tube, 1.5 mL of pure corn oil were added and the mixture was homogenized for 1 min at 8 000 rpm using a Polytron homogenizer. The protein dispersion was centrifuged at 3020 × g for 20 min after holding for 30 min at room temperature. The free oil separated after centrifugation was pipetted off and 2 mL of distilled water were added to the contents of the tube. Oil adhered to the sides of the tube was then removed with the help of a glass rod. Any oil trapped below the protein precipitate was removed by forcing it to the surface of the water. To the content of the tube was added 1 mL of 0.1 M metaphosphoric acid [$(HPO_3)_n$, 35% HPO_3, pH 2.1) which was then centrifuged at 4 200 × g for 15 min. The supernatant was pipetted off and the precipitate was washed with distilled water (3 to 4 mL) without dispersing it. Finally, the tube walls were cleaned with a cotton swab to remove any excess oil deposited. The protein precipitate was mixed well with 0.3 mL of distilled water and then 20 mL of the digestion medium (7 M urea in 50% H_2SO_4) were added to the mixture in 2 mL aliquots. The mixture was homogenized using a Polytron homogenizer for 30 s at 4 000 rpm. The homogenate was held at room temperature for 30 min and then the absorbance was read at 600 nm using the digestion mixture as blank. The aqueous supernatants removed in the previous steps were used for determination of lost protein during handling of the precipitate as given in Section 3.2.11.19 (Lowry's method; Lowry *et al.*, 1951).

The standard curve given in Figure A4 in Appendix 1 for determination of bound oil content was prepared as follows. To a series of unmodified protein (40 mg) were added 0 to 100 mg of pure corn oil (specific gravity=0.89) which was subsequently mixed with a glass rod. While the mixture was being mixed, 0.3 mL of distilled water followed by 20 mL of digestion mixture were added to the tube. The mixture was homogenized for 4 000 × g for 30 s and held at room temperature for 30 min before measuring the absorbance at 600 nm.

3.2.9.5 Whippability and foam stability

One hundred millilitres of a dispersion of protein isolates (1%, w/v) in distilled water were homogenized for 60 s using a Polytron homogenizer at 10 000 rpm. The mixture was then immediately transferred into a 250 mL measuring cylinder and the foam volume was recorded. The percentage ratio of the volume increase to that of the original volume of protein solution was calculated and expressed as foam capacity or whippability (Naczk *et al.*, 1985). Foam stability was expressed (on the basis of 100 mL of a 3%, w/v dispersion) as the volume of the foam remaining after 0.5, 5, 10, 15 and 20 min of quiescent period.

3.2.9.6 *In-vitro* digestibility

In-vitro digestibility of protein isolates was determined with trypsin-pepsin and pepsin-pancreatin according to the method of Saunders *et al.* (1973) with minor modifications. In a centrifuge tube, 1 g of protein material was suspended in 20 mL of 0.10 M HCl and mixed with 50 mg pepsin (from porcine stomach mucosa, 570 AU/mg solid) in 1 mL of 0.01 M HCl. The mixture was gently shaken at 37 °C for 48 h and then centrifuged. After removing the supernatant, solids were suspended in a solution made of 10 mL of water and 10 mL of 0.10 M sodium phosphate buffer (pH 8.0) containing 5 mg of trypsin (from porcine pancreas, 1870 BAEE units/mg solid). The mixture was gently shaken for 16 h at 23 °C in a water bath shaker. The digested mixture was then centrifuged and trichloroacetic acid (TCA) was added to the supernatant to reach a concentration of 8 M in the solution. The supernatant previously obtained from pepsin digestion was also treated in a similar manner. Precipitated proteins were removed by centrifugation at 10 000 × g for 25 min. The TCA-soluble nitrogen content of the supernatant was determined by Kjeldahl nitrogen analysis as described in Section 3.2.11.15.

For pepsin-pancreatin digestion, 250 mg of the sample were suspended in 15 mL of 0.10 M HCl containing 1.5 mg of pepsin and gently shaken for 15 min at 37 °C. Resultant solution was neutralized with 0.50 M NaOH and treated with 4 mg pancreatin (from porcine pancreas, activity equivalent to 4 × USP) in 7.5 mL of sodium phosphate buffer (0.10 M, pH 8.0). The mixture was shaken for 24 h at 37 °C in a water bath shaker and the undigested solids were separated by centrifugation, as given above. The supernatant was treated in a similar manner as described earlier for trypsin-pepsin digestion; nitrogen content was then determined by Kjeldahl analysis. *In-vitro* digestibility was expressed as percentage enzymatic digestion and as given below.

$$\text{Enzymatic digestion, \%} = \frac{\text{Nitrogen (non-protein nitrogen) released by enzyme}}{\text{Total nitrogen content of undigested sample}} \times 100$$

3.2.9.7 Solubility

To study the effect of pH and salt concentration on solubility, 1% (w/v) protein dispersions were prepared by mixing 0.25 g of the isolate with 0, 2.0 or 4.0 mL of a 25.6% (w/v) NaCl solution in order to make a final NaCl concentration of 0.0, 0.35, or 0.70 M. The pH was then adjusted with 2 M NaOH or 2 M HCl followed by addition of distilled water to reach a 25 mL volume. The protein dispersion was centrifuged at 4000 × g for 15 min. The protein content of the dispersion after centrifugation (supernatant) was determined using Lowry's method (Lowry *et al.*, 1951) as described in Section 3.2.11.19. The solubility was expressed as percent ratio of the protein content of the supernatant to that of the suspension.

3.2.9.8 Aromatic (surface) hydrophobicity

Aromatic/surface hydrophobicity of proteins was determined fluorometrically as described by Paulson and Tung (1987) for canola protein isolates. Each protein sample (2 mL) was serially diluted with 0.01 M phosphate buffer (pH 7.0) to obtain protein concentrations ranging from 1.56×10^{-2} to 5.00×10^{-2}% (w/v). Each protein concentration was prepared in two sets of test tubes and 10 μL of 1-anilino-8-naphthalene sulphonate (ANS, 8.0 mM in 0.01 M phosphate buffer, pH 7.0) were added to one set of tubes. The ANS-protein conjugate was measured using a spectrofluorometer (Perkin Elmer LS5, Perkin Elmer Co., Montréal, PQ) at 390 and 470 nm for excitation and emission, respectively. The net fluorescence intensity of each dilution was obtained by subtracting the fluorescence intensity of a protein blank measured without ANS at the same concentration. The slope of the plots of net fluorescence intensity versus protein concentration was calculated by a simple linear regression and reported as protein aromatic hydrophobicity.

3.2.10 Germination of flaxseed

Whole flaxseeds were surface sterilized by soaking in 0.5% (w/v) Javex (NaClO content of 4%, v/v) solution for 15 min, then washed well with demineralized water and spread on enamel coated plates (30 cm × 18 cm × 5 cm) with paper towels saturated with demineralized water underneath. The trays were labelled with sampling dates and arranged as for a complete randomized design (CRD, Mason *et al.*, 1989) on a table kept in the laboratory with an even light supply (375 lux). Seeds in trays were covered with paper towels and allowed to germinate. The average temperature of the room was 22±1 °C and the relative humidity was 78±2%. The trays containing seeds were supplied with ample water to maintain approximately 100% relative humidity within each tray throughout the germination period. Demineralized water was used to maintain the moisture content during germination. Samples of seedlings were withdrawn on days 0, 2, 4, 6 and 8 for further analyses. About 25 seedlings were randomly selected from each tray for each sampling day and the length of the seedlings was measured. The rest of the seedlings were lyophilized and dried samples were stored at 4±1 °C. Dried seedlings were ground using a coffee grinder to pass through

2 mm mesh and used for chemical analyses. Defatting of germinated flaxseed samples was carried out as described in Section 3.1.1.

3.2.11 Chemical analyses of flaxseed, germinated flaxseed and flax protein isolates

3.2.11.1 Amino acid composition

Samples (5 to 10 mg) of defatted meals and lyophilized protein isolates were hydrolysed in 1 mL of 6 M HCl containing 0.5% (v/v) phenol at 110 °C for 24 h under a nitrogen atmosphere (Blackburn, 1978). Hydrochloric acid in the hydrolysate was removed under vacuum and the dried sample was reconstituted with a sodium citrate buffer (0.2 M, pH 2.2) for analysis. Amino acids in the hydrolysate were separated, identified and quantified using a Beckman 121 MB amino acid analyzer (Beckman Instruments, Palo Alto, CA) equipped with a cation exchange resin column (Benson D-X 8.25, bed size 200 × 2.8 mm) at the Amino Acid Facility, Department of Biochemistry, Memorial University of Newfoundland.

To determine the content of sulphur-containing amino acids, samples were oxidized by performic acid (Blackburn, 1978) prior to their hydrolysis in a 6 M HCl solution. Cysteine and methionine were measured as cysteic acid and methionine sulphone, respectively. For tryptophan analysis, samples were prepared by hydrolysing in 1 mL of 3 M mercaptoethanesulphonic acid (Penke *et al.*, 1974) for 22 h at 110 °C under nitrogen and then neutralized with LiOH and adjusted to pH 2.2.

3.2.11.2 Ash content

Ground flaxseed samples and flaxseed protein isolates (2 to 3 g) were weighed into pre-cleaned porcelain crucibles and charred using a Bünsen burner flame. Crucibles containing the charred samples were transferred into a muffle furnace (Lab Heat, Blue M, IL) at 550 °C and maintained until the whole sample turned completely grey. Percentage ash content was calculated from the weight of the remaining residue after ashing (AOAC, 1990).

3.2.11.3 Cyanogenic glycoside content

Defatted meals (approximately 5.0 g) and protein isolates (approximately 2.5 g) were carefully weighed and extracted with boiling 80% (v/v) ethanol (1:10, w/v) over a 10 min period. After 1 min of homogenization, using a Polytron homogenizer (10 000 rpm), the homogenate was centrifuged at 4 000 × g for 10 min and the supernatant collected. The residue was re-extracted twice. Combined supernatants were evaporated at 40 °C under vacuum and the residue was dissolved in 10 mL of methanol. To this solution, 20 mL of chloroform were added and mixed well. Precipitated polar compounds were removed by centrifugation at 4 000 × g for 5 min. The recovered supernatant was evaporated to dryness under vacuum and redissolved in 4 mL of 15% (v/v) HPLC-grade methanol in water. For HPLC analysis, samples were filtered through a 45 μm nylon filter (Cameo 11, MSI, Westboro, MA). Linamarin (Sigma Chem. Co., St. Louis, MI), linustatin and neolinustatin, obtained from flaxseed according to Amarowicz *et al.* (1993a), were used as standards.

A Shimadzu (Shimadzu Co., Kyoto, Japan) HPLC system consisting of two pumps (model LC-6A) with a mixing chamber, an autoinjector (model SIL-6B), a system controller (model SCL-6B) and a chromatographic data processor (model CR501) were used. The detector used was a differential refractometer (model R401, Waters Associates, Milliford, MA). Cyanogenic glycosides of extracts were separated on a CSL-spherisorb-ODS2 reversed phase analytical column (10 µm particle size, 4.5 mm × 24 mm, Chromatographic Specialities Inc, Brockville, ON) coupled with a guard column (4.5 mm × 5 cm) of similar type. Solvent used was water/methanol (85:15, v/v); flow rate, 0.8 mL/min; injection volume, 20 µL and analysis time, 14 min.

3.2.11.4 Dietary fibre content

Dietary fibre content of samples was determined, using the analytical kit supplied by Sigma (Sigma Chem Co., St. Louise, MO), according to the method of Prosky *et al.* (1985). Determinations were replicated four times. Approximately 1 g of defatted material was added to 50 mL of a 0.05 M phosphate buffer (pH 6.0) in a 400 mL glass beaker along with 0.2 mL of α-amylase (Sigma Chem Co., St. Louise, MO) solution and mixed well. The beaker was covered with aluminium foil and placed in a boiling water bath for 30 min and the contents were mixed at 5 min intervals. The solutions were cooled to room temperature and pH was adjusted to 7.5±0.1 using 0.2 M NaOH. A 0.1 mL of protease (50 mg/mL, Sigma Chem Co., St. Louise, MO) in

0.05 M phosphate buffer (pH 6.0) was then added and incubated at 60 °C for 30 min with continuous mixing. After cooling to room temperature, the digestion mixture was adjusted to pH 4.5±0.2 with 0.2 M phosphoric acid. Amyloglucosidase (0.3 mL, Sigma Chem Co., St. Louise, MO) was then added to the sample and incubated at 60 °C for 30 min with continuous agitation. After incubation, 280 mL of 95% (v/v) ethanol at 60 °C were added to the mixture and allowed to precipitate at room temperature for 12 h. The solution was filtered through a clean pre-weighed sintered crucible (American Standards for Testing Materials: ASTM 4-5.5) under suction. The residue remained in the crucible was washed with three 20 mL portions of 78% (v/v) ethanol, two 10 mL portions of 95% (v/v) ethanol and then two 10 mL portions of acetone. Crucibles containing the washed residue were dried overnight at 105 °C in an oven and weighed after cooling. Residues left in two crucibles were used for nitrogen content determination by Kjeldahl method as described in Section 3.2.11.15 and two other crucibles were subjected to determination of ash by ignition at 525 °C for 5 h. A blank determination was carried out in a similar manner. The percentage content of total dietary fibres was calculated as given below.

Total dietary fibre, % =

$$\frac{\text{weight}_{\text{sample residue (mg)}} - \left[\dfrac{(\%\ \text{protein}_{\text{sample residue}} + \%\ \text{ash}_{\text{sample residue}})}{100} \times \text{weight}_{\text{sample residue (mg)}}\right] - \text{blank}}{\text{weight}_{\text{sample (mg)}}} \times 100$$

Where,

$$\text{blank} = \text{weight}_{\text{blank residue (mg)}} - \left[\frac{(\%\ \text{protein}_{\text{blank residue}} + \%\ \text{ash}_{\text{blank residue}})}{100} \times \text{weight}_{\text{blank residue (mg)}}\right]$$

$$\%\ \text{ash}_{\text{blank residue}} = \frac{\text{weight of ash}_{\text{blank (mg)}}}{\text{weight of residue}_{\text{blank (mg)}}} \times 100$$

$$\%\ \text{ash}_{\text{sample residue}} = \frac{\text{weight of ash}_{\text{sample (mg)}}}{\text{weight of residue}_{\text{sample (mg)}}} \times 100$$

$$\%\ \text{protein}_{\text{blank residue}} = \frac{\text{weight of protein}_{\text{blank (mg)}}}{\text{weight of residue}_{\text{blank (mg)}}} \times 100$$

$$\%\ \text{protein}_{\text{sample residue}} = \frac{\text{weight of protein}_{\text{sample (mg)}}}{\text{weight of residue}_{\text{sample (mg)}}} \times 100$$

3.2.11.5 Fatty acid composition of lipids

Fatty acid methyl esters (FAMEs) of total lipids (Section 3.2.11.10) and lipid fractions, obtained from column chromatography (Section 3.2.11.11), were prepared by transmethylation in 6% (v/v) H_2SO_4 in 99.9 mole% methanol (HPLC grade) at 65 °C for 15 h (Keough and Kariel, 1987). FAMEs were then extracted into hexane and separated using a gas chromatograph (Hewlett-Packard 5890 Series II, Hewlett-Packard, Mississuaga, ON) equipped with a fused silica capillary column (Supelcowax 10, 0.25 mm x 60 m, 0.25 µm film thickness; Supelco, Oakville, ON), a flame ionization detector and a split/splitless injector. The chromatographic parameters were: detector and injector temperatures, 250 °C; oven temperature programming, hold for 10.25 min at 220 °C then ramped to 240 °C at 30 °C/min followed by a hold period of 9 min. Total run time was 19.92 min and helium was used as a carrier gas. The FAMEs were identified by comparison with relative retention times of reference FAMEs (Supelco, Oakville, ON). The content of each identified fatty acid in the sample was calculated from the integration data of the chromatographed fatty acids.

3.2.11.6 Free amino acid (total) content

Germinated flaxseed samples (1 to 2 g) were extracted with 20 mL of 6% (v/v) perchloric acid (PCA) solution by homogenization using a Polytron homogenizer (10 000 rpm, 2 min) at 5 °C. Extracts were centrifuged at 4 100 × g for 20 min and solids were re-extracted with another 10 mL of PCA. The supernatants were combined and pH was adjusted to 10.0 using a 33% (w/v) KOH solution. The resultant solution was centrifuged to remove any precipitate and the supernatant was decanted and its pH adjusted to 2.0 with 3 M HCl. The total volume of the extract was brought to 25 mL with distilled water.

The total content of free amino acids in the final extract was determined colorimetrically using ninhydrin reagent as outlined in Section 3.2.8.2. Free amino acid content of the samples was calculated as L-leucine equivalents per g of sample using the standard curve given in Figure A5 of Appendix 1.

3.2.11.7 Free fatty acid (total) content

Total lipids (5 to 10 mg) obtained from Folch extraction (Folch *et al.*, 1957; see Section 3.2.11.10) were dissolved in 5 mL of chloroform in a 15 mL screw capped pre-cleaned glass vial. The free fatty acid content of lipids was determined colorimetrically according to the method of Lowry and Tinsley (1975). To lipids dissolved in chloroform, 2.5 mL of copper reagent (1 M triethanolamine: 1 M acetic acid: 6.5%, w/v cupric nitrate trihydrate, 9:1:10, v/v/v) were added and mixed for 2 min by vortexing. The mixture was then centrifuged for 5 min at 1 500 × g. Three millilitres of clearly separated chloroform layer were carefully transferred to another tube in order to prevent contamination from the upper aqueous layer. The chloroform layer was mixed well with 0.5 mL of sodium diethyldithiocarbamate in 2-butanol (0.1%, w/v) and the absorbance of the chloroform layer was read at 440 nm using a reagent blank containing of 3 mL of chloroform and 0.5 mL of sodium diethyldithiocarbamate in 2-butanol (0.1%, w/v). The total content of free fatty acids in the sample was determined as linoleic acid equivalents using the standard curve given in Figure A6 of Appendix 1.

3.2.11.8 Individual free amino acids and their contents

Sample preparation for determination of individual free amino acids was similar to that described in Section 3.2.11.6 for total free amino acids. The extract was filtered through a 0.45 μm nylon filter to eliminate any turbidity. One millilitre of lithiumcitrate buffer (pH 2.2, 0.3 M) was added to 2 mL of the filtered extract and the resultant solution was analyzed for individual amino acids using a Beckman 121 MB amino acid analyzer.

3.2.11.9 *In-vitro* digestibility

In-vitro digestibility of germinated flaxseed by pepsin-pancreatin was determined essentially in a similar manner to that described in Section 3.2.9.6.

3.2.11.10 Lipid (total) content

Total lipids of flaxseed were extracted according to the Folch method (Folch *et al.*, 1957). One to two grams of each sample were homogenized in 10 mL of methanol using a Polytron homogenizer at 10 000 rpm for 1 min and then for a further 2 min after addition of 20 mL of chloroform. The homogenate was then filtered under suction and the residue was re-suspended in 30 mL of chloroform/methanol (2:1, v/v) and homogenized for 3 min and filtered under suction. Residues were washed with 30 mL of the same solvent system. Filtrates were combined and mixed well with a 0.88% (w/v) KCl solution (25% of the total volume of filtrate) in a separatory funnel. The lower chloroform layer was withdrawn and washed with a methanol/water (1:1, v/v, 25% of total volume of chloroform layer) mixture. The washed chloroform layer was passed through an anhydrous sodium sulphate bed (1 cm thick). The chloroform was removed in a pre-weighed 50 mL round bottom flask using a rotary evaporator

(Rotavapor RE111, Büchi Laboratorium Technik, Schweiz, Switzerland) and the weight of the extracted oil was determined by difference. Lipid content (%) of samples was then calculated from the weights of the oil and the original sample.

3.2.11.11 Separation of major lipid classes by column chromatography

Lipid classes were separated from total lipids extracted by the Folch method according to Christie (1982). Samples (2.0 g) of total lipids (see Section 3.2.11.10) were applied onto a silicic acid column (1.25 cm internal diameter and 20 cm height; 100 mesh silicic acid powder, Mallinckrodt Canada Inc., Point Claire, PQ). The neutral lipid (NL) fraction was first eluted with chloroform (48 times of the column bed volume). Monogalactosyl diacylglycerols and digalctosyl diacylglycerols (GL) were then eluted with chloroform-acetone (50:50, v/v, 20 times the column bed volume) and acetone (48 times the column bed volume), respectively. Finally the phospholipid (PL) fraction was eluted with methanol (48 times the column bed volume). The solvents were removed from each fraction under vacuum using a rotary evaporator at 40 °C. All fractions were weighed and their weight percentage was calculated.

3.2.11.12 Identification of compounds in major lipid classes by thin-layer chromarod-Iatroscan (TLC-FID)

3.2.11.12.1 Instrumentation

The total lipids obtained from Folch extraction (Section 3.2.11.10) were chromatographed separately on Chromarod S-III and then analysed on an Iatroscan® MK-5 (Iatron Laboratories Inc., Tokyo, Japan) analyzer equipped with a flame ionization detector (FID) connected to a computer loaded with T-datascan software (Scientific Products and Equipment, Concord, ON) for data handling. The FID was operated using a hydrogen flow rate of 160 mL/min and an air flow rate of 2 000 mL/min. The scanning speed of rods was 30 s/rod. The Iatroscan was fitted with a push button switch to interrupt scanning at anytime when required, especially for partial scanning.

3.2.11.12.2 Preparation of chromarods

The chromarods were cleaned by soaking in concentrated nitric acid overnight followed by thorough washing with distilled water and then acetone before use. The rods were scanned twice to burn off any impurities left on them and to obtain a standard silica gel. Chromarods were impregnated with boric acid by dipping in a 3% (w/v) boric acid solution for 5 min and then drying at 120 °C for 5 min. Finally, rods were scanned twice to burn off any residual impurities.

3.2.11.12.3 Standards and calibration

A composite stock solution of neutral lipids [free fatty acid (FFA;oleic acid), monoacylglycerol (MAG;monoolein), diacylglycerol (DAG;diolein) and triacylglycerol (TAG;triolein) and phospho lipids (LPE;lysophosphatidylethanolamine, LPC;lysophosphatidylcholine, PA;phosphatidic acid, PC;phosphatidylcholine, PE;phosphatidylethanolamine, PI;phosphatidylinositol and PS;phosphatidylserine) were dissolved in $CHCl_3/CH_3OH$ (2:1, v/v) and stored under nitrogen at -20 °C. Different dilutions of the stock solution ranging from 0.1 to 10 µg/µL of lipid mixture were used as working standards. Before making the composite standard mixture, each compound was developed individually and run on the Iatroscan-FID to determine its purity and *Rf* value. The samples dissolved in appropriate solvents were spotted on rods using Drummond microcap disposable pipettes (Drummond Scientific Co., Broomall, PA). As soon as the samples were spotted, solvents were dried off using a stream of cold air supplied by a blow dryer. The rods containing samples were placed in a humidity tank over saturated $CaCl_2$ for 10 min and then immediately transferred to the developing tank.

3.2.11.12.4 Iatroscan (TLC-FID) analyses of flaxseed lipids

Total lipids were extracted from ungerminated and germinated flaxseeds (Folch method, 1957) as described in Section 3.2.11.10. Lipids were dissolved in chloroform/methanol (2:1, v/v) in order to obtain a concentration of 1 µg lipid/µL. The sample (1 µL/rod) was applied on nine out of the ten rods and a randomly selected rod was used for standard mixture.

Three solvent systems were employed to obtain three separate chromatograms per rod. The first development of rods was carried out in benzene/chloroform/acetic acid (70:30:2, v/v/v; Fraser *et al.*, 1985). The chromarods were then dried at 110 °C for 3 min and partially scanned to a point just beyond the monoacylglycerol peak to reveal neutral lipids. The second development was carried out in acetone (100%; Christie, 1982), chromarods were dried at 110 °C for 3 min and the scanning was done to the lowest point beyond the acetone-mobile lipid peak. Final development of chromarods was carried out twice using chloroform/methanol/water (70:30:3, v/v/v; Parrish, 1987) followed by drying at 110 °C for 5 to 7 min. The chromarods were scanned completely to reveal phospholipids and less mobile lipid components.

Peak areas of unknown compounds were calculated as weight percentages using conversion factors established with the calibration lines of the authentic standards for neutral lipids and phosholipids (Figures A7 and A8, Appendix 1). Each point on the calibration line was the mean value of 7 to 10 analyses.

3.2.11.13 Determination of mineral constituents

3.2.11.13.1 Preparation of samples for mineral analysis

Germinated, dried and ground flaxseed samples (1 to 2 g) were subjected to dry ashing in well cleaned (soaked in chromic-sulphuric acid solution for 48 h followed by thorough washing with demineralized water and igniting at 450 °C) porcelain crucibles at 550 °C in a muffle furnace. The resultant ash was dissolved in 5 mL of $HCl/H_2O/HNO_3$ (2:3:1, v/v/v) and warmed on a hot plate until brown fumes disappeared. To the remaining content in each crucible 5 mL of deionized water were added and heated until a colourless solution was obtained. The mineral solution in each crucible was transferred into a 100 mL volumetric flask by filtering through a Whatman number 42 filter paper and further diluted to 100 mL with deionized water.

3.2.11.13.2 Elemental analysis by Atomic Absorption Spectrophotometry

The concentration of elements (Ca, Cu, Fe, K, Mg, Mo, Mn, Na and Zn) in each solution, prepared as described in Section 3.2.11.13.1, was determined using a Perkin-Elmer 8650 atomic absorption spectrophotometer (Perkin Elmer Co., Montréal, PQ). The parameters used for the analysis of each element are given in Table 3.4. Calibration curves of absorbance values versus concentration of each element at appropriate concentrations (to obey Beer's-Lambert law) were constructed using their respective standards of 1 000 µg/L (Fisher scientific, Unionville, ON). A path length of 10 cm was used and concentration of each element in samples was calculated as µg per g of dry matters.

3.2.11.14 Moisture content

Flaxseed samples were ground into fine particles (passed though mesh size 1 mm) using a coffee grinder (Moulinex). Ground samples (2.0 to 3.0 g) were dried in,

Table 3.4 Analytical parameters used in atomic absorption spectrophotometry.

Element	Wavelength (nm)	Slit width (nm)	Oxidant-fuel of premix
Ca	422.7	0.7	Air-acetylene
Cu	324.8	0.7	Air-acetylene
Fe	248.3	0.2	Air-acetylene
K	766.5	0.7	Air-acetylene
Mg	285.2	0.7	Air-acetylene
Mn	279.5	0.2	Air-acetylene
Mo	313.3	0.7	N_2O-acetylene
Na	589.0	0.2	Air-acetylene
Zn	213.9	0.2	Air-acetylene

pre-weighed aluminium moisture determination pans (57 mm, Fisher Scientific Unionville, ON) in a forced-air oven at 104±1 °C for 18 h until a constant weight was obtained. Moisture content was calculated as percentage weight loss (moisture) of the samples during drying (AOAC, 1990).

3.2.11.15 Nitrogen content

Total nitrogen content of meals, SHMP-extracts, and lyophilized protein isolates was determined by Kjeldahl analysis (AOAC, 1990). Samples (100 to 250 mg of dried samples and 5 to 10 mL of liquid samples) were digested in 20 mL concentrated sulphuric acid in the presence of two catalyst pellets (Kjeltabs, Profamo Analytical Service Inc., Dorval, PQ) containing 5 g of K_2SO_4 and 0.25 g of HgO in a digester (Büchi 430, Büchi Laboratorium-Technik AG, Schweiz, Switzerland) until a clear solution was obtained. To the digested samples, 50 mL of distilled water and 150 mL of 25% (w/v) NaOH were added in a distillation tube (Büchi 321, Büchi Laboratorium-Technik AG, Schweiz, Switzerland). Samples were then steam-distilled and the distillate was collected into 50 mL of a 4% (w/v) boric acid solution containing 1 mL of indicator (N point indicator, EMSCI, Gibbstown, NJ). One hundred and fifty millilitres of distillate were collected and titrated against 0.025 M H_2SO_4 to reach the end point. A sample blank (distillate collected with distilled water and NaOH only) determination was also carried out. Nitrogen content of samples were calculated as given below.

Dried samples:

$$\%\ \text{Nitrogen} = \frac{\text{Volume}_{\mathrm{H_2SO_4}}\ (\text{sample} - \text{blank})\ \text{mL} \times \text{Normality}_{\mathrm{H_2SO_4}} \times 14.007}{\text{Sample weight mg}} \times 100$$

Liquid samples:

$$\%\ \text{Nitrogen} = \frac{\text{Volume}_{\mathrm{H_2SO_4}}\ (\text{sample} - \text{blank})\ \text{mL} \times \text{Normality}_{\mathrm{H_2SO_4}} \times 14.007 \times \text{Total volume mL}}{\text{Sample weight mg}} \times 100$$

$$\%\ \text{Crude protein} = \%\ \text{Nitrogen} \times 6.25$$

3.2.11.16 Non-protein nitrogen (NPN) content

To approximately 1 g of defatted flaxseed sample, a 12% (w/v) solution of trichloroacetic acid (TCA, 20 mL) was added and the NPN compounds were extracted using a wrist-action shaker for 30 min at room temperature. Precipitated protein and other insoluble materials were separated by centrifugation at 4 000 × g for 10 min. The supernatant was filtered through a layer of glass wool to remove any remaining suspended particles. The volume of the supernatant was recorded and its nitrogen content was determined by Kjeldahl analysis (Section 3.2.11.15). Non-protein nitrogen content of each sample was then calculated as percentage ratio of dissolved nitrogen in TCA to the total nitrogen content of the sample (Naczk *et al.*, 1985).

3.2.11.17 Phosphorus content

Samples (approximately 1 g) were digested in 10 mL of a perchloric acid/nitric acid (1:2, v/v) mixture and digested in a Kjeldahl digester until a clear solution was obtained. Twenty millilitres of demineralized water were added to digested samples and the mixture was boiled for 5 min. Subsequently, the mixture was filtered through a filter paper (Whatman number 42) and diluted to 100 mL with demineralized water (AOAC, 1990). Phosphorus content of the digest was determined colorimetrically according to the method described by Nahapetian and Bassiri (1975). To 1 mL of the diluted digest, 4 mL of demineralized water, 3 mL of 0.75 M H_2SO_4, 0.4 mL of 10% (w/v) $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and 0.4 mL of 2% (w/v) ascorbic acid were added and mixed. The solution was allowed to stand for 20 min and absorbance readings were recorded at 660 nm. The content of phosphorus in the extracts was determined using a standard curve obtained for KH_2PO_4 (Figure A9, Appendix 1) and expressed as mg phosphorus per gram of sample.

3.2.11.18 Phytic acid content

Phytic acid in the meals and protein products was extracted according to the method of Tongkonchitr *et al.* (1981) as modified by Naczk *et al.* (1986). Approximately 2 g of each meal were extracted with 40 mL of 1.2% HCl containing 10% (w/v) Na_2SO_4 for 2 h using a wrist-action shaker. The slurry was centrifuged for 20 min at 4 000 × g to separate insoluble particles. Five millilitres of the supernatant were mixed with 5 mL of distilled water and 6 mL of $FeCl_3\cdot 6H_2O$ in 0.07 M HCl solution. The mixture was then heated in a boiling water bath for 45 min and then cooled to room temperature. The resulting ferric phytate precipitate was collected by centrifugation at 4 000 × g for 15 min and the supernatant was discarded. The precipitate was mixed well with 5 mL of 4% (w/v) Na_2SO_4 in 0.07 M HCl and the mixture was centrifuged again. The recovered ferric phytate precipitate was digested using 6 mL of 1:1 (v/v) mixture of concentrated H_2SO_4 and concentrated HNO_3. The digestion was terminated when white fumes hung over the liquid. A 10 mL portion of distilled water was added to the warm digest and the solution was heated in a boiling water bath for 30 min to destroy the pyrophosphate complex and the mixture was then diluted to 100 mL with distilled water. The phosphorus content of the extract was determined as given in Section 3.2.11.17 and its phytic acid content (%) was calculated by multiplying phosphorus content by a factor of 3.55 which is derived from the empirical formula of phytic acid, $C_6P_6H_{18}O_{24}$.

3.2.11.19 Protein content (Lowry's method)

Protein solution (0.5 mL) was added to 2.5 mL mixed reagent of 2% (w/v) Na_2CO_3 in 0.1 M NaOH:0.5% (w/v) $CuSO_4 \cdot 5H_2O$ in 1% (w/v) sodium tartrate (50:1) and kept for 10 min at room temperature after thorough mixing. To the reaction mixture 0.25 mL of Folin-Ciocalteau reagent (1:1 dilution with distilled water) were added, mixed well and kept at room temperature for 30 min. Absorbance of the reaction mixture was read at 750 nm and the protein content of the sample was calculated from the standard curve prepared using bovine serum albumin (BSA) as illustrated in Figure A10 of Appendix 1.

3.2.11.20 Identification and quantification of polyamines

Defatted samples (1 to 2 g) were extracted with 10 mL of a 6% (v/v) perchloric acid by homogenization at 5 °C (10 000 rpm, 1 min), using a Polytron homogenizer. Insoluble meal particles and precipitated proteins were removed by centrifugation at 2 000 × g for 15 min. The supernatant was first filtered through a layer of glass wool and then a 0.45 μm nylon filter and diluted to 25 mL with HPLC-grade water. The polyamines in each solution were separated on a 5 μm particle size Beckman Ultrasphere C_{18}-IP analytical column (4.6 × 250 mm) coupled with a guard column (ODS-C_{18}, 5 μm, 2.1 × 70 mm). A gradient solvent system consisting of a 0.1 M sodium acetate (pH 4.5) containing 10 mM octanesulphonic acid (OSA) and 10% (v/v) methanol (solvent-A) and 0.2 M sodium acetate (pH 4.5) containing 10 mM OSA and acetonitrile (10:3, v/v) and 10% (v/v) methanol (solvent-B) was used. The gradient composition used for separation is given Table 3.5. Post-column derivatisation of separated polyamines was carried out with *o*-phthalaldehyde/2-mercaptoethanol reagents. Polyamine derivatives were detected using a fluorescence detector (Model 420, Waters, Milford, MA) and excitation at 345 nm and emission at 455 nm were monitored. A 10 μl flow cell maintained at 1 mL/min flow rate was used. Peaks were identified and recoveries calculated by spiking the sample with known amounts of polyamines agmatine, cadaverine, histamine, putrescine, spermidine and spermine

Table 3.5 Composition of solvent gradient for HPLC analysis of polyamines[1].

Programme	Time (min)	Flow rate (mL/min)	Percentage of each solvent	
			A	B
Initial	0	1.0	100	0
	12.0	1.0	100	0
	16.0	1.0	60	40
	36.0	1.0	60	40
	48.0	1.0	0	100
End	65.0	1.0	100	0
Equilibration	97.0	1.0	100	0

[1] Solvent A: 10 mM OSA in 0.1 M sodium acetate, (a); methanol (b); 9:1 (a+b=A)
Solvent B: 10 mM OSA in 0.2 M sodium acetate/acetonitrile, 10:3 (v/v), (a); methanol (b); 9:1 (a+b=B)

(Sigma Chem. Co., St. Louise, MO) which were dissolved in 0.2 M PCA. A Beckman (Beckman Scientific, Irvine, CA) HPLC system consisting of a pump (Beckman) for post-column derivatisation and two other pumps (Model 510, Waters, Milford, MA) with a mixing chamber for solvent gradient and an autosampler (Model 231-401, Mandel Scientific, Toronto, ON) was used.

3.2.11.21 Separation of protein classes based on their solubility characteristics

Protein classes of germinated and ungerminated flaxseeds were determined according to their solubility using a modified Osborne classification procedure as described by Lund and Sandstrom (1943). Defatted and dried flaxseed samples (approximately 2 g) were dispersed and extracted into 25 mL of distilled water over a 15-min period at room temperature (22 °C) using a shaking water bath. The suspension was then centrifuged at 4 000 × g for 10 min and the supernatant was recovered and saved. The residues were re-extracted two more times with the same solvent under similar conditions and recovered supernatants were combined and regarded as the water-soluble fraction. The residue was then extracted successively with 5% (w/v) NaCl, 70% (v/v) ethanol at 65 °C and 0.2% (w/v) NaOH in a similar manner as for the water-soluble fraction; respective soluble fractions were collected separately. The total nitrogen contents of the supernatants collected and the residue left after sequential extractions were determined using the Kjeldahl method as described in Section 3.2.11.15. The content of each protein fraction was calculated as a percentage of the total nitrogen content (as sum of nitrogen content of all fractions including residue) of the meal.

3.2.11.22 Soluble sugar content

Defatted meals (approximately 5.0 g) and protein isolates (approximately 2.5 g) were extracted with 80% (v/v) boiling ethanol (1:10, w/v) over a 10 min period. After 1 min of homogenization using a Polytron at 10 000 rpm, the homogenate was centrifuged at 4 000 × g for 10 min and the supernatant was collected. The residue was re-extracted two more times in a similar manner. Combined supernatants were diluted to 100 mL with 80% (v/v) ethanol. Aliquots of the extracts were used for determination of sugar content by the anthrone method as described in Section 3.2.2.2. The content of total soluble sugars was expressed as mg D-glucose equivalents per gram of sample (Shahidi *et al.*, 1990).

3.2.11.23 Composition of soluble sugars

Soluble sugars were extracted into 80% (v/v) ethanol as described in Section 3.2.11.22. The extracts were combined and evaporated to dryness under vacuum using a rotary evaporator and the resultant residues were dissolved in 1 mL of methanol. Sugars in the extracts were separated on a LiChroCART (Merck, 4 × 250 mm, 7 μm) analytical column using acetonitrile/water (77:23, v/v) as the mobile phase. The system used for the analysis was a Shimadzu HPLC system equipped with two LC-6A pumps, RID-6A refractive index detector and a C-R4A Chromatopac for data handling. A 20 μL sample and a flow rate of 1 mL/min were used for the analysis. The sugars in the extract were detected by the refractive index detector and tentatively identified by comparing their retention times with those of known standards (Sigma Chem. Co., St. Louise, MO).

3.2.11.24 Determination of trypsin inhibitors

The germinated, dried and ground flaxseed samples were extracted with a 0.05 M potassium phosphate buffer containing 0.5 M NaCl (pH 8.0, 1:50, w/v) for 2 h using a water bath shaker maintained at 22±1 °C (Roozen and de Groot, 1991). The suspension was centrifuged for 15 min at 25 000 × g and 5 °C. Affinity chromatography was used to determine trypsin inhibitors in the supernatants. Trypsin-sepharose 4B (cyanogen bromide activated, Sigma Chem Co., St. Louise, MO) suspended in 0.1 M NaCl was packed in 5 mL Spectra/Chrom (Fisher Scientific, Unionville, ON) disposable columns (9 mm internal diameter, 2 mL gels) and washed with 20 mL of a 0.05 M phosphate buffer at pH 8.0. Twenty millilitres of the extract were applied onto the column and eluted first with 20 mL of a phosphate buffer (pH 8.0) followed by 20 mL of 0.05 M acetate buffer containing 0.5 M NaCl (pH 5.2) and 20 mL of 0.05 M glycine-HCl buffer containing 0.5 M NaCl (pH 3.0). The last eluate with glycine-HCl buffer was collected into a 25 mL volumetric flask and diluted with the same buffer. The protein content of the eluate was determined using Lowry's method (Section

3.2.8.2) and a bovine serum albumin (BSA) standard curve (Figure A10, Appendix 1). The content of trypsin inhibitor (TI) in samples was calculated as mg TI per gram of crude protein as follow:

$$\text{mg Trypsin inhibitor/ g crude protein} = \frac{a \times 25 \times V_1}{1000 \times V_2 \times g \times M}$$

Where, a = concentration of trypsin inhibitor in the eluate (µg/mL)

V_1 = volume of phosphate buffer used to extract sample (mL)

V_2 = volume of extract eluted on the column (mL)

g = weight of sample used for extraction (g)

M = weight (g) of crude protein per g of sample

3.2.12 Evaluation of nutritional parameters of germinated flaxseed

The amino acid composition of samples determined according to the procedure described in Section 3.2.11.1 was used for calculation of the nutritional value of flax proteins as summarised below.

(i) The proportion of essential amino acids (E) to the total amino acids (T) of the protein:

$$\text{E/T, \%} = \frac{\text{Ile} + \text{Leu} + \text{Lys} + \text{Met} + \text{Cys} + \text{Phe} + \text{Tyr} + \text{Thr} + \text{Trp} + \text{Val}}{\text{Ala} + \text{Asp} + \text{Arg} + \text{Gly} + \text{Glu} + \text{His} + \text{Ile} + \text{Leu} + \text{Lys} + \text{Met} + \text{Cys} + \text{Phe} + \text{Tyr} + \text{Pro} + \text{Ser} + \text{Thr} + \text{Trp} + \text{Val}}$$

(ii) $$\text{Amino acid score} = \frac{\text{mg of amino acid per g test protein}}{\text{mg of amino acid per g of FAO/WHO standard pattern}} \times 100$$

Limiting essential amino acids were determined based on their amino acid scores. The first limiting amino acid is the one with least amino acid score. Essential amino acid (g amino acid/16 g N) pattern of the FAO/WHO standard protein is Ile=4.00, Leu=7.04, Lys=5.44, Met+Cys=3.52, Phe+Tyr=6.08, Thr=4.00, Trp=0.96 and Val=4.96.

(iii) Biological value (BV)

The following regression equation (Morup and Olesen, 1976) was used for prediction of BV.

$$BV = 10^{2.15} \times q_{Lys}^{0.41} \times q_{Phe+Tyr}^{0.60} \times q_{Met+Cys}^{0.77} \times q_{Thr}^{2.4} \times q_{Trp}^{0.21}$$

Where,

$$q_i = \frac{ai_{\text{sample}}}{ai_{\text{reference}}} \qquad \text{for} \quad ai_{\text{sample}} \leq ai_{\text{reference}}$$

$$q_i = \frac{ai_{\text{reference}}}{ai_{\text{sample}}} \qquad \text{for} \quad ai_{\text{sample}} \geq ai_{\text{reference}}$$

ai = mg of the amino acid per g of total essential amino acids

(iv) Computed protein efficiency ratio (C-PER)

The C-PER values were calculated from the essential amino acid composition and *in vitro* digestibility data according to the procedure of Satterlee *et al.* (1982). Each amino acid was calculated with respect to FAO/WHO standard values and *in vitro* digestibility of the protein as [(g amino acid/16 g N) ÷ FAO/WHO standard value] × *in vitro* digestibility (%) and considered as %FAO/WHO. Standard amino acid values of FAO/WHO are given in Section 3.2.12 (ii). When combining Met+Cys and Phe+Tyr, Cys and Tyr were considered to be 50% of Met+Cys and Phe+Tyr totals, respectively. Casein (essential amino acid composition is Ile=5.40, Leu=9.50, Lys=8.50, Met+Cys=3.50, Phe+Tyr=11.10, Thr=4.40, Trp=1.4 and Val=6.3 g aa/16 g N; *in vitro* digestibility, 80.80%) was used as the reference protein. The above calculated values were rounded to the nearest integer and any value above 100 was considered as 100. Then the values of X and Y were calculated as X = [(1 ÷ %FAO/WHO for each amino acid) (W value)] and $Y = \sum$ W values. The W values used in the calculation were decided according to the following given by Satterlee *et al.* (1982).

% FAO/WHO	W value
≥100	1
91-99	2
81-90	2.83
71-80	4
61-70	5.66

51-60	8
41-50	11.31
31-40	16
21-30	22.63
11-20	32
0-10	45.25

Then the essential amino acid score was calculated as *Y/X* separately for sample and casein. The ratio of the essential amino acid score of the sample to the amino acid score of casein was also determined. The *Z* value was computed by multiplying the ratio of essential amino acids of sample to those of casein by a factor of 2.5. Then the 4 discriminant values to determine the group for sample classification was determined using the following discriminant equations.

Group 1
= −671.8418 − 6.57689(Lys) + 3.56696(Met+Cys) + 13.10145(Thr) + 2.54503(Ile) + 16.99881(Leu) − 0.43395(Val) − 11.5244(Phe+Tyr) + 31.55321(Trp) + 14.59278(Digestibility)

Group 2
= −666.4492 − 2.78584(Lys) + 5.17441(Met+Cys) + 13.08564(Thr) + 4.61808(Ile) + 16.22603(Leu) − 1.63223(Val) − 10.13673(Phe+Tyr) + 32.60196(Trp) + 14.11668(Digestibility)

Group 3
= −619.0813 − 3.13909(Lys) + 4.26918(Met+Cys) + 10.00988(Thr) + 1.42144(Ile) + 15.7547(Leu) − 5.6604(Val) − 11.28705(Phe+Tyr) + 30.49168(Trp) + 13.79953(Digestibility)

Group 4
= −744.7122 − 0.37674(Lys) + 6.03697(Met+Cys) + 11.51527(Thr) + 1.63251(Ile) + 17.29687(Leu) − 3.0294(Val) − 11.5033(Phe+Tyr) + 37.88725(Trp) + 14.68169(Digestibility)

Finally the group number that gave the largest value in the discriminant equations was used as the same group number to select correct C-PER equation. The equations used for calculation of C-PER are as below.

Group 1: $\text{C-PER} = 1.12683 - 1.61426(Z) + 0.99306(Z^2)$

Group 2: $\text{C-PER} = -7.25391 + 8.14063(Z) + 1.79517(Z^2)$

Group 3: $\text{C-PER} = 4.30469 - 1.99609(Z) + 0.45996(Z^2)$

Group 4: $\text{C-PER} = 12.75 - 8.21484(Z) + 1.66016(Z^2)$

3.2.13 Statistical analysis

All experiments carried out in this study were replicated three times. Mean values with standard deviations (SD) were reported when and where necessary. Analysis of variance (ANOVA) was performed and differences in mean values were determined using Tukey's studentized test at $p<0.05$ and employing ANOVA and TUKEY procedures of statistical analytical system (SAS, 1990), respectively. Simple linear and multiple regression analyses were also performed using the same software in the GLM and RSREG procedures, respectively. Section 3.2.6.2 explains data analysis carried out for the optimization study. Experimental designs used in the study are described in appropriate sections.

CHAPTER 4

RESULTS AND DISCUSSION

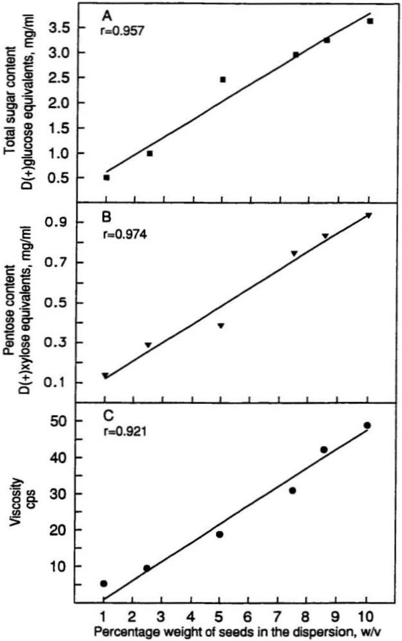
4.1 Flaxseed mucilage and its removal

4.1.1 Contents of total sugars, pentose sugars and viscosity values of mucilage extracts prepared from untreated flaxseed

The water extract of whole flaxseeds contained seed coat polysaccharides known as mucilage. Mazza and Biliaderis (1989), Bhatty and Cherdkiatgumchai (1990) and Fedeniuk and Biliaderis (1994) have reported that water is the most suitable medium for extraction of flaxseed mucilage intended for further studies. Figure 4.1 shows that the contents of total sugars and pentoses per unit volume of the aqueous extract was increased as the amount of seed used in the preparation of mucilage was increased. Total contents of sugars and pentoses were positively correlated ($r = 0.974$ and 0.957, respectively; Figure 4.1A and 4.1B) with the amount of seeds used for extract preparation. The water soluble polysaccharides of flaxseed are mainly composed of glucose (29%), xylose (23%), galactose (19%), rhamnose (14%), arabinose (11%) and fucose (3.5%) (Oomah *et al.*, 1995). Therefore, the amount of polysaccharides in the water extract is related to the content of sugars. Since pentoses constitute only a fraction of total sugars of the hydrolysed water-soluble polysaccharides, their content was lower than that of total sugars.

Viscosity values of mucilage extracts correlated with the weight of seeds used for their preparation ($r = 0.921$; Figure 4.1C). Polysaccharides extracted from seeds into water are the main factor contributing to increased viscosity values of extracts

Figure 4.1 Relationship between percentage weight of seeds used in the preparation of extract and contents of total sugars (A), pentoses (B) and viscosity values (C) of the mucilage extracts prepared from untreated flaxseed.


A
r=0.957
Total sugar content
D(+)glucose equivalents, mg/ml
3.5
3.0
2.5
2.0
1.5
1.0
0.5
B
r=0.974
Pentose content
D(+)xylose equivalents, mg/ml
0.9
0.7
0.5
0.3
0.1
C
r=0.921
Viscosity
cps
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
Percentage weight of seeds in the dispersion, w/v

similar to results obtained for total sugars and pentose contents in the extracts. Mazza and Biliaderis (1989) have reported that as the mucilage concentration increased the viscosity of the solution was also increased. Bhatty (1993b) has also reported that the viscosity values increased as the weight of flaxseed hulls increased in the dispersion and noted that the viscosity of mucilage extracts in water provides an indirect measure of mucilage content of flaxseed. Thus, the total sugar and pentose contents as well as viscosity values may be used as indices to measure the content of polysaccharides in the aqueous extracts of flaxseed.

4.1.2 Effect of soaking and enzyme treatments on contents of total sugar, pentose and viscosity values of flaxseed mucilage extracts

The total sugar and pentose contents as well as viscosity values of mucilage extracts prepared from seeds subjected to prior soaking, Celluclast® 1.5L, Pectinex™ Ultra SP and Viscozyme® L treatments are given in Tables 4.1, 4.2, 4.3 and 4.4, respectively. Mucilage extracts of soaked and enzyme-treated seeds had lower contents of total sugars and pentoses as well as viscosity values than those of the untreated seeds. An increase in the concentration of sodium bicarbonate in the soaking solution or soaking time resulted in further lowering of seed coat polysaccharides as reflected in the numerical values of parameters used above. The combined effect of sodium bicarbonate concentration and soaking time was also significant ($p<0.05$) in reducing the contents of total sugars and pentoses.

Table 4.1 Total sugar and pentose contents as well as viscosity values of mucilage extracts from flaxseed subjected to soaking treatment[1].

Treatment	Total sugar content[2] (mg/ml)	Pentose content[3] (mg/ml)	Viscosity (cps)
NaHCO$_3$ 0.00 M (pH=6.8)			
3 h	2.60±0.02^{a}	0.80±0.06^{a}	8.25±0.43^{a}
6 h	2.35±0.12^{b}	0.69±0.03^{b}	7.50±0.50^{a}
12 h	2.05±0.07^{cd}	0.55±0.03^{c}	5.67±0.29^{bc}
NaHCO$_3$ 0.05 M (pH=8.2)			
3 h	2.17±0.05^{c}	0.69±0.03^{b}	6.33±0.29^{b}
6 h	1.92±0.06^{d}	0.47±0.01^{de}	5.17±0.29^{cd}
12 h	1.55±0.06^{ef}	0.16±0.01^{f}	3.41±0.14^{e}
NaHCO$_3$ 0.10 M (pH=8.4)			
3 h	2.10±0.03^{cd}	0.54±0.03^{cd}	5.00±0.30^{cd}
6 h	1.65±0.05^{e}	0.44±0.02^{e}	4.42±0.38^{d}
12 h	1.45±0.03^{f}	0.13±0.01^{f}	2.83±0.14^{e}

[1] Mean ± SD (three soaking treatments)
Means followed by different superscripts within a column are significantly ($p<0.05$) different from one another

[2] As D-glucose equivalents

[3] As D-xylose equivalents

Table 4.2 Total sugar and pentose contents as well as viscosity values of mucilage extracts from Celluclast® 1.5L treated (1:5 w/v, 0.01 M acetate buffer, pH=4.0, 50 °C) flaxseed[1].

Treatment	Total sugar content[2] (mg/ml)	Pentose content[3] (mg/ml)	Viscosity (cps)
Celluclast® 1.5L, 0 mg protein/100 g			
1 h	2.62±0.18^{a}	0.48±0.02^{a}	6.58±0.14^{a}
3 h	2.15±0.15^{b}	0.35±0.01^{b}	6.00±0.10^{b}
6 h	2.04±0.06^{b}	0.32±0.01^{bc}	5.50±0.15^{c}
Celluclast® 1.5L, 22.5 mg protein/100 g			
1 h	2.57±0.05^{b}	0.34±0.02^{bc}	5.25±0.10^{c}
3 h	1.65±0.08^{c}	0.27±0.01^{d}	4.50±0.20^{d}
6 h	1.65±0.08^{c}	0.18±0.01^{e}	3.92±0.14^{e}
Celluclast® 1.5L, 45.0 mg protein/100 g			
1 h	2.14±0.07^{b}	0.30±0.01^{cd}	4.25±0.10^{d}
3 h	1.77±0.09^{c}	0.17±0.01^{e}	3.67±0.14^{ef}
6 h	1.62±0.02^{c}	0.16±0.01^{e}	3.58±0.14^{f}

[1] Mean ± SD (three enzyme treatments)
Means followed by different superscripts within a column are significantly ($p<0.05$) different from one another

[2] As D-glucose equivalents

[3] As D-xylose equivalents

Table 4.3 Total sugar and pentose contents as well as viscosity values of mucilage extracts from Pectinex™ Ultra SP treated (1:5 w/v, 0.01 M acetate buffer, pH=4.0, 30 °C) flaxseed[1].

Treatment	Total sugar content[2] (mg/ml)	Pentose content[3] (mg/ml)	Viscosity (cps)
Pectinex™ Ultra SP, 0 mg protein/100 g			
1 h	2.97 ± 0.20^{a}	0.21 ± 0.02^{a}	6.75 ± 0.10^{a}
3 h	2.62 ± 0.11^{a}	0.19 ± 0.01^{a}	6.25 ± 0.25^{b}
6 h	2.42 ± 0.11^{bc}	0.15 ± 0.03^{b}	5.83 ± 0.14^{b}
Pectinex™ Ultra SP, 22.5 mg protein/100 g			
1 h	2.27 ± 0.13^{c}	0.13 ± 0.02^{c}	5.25 ± 0.10^{c}
3 h	1.79 ± 0.08^{de}	0.11 ± 0.01^{de}	3.75 ± 0.25^{d}
6 h	1.65 ± 0.08^{de}	0.08 ± 0.01^{f}	3.75 ± 0.20^{d}
Pectinex™ Ultra SP, 45.0 mg protein/100 g			
1 h	1.92 ± 0.15^{d}	0.12 ± 0.03^{cd}	3.83 ± 0.14^{d}
3 h	1.65 ± 0.10^{de}	0.09 ± 0.02^{ef}	3.58 ± 0.14^{d}
6 h	1.59 ± 0.05^{e}	0.08 ± 0.01^{f}	3.42 ± 0.14^{d}

[1] Mean ± SD (three enzyme treatments)
Means followed by different superscripts within a column are significantly ($p<0.05$) different from one another

[2] As D-glucose equivalents

[3] As D-xylose equivalents

Table 4.4 Total sugar and pentose contents as well as viscosity values of mucilage extracts from Viscozyme® L treated (1:5 w/v, 0.01 M acetate buffer, pH=4.00, 40 °C) flaxseed[1].

Treatment	Total sugar content[2] (mg/ml)	Pentose content[2] (mg/ml)	Viscosity (cps)
Viscozyme® L, 0 mg protein/100 g			
1 h	2.97±0.20^{a}	0.19±0.01^{a}	7.50±0.40^{a}
3 h	2.70±0.08^{ab}	0.15±0.04^{b}	5.50±0.10^{b}
6 h	2.56±0.07^{b}	0.11±0.01^{c}	5.17±0.14^{c}
Viscozyme® L, 22.5 mg protein/100 g			
1 h	2.47±0.09^{b}	0.10±0.01^{cd}	3.75±0.10^{d}
3 h	1.76±0.16^{c}	0.08±0.00^{de}	3.25±0.10^{e}
6 h	1.73±0.01^{c}	0.07±0.01^{ef}	3.25±0.20^{e}
Viscozyme® L, 45.0 mg protein/100 g			
1 h	2.63±0.24^{b}	0.10±0.01^{cd}	3.75±0.10^{d}
3 h	1.74±0.04^{c}	0.08±0.01^{de}	3.25±0.20^{e}
6 h	1.71±0.07^{c}	0.05±0.01^{f}	3.17±0.14^{e}

[1] Mean ± SD (three enzyme treatments)
Means followed by different superscripts within a column are significantly ($p<0.05$) different from one another

[2] As D-glucose equivalents

[3] As D-xylose equivalents

Carbohydrase-assisted degradation of flax mucilage at different enzyme concentrations and reaction times resulted in a decrease in the content of seed coat mucilage. Treated seeds contained significantly ($p<0.05$) lower amount of total sugars and pentoses as well as viscosity values when compared with their respective controls. Treatment of seeds with Celluclast® 1.5L at 45.0 mg protein/100 g seed for 3 h or 22.5 mg protein/100 g for 6 h conferred a similar effect in reducing the mucilage content of seeds. The effect of Pectinex™ Ultra SP and Viscozyme® L was similar to that of Celluclast® 1.5L. Treating of seeds for 3 h with Viscozyme® L at 22.5 mg protein/100 g level lowered the total sugar and pentose content as well as viscosity values of mucilage extracts similar to those after 3 or 6 h treatment with 45 mg protein/100 g. Therefore, Viscozyme® L at 22.5 mg protein/100 g treated seeds were used for SEM and nitrogen solubility studies.

Polysaccharides have a high affinity for water and are dissolved in it by continuous hydration which replaces polysaccharide-polysaccharide interactions with polysaccharide-water binding. Branching of polysaccharides greatly reduces the possibility of intermolecular association and enhances their solubility (Whistler, 1973). Therefore, branched polysaccharides of flaxseed coat (Muralakrishna *et al.*, 1987; Cui *et al.*, 1994) get solvated during the soaking process and finally get into the solution. In this study, as the soaking time increased the solubilisation of polysaccharides was also improved. The mild alkali pH of the bicarbonate soaking medium may give rise to depolymerization of polysaccharides (Bemiller, 1973) and enhanced solubility.

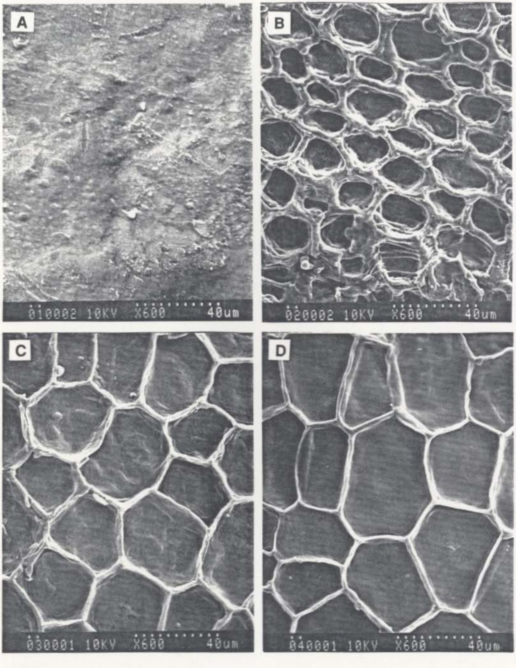
Therefore, presence of $NaHCO_3$ in the soaking medium enhanced the removal of mucilage from seeds as compared with that of water alone. Further washing with water removed more of the polysaccharides present in flaxseed coats.

Carbohydrases have been used to degrade cell wall polysaccharides of oilseeds for better recovery of oil (Olsen, 1988; Sosulski *et al.*, 1988; Dominguez *et al.*, 1994). The primary modes of actions of the three enzymes used in this study are summarised in Table 3.1. The level of enzymes used and the reaction time employed in this study was sufficient enough to degrade only the polysaccharides deposited on the outer surface of the seed coats and not the cell walls. Viscozyme® L, a multi-active enzyme, was more efficient than the other two enzymes tested in removing flaxseed mucilage (Tables 4.2, 4.3 and 4.4), perhaps due to its mode of activity. Soaking of flaxseed in a bicarbonate solution (0.10 M, 12 h) provided a cost-effective method to remove mucilage of the seed coat as compared to the enzyme treatment procedure. Furthermore, the soaking process has the added advantage that the isolated mucilage may be recovered in the intact form for possible uses in other applications.

4.1.3 Seed coat microstructure

The scanning electron micrographs of seed coat surfaces of untreated as well as water (12 h), sodium bicarbonate (0.10 M, 12 h)-soaked and Viscozyme® L (22.5 mg protein/100 g, 3 h)-treated flaxseeds are shown in Figure 4.2. The cells of the outermost layer of untreated seed coats (Figure 4.2A) were not distinctly visible as they

Figure 4.2 Scanning electron micrographs of untreated (A); water, 12 h (B); sodium bicarbonate, 0.10 M/12 h (C) and Viscozyme® L, 22.5 mg protein/100 g, 3 h (D) treated seed coat fragments (magnification × 600).


A
010002 10KV X600 40um
B
020002 10KV X600 40um
C
030001 10KV X600 40um
D
040001 10KV X600 40um

were covered with polysaccharides. For treated seeds, (Figure 4.2B, 4.2C and 4.2D) defined cell structures were clearly noted. The hollow cells with visible walls indicated that most cellular contents were removed by soaking or enzyme treatment. Bhatty (1993b) has also reported a contrasting structural difference in flaxseed coat cells after soaking of hull fragments in water. The cells of seeds soaked in water retained some of the cellular contents as compared with 0.10 M $NaHCO_3$-soaked and Viscozyme® L-treated seeds which appeared completely empty.

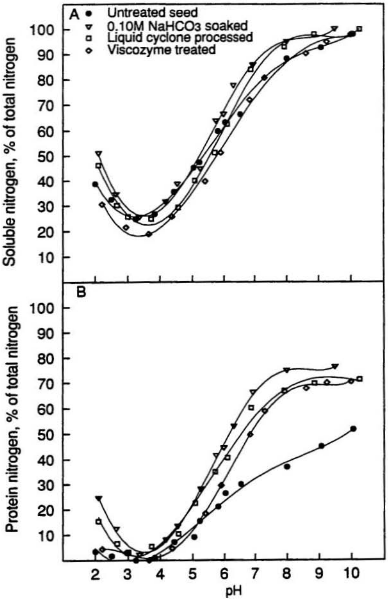
According to Boesewinkel (1984) the outermost layer of the flaxseed coat, or the epidermis, is composed mainly of cells that contain mucilage as their secondary cell wall material. The shape of mucilage cells may vary from rectangular to polygonal which is characteristic to the variety (Freeman, 1992). The seed variety in this study had mostly pentagonal and hexagonal cells which were clearly visible after soaking or enzyme treatment. The mucilage was solubilized and removed by water from the epidermis during the treatments used. The thick cuticle which covers the seed coat and is responsible for its shiny appearance (Freeman, 1992) was also removed due to the volume expansion of mucilage upon water absorption. Loss of cuticle and mucilage from flaxseed gave rise to loss of their sheen appearance and slipperiness after soaking or enzyme treatment. Flaxseed (variety, Somme) had a slippery surface with a shiny brown coloured coat before the treatments. Although it might have been expected that Viscozyme® L would solubilize cell walls, the concentration and reaction time of the enzyme were insufficient to break complex constituents of cell walls and their cellular structure remained intact. This is apparent when the outermost seed coat microstructure is compared before and after soaking or enzyme treatment (Figure 4.2).

4.1.4 Nitrogen solubility and protein recovery of flax meal

The percentage soluble nitrogen content of untreated, $NaHCO_3$ (0.10 M, 12 h)-soaked, Viscozyme® L (22.5 mg protein/100 g, 3 h)-treated defatted meals and liquid cyclone processed flours at different pH conditions is given in Figure 4.3A. Approximately 50% of total nitrogen content of all meals examined was soluble at pH values between 6 and 7. The minimum solubility range was at pH 3.4 to 3.6 and an increase in nitrogen solubility was evident below pH 3.0. This concurs with previous results reported from our laboratory (Wanasundara and Shahidi, 1994b). Flours obtained from liquid cyclone-processed seeds also exhibited similar solubility values to those obtained for treated seeds. The flour so obtained was free of hulls and therefore, almost devoid of seed coat polysaccharides.

The recovery of flaxseed proteins (Figure 4.3 B) from untreated defatted seeds was 45 to 50% at pH >7.0. This was improved to over 70% for the $NaHCO_3$ (0.10 M, 12 h)-treated seeds (Figure 4.3B). Higher amounts of protein were recovered from meals at high pH values as more nitrogen was solubilized. However, meals from untreated seeds had lower protein nitrogen values at alkali pH range when compared with treated seeds or liquid cyclone-processed flours containing low amounts of polysaccharides. The low mucilage content of treated seeds must have facilitated the

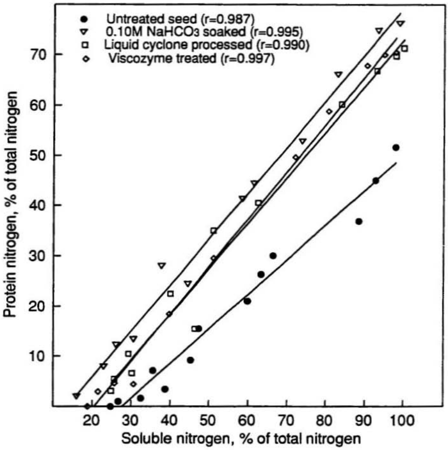
Figure 4.3 Percentage of soluble nitrogen (A) and protein nitrogen (B) of untreated, liquid cyclone processed, sodium bicarbonate (0.10 M, 12 h) and Viscozyme® L (22.5 mg protein/100 g, 3 h)-treated flax meal as affected by pH of the extraction medium.


A
● Untreated seed
▽ 0.10M NaHCO3 soaked
□ Liquid cyclone processed
◇ Viscozyme treated
Soluble nitrogen, % of total nitrogen
100
90
80
70
60
50
40
30
20
10
B
Protein nitrogen, % of total nitrogen
100
90
80
70
60
50
40
30
20
10
2
3
4
5
6
7
8
9
10
pH

precipitation of proteins upon lowering of pH to the minimum solubility pH. Extracts of untreated flax meal were viscous due to solubilisation of seed coat polysaccharides which interfered with adequate precipitation of protein aggregates formed at the minimum solubility pH. To obtain 50% recovery of proteins, untreated flax meals had to be extracted at pH values above 9.5 (53% maximum nitrogen recovery as proteins). In the pH range of 7.0 to 8.0, 75, 68 and 65% of protein nitrogens were recovered from the $NaHCO_3$ (0.10 M, 12 h)-soaked, liquid cyclone-processed and Viscozyme® L (22.5 mg protein/100 g, 3 h)-treated flax meals, respectively. Therefore, removal or reduction of seed coat polysaccharides enhances the recovery of protein nitrogen from flaxseed meals.

Approximately 18 to 20% of solubilized nitrogenous compounds in flax meals were not recovered as protein in the pH range studied. This is comparable to the amount of non-protein nitrogen present in flaxseed which were not precipitated by acids (Smith, *et al.*, 1946; Sosulski and Bakal, 1969; Wanasundara and Shahidi, 1994b). Figure 4.4 shows the relationship between the contents of soluble nitrogen and protein nitrogen of flax meals in this study. Table 4.5 summarises the relationships between soluble and protein nitrogens of flax meals. These results, together with those illustrated in Figure 4.4, indicate that 16.4 to 26.6% of soluble nitrogens in different flax meals are not recoverable as proteins. Therefore, use of the total nitrogen content of flaxseed extracts to represent total protein results in overestimation of their recoverable protein content. Non-protein nitrogen compounds of oilseeds include free

Figure 4.4 Relationship of soluble nitrogen and protein nitrogen of untreated, liquid cyclone processed, sodium bicarbonate (0.10 M, 12 h) and Viscozyme® L (22.5 mg protein/100 g, 3 h)-treated flax meals.



● Untreated seed (r=0.987)
▽ 0.10M NaHCO3 soaked (r=0.995)
□ Liquid cyclone processed (r=0.990)
◇ Viscozyme treated (r=0.997)
Protein nitrogen, % of total nitrogen
10
20
30
40
50
60
70
20
30
40
50
60
70
80
90
100
Soluble nitrogen, % of total nitrogen

Table 4.5 Relationship of soluble and protein nitrogens of low-mucilage and untreated flax meals.

Source	Regression equation
Untreated flax meal	$\text{Nitrogen}_{\text{protein}}\ (\%) = 0.732 \times \text{Nitrogen}_{\text{soluble}}\ (\%) - 19.463$
Liquid cyclone processed flax flour	$\text{Nitrogen}_{\text{protein}}\ (\%) = 0.890 \times \text{Nitrogen}_{\text{soluble}}\ (\%) - 19.199$
Sodium bicarbonate soaked flax meal (0.10 M, 12 h)	$\text{Nitrogen}_{\text{protein}}\ (\%) = 0.895 \times \text{Nitrogen}_{\text{soluble}}\ (\%) - 14.769$
Viscozyme® L treated flax meal (22.5 mg protein/100 g, 3 h)	$\text{Nitrogen}_{\text{protein}}\ (\%) = 0.895 \times \text{Nitrogen}_{\text{soluble}}\ (\%) - 19.199$

amino acids, peptides and other soluble nitrogen containing compounds of the seed (Bhatty and Finlayson, 1973).

The present study clearly demonstrates that removal of seed coat polysaccharides has a major effect in enhancing protein yield of flax meal via solubilisation processes. Enzyme or soaking pre-treatments did not disintegrate seeds and may be considered as low-cost processes when compared with mechanical operations such as the liquid cyclone process. The pre-treated seeds may be subjected to the conventional solvent extraction process and proteins from meals may be recovered by standard practices such as alkali solubilisation and isoelectric precipitation.

4.2 Optimization of protein extraction from low-mucilage flax meal with sodium hexametaphosphate (SHMP)

Solubility of oilseed proteins is affected by pH, temperature and solute concentration of the medium, meal-to-solvent ratio, particle size, etc. (Sosulski and Bakal, 1969; Prakash and Narasinga Rao, 1986). Therefore, it is necessary to consider all these factors collectively as process variables in optimizing protein extraction for product preparation.

4.2.1 Diagnostic checking of fitted models

Experimental values obtained for responses Y_1 (% nitrogen extracted) and Y_2 (% protein recovered) for twenty design points are given in Table 4.6. Multiple regression coefficients obtained by employing a least squares technique to predict

Table 4.6 Central Composite Rotatable Design arrangement of independent variables and responses obtained[1].

Design point	Independent variables[2]			Response variables	
	pH (X_1)	Meal-to-solvent ratio (X_2)	SHMP concentration (X_3)	% Nitrogen extracted (Y_1)	% Protein recovered (Y_2)
1	-1	-1	-1	44.59	23.00
2	-1	+1	-1	50.20	29.90
3	-1	-1	-1	51.34	30.40
4	-1	+1	+1	60.37	38.20
5	+1	-1	+1	61.99	41.60
6	+1	+1	-1	70.26	48.92
7	+1	-1	-1	70.27	49.50
8	+1	+1	+1	74.14	53.82
9	-1.68	0	+1	36.99	15.79
10	+1.68	0	0	75.20	56.70
11	0	-1.68	0	60.89	39.98
12	0	+1.68	0	64.11	43.98
13	0	0	0	54.10	33.70
14	0	0	-1.68	68.70	49.50
15	0	0	+1.68	70.25	49.25
16	0	0	0	73.10	52.20
17	0	0	0	69.93	48.91
18	0	0	0	69.27	48.27
19	0	0	0	73.97	52.60
20	0	0	0	74.39	53.10

[1] Non-randomized, average value of duplicate determinations except design points 16 to 20

[2] Coded symbols and levels of independent variables refer to Figure 3.3 and Table 3.3

quadratic polynomial models for Y_1 and Y_2 are summarised in Table 4.7. Examination of these parameters (coefficients) with the t-test indicated that all linear and quadratic terms were significant ($p<0.05$) but interaction terms were not. The results of analysis of variance (ANOVA) of the models are shown in Table 4.8. The models fitted for Y_1 and Y_2 were significant ($p<0.01$) by the F-test. Both models predicted were adequate as indicated by error analysis that showed non-significant ($p<0.01$) lack of fit. The regression models for data were highly significant ($p<0.01$) with satisfactory coefficients of determination, $R^2=0.95$ and 0.96 for Y_1 and Y_2, respectively, which is the proportion of variability of the data accounted for the model. The contribution of linear and quadratic terms to the model was 0.69 and 0.26 for Y_1 and 0.73 and 0.23 for Y_2, respectively. The coefficients of variation (CV) were less than 10% (Table 4.7) indicating that the models are reasonably reproducible (Montgomery, 1984). The coefficients determined by the predicted models (Table 4.7) for Y_1 and Y_2 are given below.

$$\begin{aligned} Y_1 = {} & -159.434 + 41.620X_1 + 2.098X_2 + 12.212X_3 - 2.260X_1^2 - 0.028X_2^2 \\ & - 1.742X_3^2 - 0.021X_1X_2 - 0.265X_1X_3 - 0.008X_2X_3 \end{aligned}$$

$$\begin{aligned} Y_2 = {} & -177.455 + 40.407X_1 + 2.168X_2 + 11.297X_3 - 2.162X_1^2 - 0.028X_2^2 \\ & - 1.625X_3^2 - 0.026X_1X_2 - 0.161X_1X_3 - 0.018X_2X_3 \end{aligned}$$

The two models indicated that pH (X_1) was a significant variable with the most linear effect on both the soluble and protein nitrogen content of low-mucilage flax meal

Table 4.7 Regression coefficients of predicted quadratic polynomial model for two response variables.

Coefficients[1]	% Nitrogen extracted (Y_1)	Standard error of Y_1	% Protein recovered (Y_2)	Standard error of Y_2
Linear				
β_0	-159.434***	30.886	-177.446***	29.827
β_1	41.620***	6.373	40.407***	6.154
β_2	2.098**	0.805	2.168**	0.777
β_3	12.212**	4.974	11.297**	4.803
Quadratic				
β_{11}	-2.260***	0.387	-2.162***	0.373
β_{22}	-0.028***	0.009	-0.028***	0.008
β_{33}	-1.742***	0.448	-1.625***	0.433
Interaction				
β_{12}	-0.021	0.078	-0.026	0.075
β_{13}	-0.265	0.520	-0.161	0.503
β_{23}	-0.008	0.078	-0.018	0.075
β_{123}	-	-	-	-
R^2	0.952		0.957	

[a] Coefficients refer to the model given in the Section 3.2.6.2

** Significant at 0.05 probability level

*** Significant at 0.01 probability level

Table 4.8 ANOVA for the predicted models.

Source	% Nitrogen extracted (Y_1)				% Protein recovered (Y_2)			
	df	SS	F ratio	*p* value	df	SS	F ratio	*p* value
Total regression	9	2191.643	22.209	0.000	9	2294.922	24.935	0.000
Linear	3	1599.725	48.632	0.000	3	1751.878	57.104	0.000
Quadratic	3	588.180	17.881	0.000	3	540.272	17.611	0.000
Crossproduct	3	3.742	0.114	0.950	3	2.773	0.001	0.964
Residual (error)	10	109.648			10	102.262		
Lack of fit	5	84.243	3.316	0.107	5	79.433	3.480	0.099
Pure error	5	25.405			5	22.828		
Response mean ± SD	63.704 ± 3.311				42.966 ± 3.198			
CV (%)	5.198				7.443			

as it had the largest linear coefficient (Table 4.7). The positive coefficients for X_1, X_2 and X_3 also indicated that their linear effects would increase the responses of Y_1 and Y_2; however, pH and concentration of SHMP had a more positive effect on responses than meal-to-solvent ratio. Results provided in Tables 4.6 and 4.7 suggest that linear and quadratic effects of the three variables are the primary determining factors of the responses (percentages of solubilized nitrogen and recovered protein) and no statistically significant interaction exists between any two of the three factors; pH, meal-to-solvent ratio or concentration of SHMP.

4.2.2 Response surface plotting and optimization based on canonical analysis

Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models (Table 4.7) were chosen as the axes (pH and concentration of SHMP) for the response surface plots. The relationship between independent and dependant variables are shown in the three-dimensional representation of the response surfaces generated for the models developed (Figures 4.5 and 4.6).

Canonical analysis was performed on the predicted quadratic polynomial models to examine the overall shape of the response surface curves and to characterize the nature of the stationary points. Results of canonical analysis of the response surfaces are given in Table 4.9. Canonical form of equations demonstrating the nature of responses were as follows:

Figure 4.5 Response surfaces for the effect of pH and SHMP concentration on soluble nitrogen content of low-mucilage flax meal (A: meal-to-solvent ratio=1:33; B: SHMP concentration=2.75%).

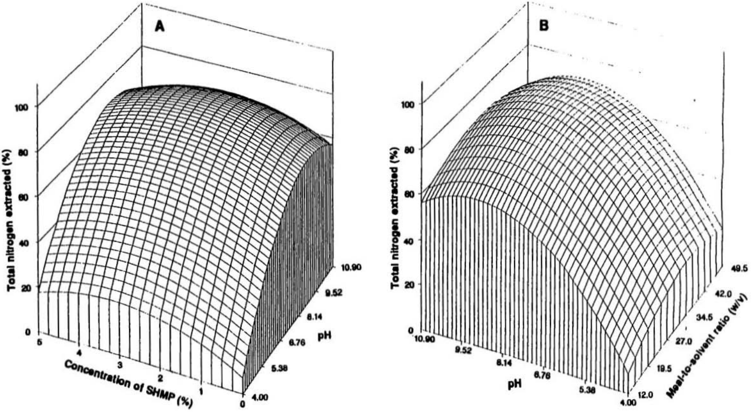

A
Total nitrogen extracted (%)
0
20
40
60
80
100
Concentration of SHMP (%)
5
4
3
2
1
0
pH
4.00
5.38
6.76
8.14
9.52
10.90
B
Total nitrogen extracted (%)
0
20
40
60
80
100
pH
10.90
9.52
8.14
6.76
5.38
4.00
Meal-to-solvent ratio (w/v)
12.0
19.5
27.0
34.5
42.0
49.5

Figure 4.6 Response surfaces for the effect of pH and SHMP concentration on recovered protein content of low-mucilage flax meal (A: meal-to-solvent ratio=1:33; B: SHMP concentration=2.8%).

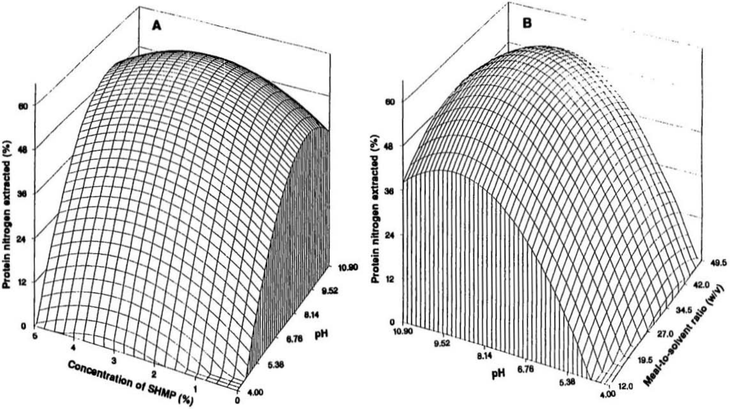

A
Protein nitrogen extracted (%)
0
12
24
36
48
60
Concentration of SHMP (%)
5
4
3
2
1
0
pH
4.00
5.38
6.76
8.14
9.52
10.90
B
Protein nitrogen extracted (%)
0
12
24
36
48
60
pH
10.90
9.52
8.14
6.76
5.38
4.00
Meal-to-solvent ratio (w/v)
12.0
19.5
27.0
34.5
42.0
49.5

Table 4.9 Canonical analysis of response surfaces.

Response variable	Critical values of independent variables			Predicted value (%)	Stationary point
	pH (X_1)	Meal-to-solvent ratio (X_2)	SHMP %, w/v (X_3)		
% Nitrogen extracted (Y_1)	8.89	1:33.6	2.75	77.60	Maximum
% Protein recovered (Y_2)	9.04	1:33.3	2.85	57.46	Maximum

$$Y_1 = 77.602 - 14.487w_1^2 - 7.906w_2^2 - 8.806w_3^2$$

$$Y_2 = 57.459 - 13.822w_1^2 - 7.773w_2^2 - 8.426w_3^2$$

where w_1, w_2 and w_3 are the axes of X_1, X_2 and X_3 considering the stationary point as the origin for the new coordinate system. The new coordinate system was calculated from partial derivatives of the quadratic response equation with respect to each X, setting the derivatives equal to zero, and then simultaneously solving the 3 equations to obtain X (Montgomery, 1984; Cornell, 1992). The coefficients of w_1, w_2 and w_3 are the eigan values of the symmetric matrix containing the estimated quadratic coefficients for X_1, X_2 and X_3, as the main diagonal elements, respectively. The resultant absolute value of X_1 was the largest and indicated that the quadratic effect of the response surface was very sensitive to changes of X_1 (pH) for both responses, Y_1 and Y_2. This also indicates that the contours should be ellipsoidal with the major axis towards w_2 (Figure 4.7). All the eigan values obtained for the analysis were negative, thus indicating that the stationary point for each response was a maximum (Cornell, 1992). The critical values of the three variables are as shown in Table 4.9 and the stationary points are located in the experimental region. The contour plots derived from the results of canonical analysis showed ellipsoidal contours at the maximum region (Figure 4.7A and B). Verification experiments, carried out within the experimental limits, showed reasonably close values to the predicted ones (Table 4.10) and further confirmed the adequacy of the predicted model. Detailed data analysis obtained from

Figure 4.7 Contour plots obtained from canonical analysis of quadratic polynomial equation predicted for soluble nitrogen (A) and recovered protein (B) of low-mucilage flax meal.

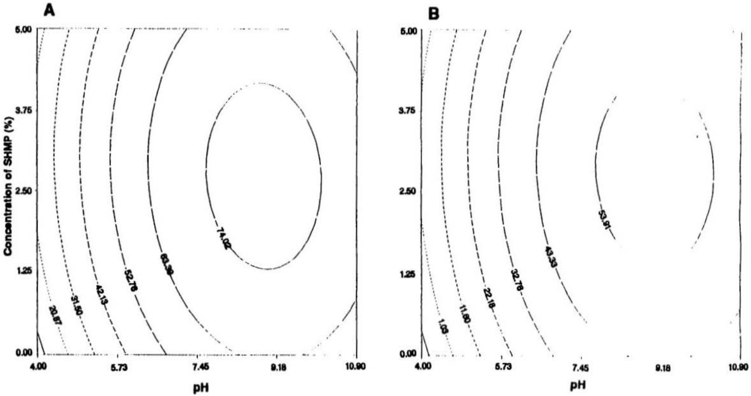

A
B
Concentration of SHMP (%)
5.00
3.75
2.50
1.25
0.00
4.00
5.73
7.45
9.18
10.90
pH
20.87
31.50
42.13
52.76
63.39
74.02
1.03
11.60
22.18
32.76
43.33
53.91

Table 4.10 Observed and predicted values of Y_1 and Y_2 of the verification points.

pH (X_1)	Meal-to-solvent ratio, w/v (X_2)	Concentration of SHMP, % (w/v) (X_3)	% Nitrogen extracted (Y_1)		% Protein recovered (Y_2)	
			Observed[1]	Predicted[2]	Observed[1]	Predicted[2]
5.5	1:25	1.5	47.62±1.62	45.083	25.04±1.91	23.803
6.5	1:35	2.5	64.67±1.00	64.603	46.22±1.78	43.415

[1] Mean values of triplicate determinations

[2] Calculated using the predicted equations given in Section 4.2.1

SAS program for optimization study is presented in Appendix 2.

The results of one-factor-at-a-time experiments do not reflect actual changes in the environment as they ignore interactions between factors which are present simultaneously. The response surface methodology can describe concomitant effects more fully and helps in more accurate optimization of factors. The central composite rotatable design (CCRD) is the preferred class of experimental design for fitting polynomial models to analyse response surfaces of multi-factor combinations. The design is considered rotatable because the variance of the predicted response, Y, at the point X is a function only of the distance of the point from the design centre irrespective of the direction. This implies that the variance contours of predicted response are concentric circles. Also a rotatable design has the property that the variance of predicted response does not change when the design is rotated about the centre point ([0,0,...0]; Montgomery, 1984). CCRD with response surface methodology (RSM) is a very effective tool for reducing the number of experimental combinations required without compromising the validity of results in studies where a large number of independent variables are included. To further improve the extraction yields of proteins in this design, other variables such as temperature, time and number of extractions may be included.

Flaxseed proteins are composed mainly of albumins and globulins (95% of total proteins) and their solubility depends on the pH of the extraction medium and solute concentration (Oomah *et al.,* 1993). Since flax meal contains a high level of non-protein nitrogen (Wanasundara and Shahidi, 1994b; Oomah and Mazza, 1993), it is unreasonable to assume that all solubilized nitrogen could be recovered from the extraction medium as protein as clearly documented in the Section 4.1.4. Therefore, in this study the protein nitrogen content was considered. Within the ranges of the variables studied, the maximum nitrogen solubilisation observed by a single extraction step was 77%, over 57% of which was recovered as protein and the other 20% accounted for non-recoverable nitrogen compounds may be non-protein nitrogen compounds.

4.3 Extraction of flaxseed proteins with sodium hexametaphosphate (SHMP)

Polyphosphates are extensively used in the food industry as functional ingredients to control pH, as bactericidal agents or for complexing and dispersing of proteins. Spinelli and Koury (1970) have demonstrated the recovery of soluble fish proteins from solutions by complexation with SHMP in order to prepare fish protein concentrates. Bhatty *et al.* (1968) have used phosphates to complex and extract oilseed proteins. Use of SHMP for extraction of proteins from cottonseed (Shemar *et al.*, 1973) and canola (Thompson *et al.*, 1976) has also been reported. According to the present study flaxseed proteins may also be extracted via SHMP-complexation. The amount of protein nitrogen extracted at the optimum pH, SHMP concentration and meal-to-solvent ratio was much higher than the value (15%) obtained by Sosulski and Bakal (1969) for untreated flaxseed meal using 0.2% NaOH at meal-to-solvent ratio of

1:20. The protein isolates so prepared had a pale yellow colour compared to the brown colour reported for isolates prepared by alkali solubilisation (Sosulski and Bakal, 1969).

Polyphosphates are approved food additives that improve functional characteristics of foods and participate in metal ion chelation, surface sorption, polyelectrolyte behaviour, stabilisation of colloidal suspensions and nutritional supplementation of phosphorus (Van Wazer, 1971). Phosphate ions can increase solubility of proteins at both above and below their isoelectric pH (Molins, 1991). Phosphates at pH values above the isoelectric point of proteins are in the ionized form and remain bound to proteins and other positively charged groups that may generally bind to protein molecules. The phosphate-protein interaction depends on changes in the net charge of protein brought about by change of pH or addition of cations. When the net charge of protein is non-zero, repulsion between the groups and chains carrying the same charge forces the protein structure to open up and make more sites available for interaction with the surrounding water molecules. As a consequence, protein molecules become soluble. Positively charged groups on protein molecules also interact with polyanionic phosphates, thus the increased net negative charge of protein molecules would enhance their solubility in water (Molins, 1991).

4.4 Chemical composition of flaxseed protein isolate

Table 4.11 presents the chemical composition of low-mucilage flaxseed, prepared by soaking 12 h in a 0.10 M $NaHCO_3$ solution and the protein isolate prepared

Table 4.11 Chemical composition of low-mucilage flaxseed (0.10 M $NaHCO_3$, 12 h soaked) and its protein isolate prepared via SHMP extraction (%, unless stated)[1].

Component	Seed	Protein isolate
Ash	2.41±0.50	9.53±0.50
Crude protein[2]	18.98±1.40	78.12±2.17
Dietary fibre	12.51±2.43	-
Lipid	49.90±2.46	7.64±0.60
Moisture	5.55±0.45	trace
Non-protein nitrogen (% of total N)[3]	14.67±1.20	-
Phytic acid[3]	1.10±0.08	2.11±0.30
Cyanogenic glycosides[3]		
Linustatin	2.70±0.19	ND[4]
Neolinustatin	3.09±0.28	ND[4]
Total phosphorus (mg P/g material)[3]	2.82±0.50	9.50±1.91
Total soluble sugars[3]	5.03±0.95	trace

[1] Mean ± SD (three samples)
[2] % N × 6.25
[3] based on defatted dry material
[4] Not detected

via SHMP complexation procedure. Crude protein content of the seeds (19.84±0.34%) was within the average range reported for flaxseed (Oomah and Mazza, 1993). The content of ash, crude protein and other components were increased when seeds were soaked in the bicarbonate solution and this may be due to a concentration effect brought about by the removal of seed coat polysaccharides. The protein isolate (protein content 78%) was prepared via three consecutive extractions and subsequent lyophilization. Incomplete recovery of proteins may be, in part, due to their loss during the washing process. The protein content of isolates prepared from low-mucilage flaxseed was lower than that of isolates from cottonseed (Shemar *et al.*, 1973), where approximately 93% protein was isolated using a 2% (w/v) SHMP solution at 60 °C over a 30-min period and a meal-to solvent ratio of 1:50 (w/v). However, results were similar to those of Thompson *et al.* (1976) for canola protein isolate (73% protein) prepared via SHMP extraction (2% SHMP, w/v; 25 °C; meal-to-solvent ratio of 1:10; 30 min).

The ash content of the protein isolate was three times higher than that of the seed, perhaps due to salt formation during protein precipitation. The high total phosphorus content (0.95%) of the isolate may also be due to the presence of residual SHMP in the products. However, the content of phosphorus present is well below the acceptable level of 4% present in lactalbumin phosphate which is a commercially available food ingredient (Thompson *et al.*, 1976). The content of phytic acid in the isolates was higher than that in the seeds. The dietary fibre content of flaxseed is higher than other oilseeds due to the presence of mucilage in its seed coats. Removal of mucilage by soaking reduced the dietary fibre content of the seeds.

4.4.1 Amino acid composition

The amino acid composition of low-mucilage flaxseed (0.10 M Na_2CO_3, 12 h soaked) and the protein isolate prepared via SHMP complexation are presented in Table 4.12. The contents of individual amino acids in flax protein isolates were within the range reported in previous studies (Bhatty and Cherdkiatgumachai, 1992; Wanasundara and Shahidi, 1994b). Tryptophan and lysine were the first limiting amino acids of flaxseed and its protein isolate, respectively. The content of lysine in the isolate was lower than that in the seed, similar to that reported by Sosulski and Sarwar (1973) for flax protein isolates prepared by alkali solubilisation. The percentage ratios of essential to total amino acids (%E/T) for the seed and protein isolate were well above 36% reported for ideal proteins as recommended by FAO/WHO (1973).

The data on the amino acid composition of flax protein isolates may indicate the potential nutritive value of this source of protein, however, the availability of these amino acids to the body is also a determining factor in protein quality assessment. Digestibility of a protein is a major factor affecting its bioavailability (Robaidek, 1983). The C-PER are obtained from essential amino acid composition and *in vitro* digestibility (with pepsin-trypsin) data and serve as an alternative method to rat bioassay for protein quality evaluation (Satterlee *et al.*, 1982) in the food industry. The C-PER value for flax protein isolate was in the range of values reported for textured

Table 4.12 Amino acid composition (g/16g N) of low-mucilage flaxseed and flax protein isolate prepared via SHMP complexation.

Amino acid	Low-mucilage flaxseed (0.10 M $NaHCO_3$, 12 h soaked)		Standard protein[1]
	Meal	Protein isolate	
Essential amino acids			
Histidine	2.50 ± 0.11	2.18 ± 0.02	-
Isoleucine	4.54 ± 0.30	4.41 ± 0.48	4.00
Leucine	6.54 ± 0.26	6.28 ± 0.53	7.04
Lysine	4.55 ± 0.18	3.62 ± 0.06[4]	5.44
Methionine+Cysteine	6.09 ± 0.02	5.06 ± 0.07	3.52
Phenylalanine+Tyrosine	7.50 ± 0.08	6.90 ± 0.01	6.08
Threonine	4.37 ± 0.20	4.79 ± 0.08	4.00
Tryptophan	0.70 ± 0.02[4]	1.55 ± 0.03	0.96
Valine	5.46 ± 0.06	4.29 ± 0.05	4.96
Non-essential amino acids			
Alanine	5.02 ± 0.10	4.54 ± 0.06	-
Aspartic acid+asparagine	10.65 ± 0.43	10.33 ± 1.20	-
Arginine	10.03 ± 0.50	10.21 ± 1.01	-
Glycine	7.44 ± 0.82	5.54 ± 0.08	-
Glutamic acid+glutamine	20.76 ± 1.09	19.99 ± 2.18	-
Proline	3.98 ± 0.23	3.77 ± 0.10	-
Serine	5.02 ± 0.09	4.67 ± 0.09	-
Total essential amino acids[2]	39.75	36.90	36.00
E/T %	37.80	37.60	-
Amino acid score	73.00	67.00	-
In vitro digestibility[3]	85.40	89.00	-
BV	85.50	86.50	-
C-PER	1.95	2.12	-

[1] FAO/WHO (1973)

[2] Histidine not included

[3] *In vitro* digestibility with pepsin-trypsin

[4] Primary limiting amino acid

vegetable proteins (2.07, Satterlee *et al.*, 1982) and casein (2.5, El-Mahdy and El-Sebaiy, 1985). The calculated biological value of the protein isolate was 86.50, which is a reasonable value for a plant protein (rice, 81%; wheat, 57%; bean, 72%; egg, 100%; Morup and Olesen, 1976). Among non-essential amino acids, aspartic acid (including asparagine), arginine and glutamic acid (including glutamine) were present in comparatively higher amounts than other amino acids normally available in seed proteins (Youle and Huang, 1981).

4.5 Gel electrophoresis of flax protein isolates

The non-denaturating polyacrylamide gel electrophoresis (NPAGE) pattern of the protein isolate prepared via SHMP complexation and total protein of flax are shown in Figure 4.8. The NPAGE pattern of the isolate showed two broad bands compared to the single broad band resulted for total protein. NPAGE separates proteins based on their negative charge and size without having any denaturation effect (Hames, 1981).

When proteins were electrophoresed by SDS-PAGE a more complex pattern was observed (Figure 4.9). Most polypeptides bind SDS in a constant ratio such that they have essentially identical charge densities and migrate in the polyacrylamide gel according to their size (Hames, 1981). The total proteins of flaxseed as such and protein isolates thereof, had similar band patterns with estimated molecular weights ranging between 14 and 45 kDa. These observations agreed well with the results of

Figure 4.8 The NPAGE of flaxseed proteins (A, total seed proteins; B and C, SHMP-protein isolates).

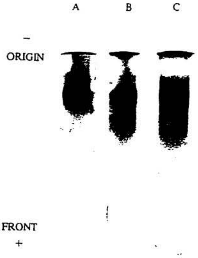
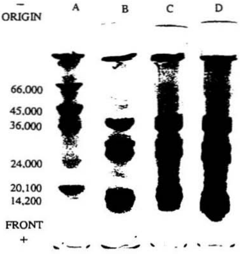
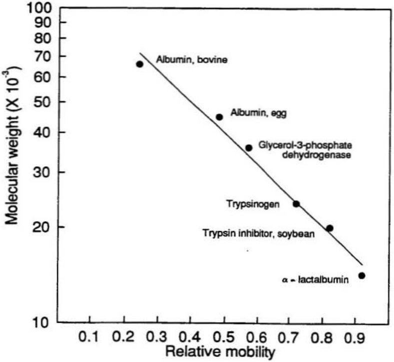

A
B
C
–
ORIGIN
FRONT
+

Figure 4.9 The SDS-PAGE of flaxseed proteins (A, molecular weight markers; B, total seed proteins; C and D, SHMP-protein isolates).


−
ORIGIN
A
B
C
D
66,000
45,000
36,000
24,000
20,100
14,200
FRONT
+


Molecular weight (X 10^{-3})
100
90
80
70
60
50
40
30
20
10
Albumin, bovine
Albumin, egg
Glycerol-3-phosphate dehydrogenase
Trypsinogen
Trypsin inhibitor, soybean
α - lactalbumin
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Relative mobility

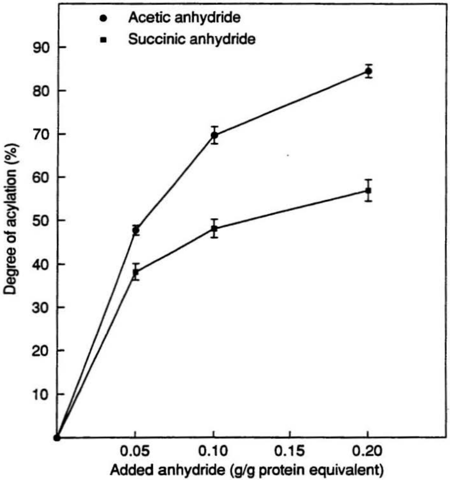
Dev and Sienkiewicz (1987) who showed that the major flax protein is composed of nine bands in the SDS-PAGE with four being predominant over the others in terms of relative intensities. Similar subunit patterns in the SHMP-protein isolate and the total proteins of flaxseed suggest that SHMP has possibly isolated total protein of the seed. Majority of these bands represent subunits of the high molecular mass protein fraction/s (Dev and Sienkiewicz, 1987).

4.6 Acylation of flaxseed protein isolate

Treatment of flaxseed protein isolate with increasing quantities of acetic or succinic anhydride progressively acylated the free amino groups of seed proteins. The degree of acylation of flax protein isolate was determined as percentage loss of ε-amino groups of lysine residues (Figure 4.10). Acetylation of cationic amino groups renders them electrically neutral while succinylation adds a negative charge to the protein molecule (Figure 2.5). Due to the high nucleophilic character and steric hindrance the ε-amino group of lysine residues are the most reactive towards acylation, thus the degree of protein modification is commonly expressed as the percentage of blocked amino groups of lysine.

The extent of acetylation (47.4, 70.0 and 84.5%) was higher than succinylation (38.2, 45.5 and 56.9%) at the three concentrations of anhydride reacted; that is 0.05, 0.10 and 0.20 g anhydride/g protein equivalent, respectively. [In the rest of the text these products are identified as 5, 10 and 20% AA and 5, 10 and 20%SA for the

Figure 4.10 Degree of acylation of flaxseed protein isolates.


● Acetic anhydride
■ Succinic anhydride
Degree of acylation (%)
10
20
30
40
50
60
70
80
90
0.05
0.10
0.15
0.20
Added anhydride (g/g protein equivalent)

acetylated and succinylated protein isolates, respectively.] Nitecka *et al.* (1986) have also reported that acetic anhydride is more effective than succinic anhydride in blocking ε-amino groups of rapeseed proteins. Results of this study indicated that all free amino groups were not acylated. Incomplete acylation of reactive amino acid residues by an acetyl, succinyl or any other acyl group is common for storage proteins of oilseeds (Child and Parks, 1976; Franzen and Kinsella, 1976a; Schwenke *et al.*, 1993; Ponnampalam *et al.*, 1990).

4.7 Effect of acylation on functional properties

4.7.1 Effect of acylation on colour of flax protein isolates

Flaxseed protein isolates prior to acylation had a light yellow colour and a fluffy texture. Table 4.13 shows that Hunter **L** and **b** values changed with the increased degree of acylation of flax protein isolates. The increase in Hunter **L** value from 68.8 to 74.2 indicated a lighter colour for the products compared to the unmodified isolate. The increase in Hunter **L** value at the highest degree of succinylation (56.9%) was larger than the highest degree of acetylation (84.5%). A decrease in Hunter **b** values of the acylated products indicates a decrease in their yellow colour which was also noticeable by the naked eye. Flax protein isolates with the highest degree of succinylation (20%SA) had the highest **L** and the lowest **b** values and were almost white in colour. The **a** values of the protein products did not show a pattern of change. No off-odours were detected in any of these products.

Table 4.13 Hunter **L, a, b** colour values of modified and unmodified flax protein isolates[1].

Treatment	Hunter **L**	Hunter **a**	Hunter **b**
Unmodified	68.8±0.8^{a}	1.0±0.1	26.5±0.9^{c}
Modified			
Acetic anhydride (g/g protein equivalents)			
0.05	69.2±1.0^{a}	1.7±0.1	23.6±0.8^{ab}
0.10	70.0±2.2^{ab}	1.2±0.2	23.6±0.9^{ab}
0.20	71.8±1.0^{b}	0.9±0.1	22.3±0.9^{ab}
Succinic anhydride (g/g protein equivalents)			
0.05	72.0±0.9^{b}	0.5±0.1	23.9±0.8^{ab}
0.10	72.9±1.2^{bc}	0.4±0.1	22.6±1.1^{a}
0.20	74.2±0.8^{c}	1.0±0.2	21.3±0.8^{a}

[1] The Colorimet unit was calibrated using a white tile with Hunter values of **L**=94.5±0.2, **a**=-1.0±0.1, **b**=0.0±0.2
Mean ± SD of three samples
Means followed by different superscripts within a column are significantly (p<0.05) different from one another

4.7.2 Effect of acylation on emulsifying properties of flax protein isolates

Emulsifying activities (EA) of acetylated and succinylated flax protein isolates, measured as a function of pH and NaCl concentration, are presented in the form of response surfaces in Figures 4.11 and 4.12, respectively. The EA of unmodified flax protein isolate was increased as the pH and concentration of NaCl in the medium increased. The resultant response surfaces for acetylated proteins were different than response surfaces for succinylated proteins, thus indicating that the effect of pH and NaCl concentration on EA was different for the acyl group involved in modification of protein. As a general trend, EA showed a decrease when degree of acetylation increased from 0.0 to 84.5% (Figure 4.11B, C and D). For a given degree of acetylation, EA decreased as the NaCl concentration in the medium increased from 0.0 to 0.70 M (Figure 4.11B and D). As the pH of the medium changed to acidic or basic, EA of the acetylated proteins was lowered compared to the EA at the neutral pH.

Succinylation showed an increasing influence on the EA of protein isolates (Figure 4.12A, B, C and D). As the degree of succinylation increased a higher EA was observed, in contrast to the acetylated flax protein isolates. Increase in pH resulted in an increase in EA, but an increase in the NaCl concentration in the medium gave lower EA values for succinylated flax protein products (Figure 4.12B). The response surfaces obtained for succinylated products showed that as the degree of succinylation increased the EA improved at low pH values (Figure 4.12B, C and D).

The emulsion stability (ES) of the prepared products is given in Figures 4.13

Figure 4.11 Emulsifying activity (measured as absorbance at 500 nm) of acetylated flax protein isolates as a function of pH and NaCl concentration: (A) unmodified, (B) 5%AA, (C) 10%AA and (D) 20%AA.

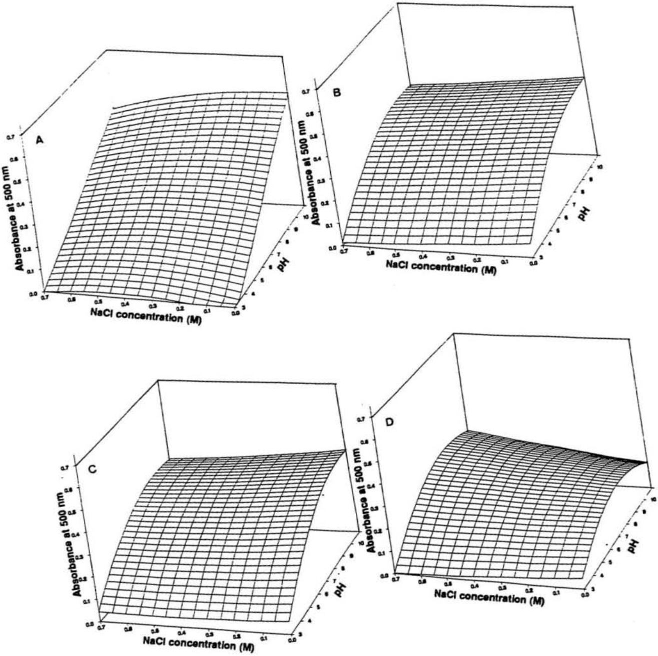
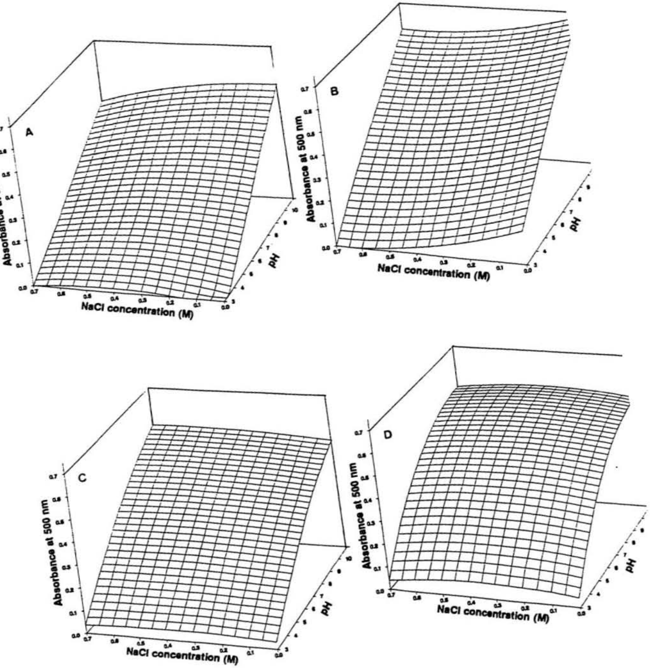

A
Absorbance at 500 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
NaCl concentration (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pH
3
4
5
6
7
8
9
10
B
Absorbance at 500 nm
NaCl concentration (M)
pH
C
Absorbance at 500 nm
NaCl concentration (M)
pH
D
Absorbance at 500 nm
NaCl concentration (M)
pH

Figure 4.12 Emulsifying activity (measured as absorbance at 500 nm) of succinylated flax protein isolates as a function of pH and NaCl concentration: (A) unmodified, (B) 5%SA, (C) 10%SA and (D) 20%SA.


A
B
C
D
Absorbance at 500 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
NaCl concentration (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pH
3
4
5
6
7
8
9
10

and 4.14 as half-life of the emulsion (time required to reduce the absorbance at 500 nm by 50%) for acetylated and succinylated derivatives, respectively. Emulsion stability increased with the degree of acetylation (Figure 4.13B and C) and succinylation (Figure 4.14 B, C and D) except at the highest degree of acetylation (Figure 4.13D). As the pH increased ES was improved; however, increased NaCl concentration in the medium did not give a similar effect for the acylated products.

4.7.3 Effect of acylation on fat binding capacity of flax protein isolates

The fat binding capacity (FBC) of flax protein products are presented in Table 4.14. Unmodified flax protein isolate was able to bind 93.0 ± 1.7 ml of corn oil per 100 g of material. Acylation changed FBC of the flax protein isolate. The highest FBC was observed at the lowest degree of acetylation (47.7%) and the lowest value was obtained at the highest degree of succinylation (56.9%). Succinylated protein products (at all degrees of succinylation) had lower FBCs than the acetylated products.

4.7.4 Effect of acylation on foaming properties of flax protein isolates

The foaming capacity and foam stability of flax protein isolates are presented in Table 4.15. The highest foaming capacity was observed for the unmodified protein isolate and the isolates with the lowest degree of modification. As the degree of acetylation or succinylation increased the foaming capacity decreased and isolates with the highest degree of acylation had the lowest foaming capacity. The foams of

Figure 4.13 Emulsion stability (time required to reduce the absorbance at 500 nm by 50%) of acetylated flax protein isolates as a function of pH and NaCl concentration: (A) unmodified, (B) 5%AA, (C) 10%AA and (D) 20%AA.

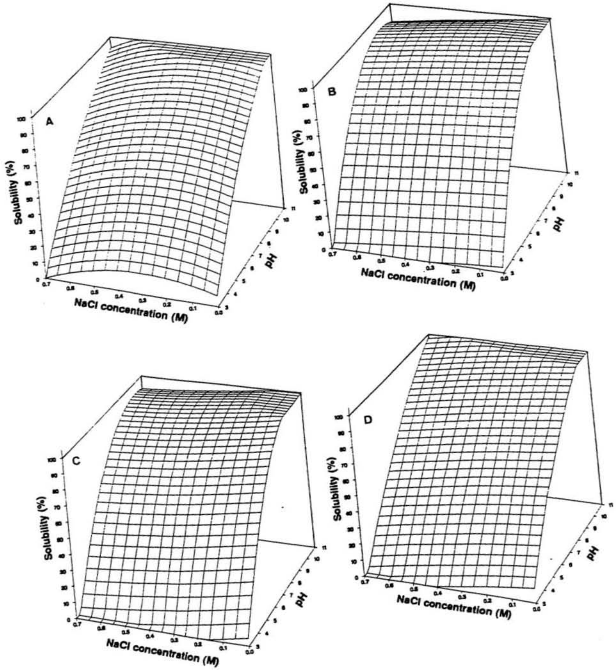
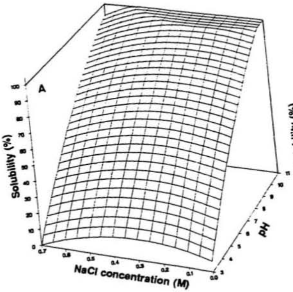
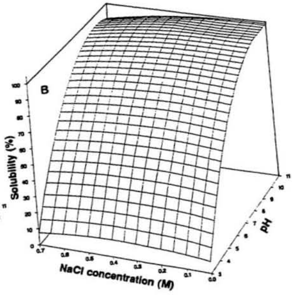
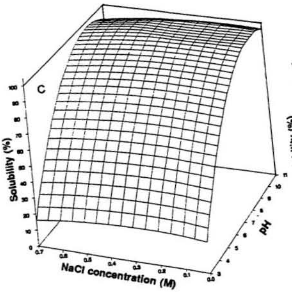

A
Solubility (%)
0
10
20
30
40
50
60
70
80
90
100
NaCl concentration (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pH
3
4
5
6
7
8
9
10
11
B
Solubility (%)
NaCl concentration (M)
pH
C
Solubility (%)
NaCl concentration (M)
pH
D
Solubility (%)
NaCl concentration (M)
pH

Figure 4.14 Emulsion stability (time required to reduce the absorbance at 500 nm by 50%) of succinylated flax protein isolates as a function of pH and NaCl concentration: (A) unmodified, (B) 5%SA, (C) 10%SA and (D) 20%SA.


A
Solubility (%)
100
90
80
70
60
50
40
30
20
10
0
NaCl concentration (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pH
3
4
5
6
7
8
9
10
11


B
Solubility (%)
100
90
80
70
60
50
40
30
20
10
0
NaCl concentration (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pH
3
4
5
6
7
8
9
10
11


C
Solubility (%)
100
90
80
70
60
50
40
30
20
10
0
NaCl concentration (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pH
3
4
5
6
7
8
9
10
11

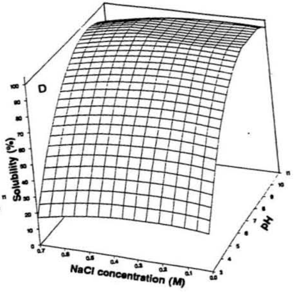

D
Solubility (%)
100
90
80
70
60
50
40
30
20
10
0
NaCl concentration (M)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pH
3
4
5
6
7
8
9
10
11

Table 4.14 Fat binding capacity of acylated flax protein isolates[1]

Treatment	Fat binding capacity (ml/100g)
Unmodified	93.0 ± 1.7^{d}
Modified	
Acetic anhydride (g/g protein equivalents)	
0.05	105.0 ± 2.1^{f}
0.10	98.7 ± 1.6^{e}
0.20	94.7 ± 1.3^{d}
Succinic anhydride (g/g protein equivalents)	
0.05	82.5 ± 1.9^{c}
0.10	72.6 ± 1.4^{b}
0.20	63.9 ± 1.5^{a}

[1] Mean ± SD of three samples
Means followed by different superscripts within a column are significantly (p<0.05) different from one another

Table 4.15 Foaming properties of modified and unmodified flax protein isolates[1]

Treatment	Foam expansion (%)[2]	Foam stability (%)[3]
Unmodified	112.0 ± 5.0^{d}	51.0 ± 3.0^{e}
Modified		
Acetic anhydride (g/g protein equivalents)		
0.05	97.0 ± 2.5^{d}	36.1 ± 2.0^{c}
0.10	92.5 ± 1.0^{c}	28.5 ± 1.0^{b}
0.20	74.5 ± 1.0^{b}	14.6 ± 1.0^{a}
Succinic anhydride (g/g protein equivalents)		
0.05	98.2 ± 2.0^{d}	44.3 ± 1.0^{d}
0.10	88.1 ± 3.4^{c}	40.3 ± 1.4^{c}
0.20	66.9 ± 2.0^{a}	16.0 ± 2.0^{a}

[1] Mean ± SD of three samples
Means followed by different superscripts within a column are significantly ($p<0.05$) different from one another

[2] At pH 7.0, percentage volume increase after whipping 50 ml of 1% (w/v) protein solution

[3] Foam remained after 15 min as a percentage of original foam volume

acylated flax protein isolates were less stable than unmodified isolates (Table 4.15). The foam stabilities of the modified protein isolates were parallel to the pattern of foaming capacity.

4.6.5 Effect of acylation on the solubility of flax protein isolates

Figures 4.15 and 4.16 represent the response surfaces of acetylated and succinylated protein isolates due to solubility changes with pH and NaCl concentration, respectively. Both acetylation (Figure 4.15B, C and D) and succinylation (Figure 4.16B, C and D) increased the solubility of isolated proteins compared to their unmodified counterpart. A decreased solubility was observed at acidic (low) pH values for all protein isolates and their modified products. Increased concentration of NaCl had a negative effect on the solubility of modified protein products which was clear at high pH values. Compared to the acetylated or unmodified proteins succinylated products exhibited marked solubility increases at pH values between isoelectric pH and pH 7.0, similar to that observed for other plant proteins such as pea (Johnson and Brekke, 1983), canola (Paulson and Tung, 1987) and soy (Franzen and Kinsella, 1976a). The general trend of protein solubility was similar to those of other acylated plant protein isolates (Shukla, 1982). The effect of NaCl on unmodified protein isolates was to decrease their solubility in the acidic and alkaline pH region, perhaps due to the salting-in and salting-out effects, respectively.

Figure 4.15 Solubility of acetylated flaxseed protein isolate as a function of pH and NaCl concentration: (A) unmodified, (B) 5%AA, (C) 10%AA and (D) 20%AA.

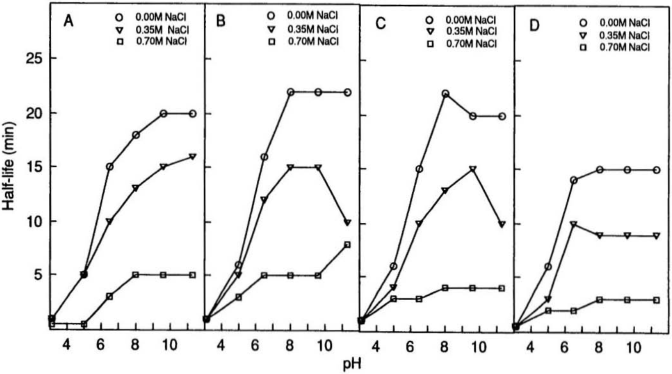
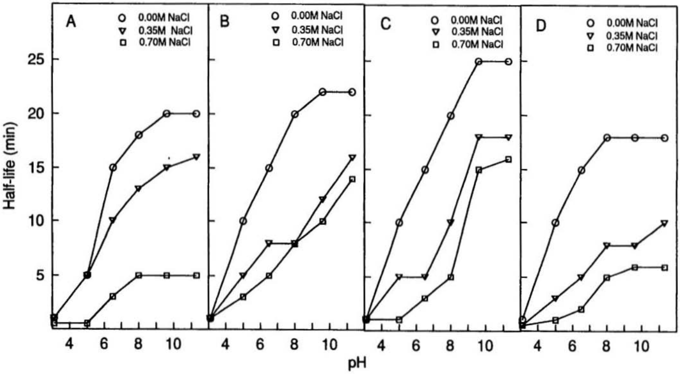

A
B
C
D
○ 0.00M NaCl
▽ 0.35M NaCl
□ 0.70M NaCl
Half-life (min)
25
20
15
10
5
4
6
8
10
pH

Figure 4.16 Solubility of succinylated flaxseed protein isolate as a function of pH and NaCl concentration: (A) unmodified, (B) 5%SA, (C) 10%SA and (D) 20%SA.


A
B
C
D
○ 0.00M NaCl
▽ 0.35M NaCl
□ 0.70M NaCl
Half-life (min)
5
10
15
20
25
4
6
8
10
pH

4.7.6 Effect of acylation on overall functional properties of flax protein isolates

Structural changes of proteins such as those in molecular mass (dissociation), shape (unfolding) and charge due to acetylation or succinylation have been used to explain variations in functional properties of chemically modified storage proteins of seeds. These alterations change hydration properties and surface activities of proteins and affect their solubility and surface activity-related properties (emulsifying and foaming) of the native proteins.

According to Habeeb *et al.* (1958) replacement of short range attractive forces (ammonium-carboxyl) with short range repulsive forces (succinate carboxyl-carboxyl) due to succinylation may alter the molecular conformation. The resultant negative charge, in combination with the electrostatic repulsion due to the introduction of succinate anion, causes looser texture, higher bulk density and lighter colour of the succinylated proteins than their unmodified counterparts. Light coloured products have been obtained from succinylation of fish (Groninger, 1973), leaf (Franzen and Kinsella, 1976b) and soybean (Melnychym and Stapley, 1973) proteins. The exact mechanism by which this change is brought about remains unknown. According to Franzen and Kinsella, (1976a,b) inclusion of water molecules is facilitated due to the repulsion of adjacent polypeptide molecules thus increasing the solubility of succinylated seed proteins.

Studies of Gueguen *et al.* (1990) on rapeseed globulin suggest that succinylation induces a stepwise dissociation of 12S globulin and leads to the unfolding of its subunits. The combination of intra- and inter-molecular charge repulsion also promotes protein unfolding and induces fewer protein-protein and more protein-water interactions. The unfolding of the protein molecule, and dissociation into subunits, shifts the isoelectric point of the proteins to lower values, thus making the acylated proteins more soluble in acidic pH range as compared with their unmodified counterparts. As the net negative charge is proportional to the extent of succinylation, solubility of flax protein isolates would increase as the number of succinylated groups increase (Figure 4.16 A,B,C and D). Solubility is a very important property of functional proteins as it is a critical prerequisite for using proteins in beverages, fluid foods and for emulsion and foam formation. In fact, solubility reflects the balance of charge and hydrophobicity of the protein molecules (Hayakawa and Nakai, 1985) which affects their interaction with the solvent and other protein molecules (Nakai *et al.*, 1991).

Acetylation reduces the extent of electrostatic attraction between neighbouring cationic amino and anionic carboxyl groups due to substitution of amino groups with neutral acetyl groups. As a result, acetylated flax proteins behave differently from succinylated products and exhibited lower solubility when compared to their succinylated counterparts.

Succinylation has been reported to improve the emulsifying properties of oilseed proteins (Child and Parks, 1976; Franzen and Kinsella, 1976a,b; Thompson and Cho, 1984; Paulson and Tung, 1988). As a reflection of increased solubility and looser structure of succinylated proteins, diffusion/migration of the protein molecules to the oil-water interface and rearrangement within the interfacial film is facilitated (Waniska and Kinsella, 1979). Therefore, good solubility of a protein is essential for formation of emulsions (Nakai and Li-Chan, 1988). Pearce and Kinsella (1978) reported that succinylation of yeast proteins affords smaller droplets in oil-in-water emulsions as compared to unmodified proteins; the decrease in droplet size increases interfacial area and hence increases the emulsifying ability of proteins. Watanabe and Arai (1982) have also reported that increased surface area increases surfactant properties of proteins. As the protein becomes more soluble, it forms layers around the fat globule and facilitates association with the aqueous phase which encloses the fat globule and renders the emulsion more stable and resistant to coalescence (Halling, 1981). A positive relationship exists between the solubility and ability of succinylated plant proteins to emulsify oil (Franzen and Kinsella, 1976a,b; Thompson and Cho, 1984; Ponnampalam *et al.*, 1990). However, emulsifying properties of acylated proteins do not depend solely on solubility. The hydrophilic-lipophilic balance (HLB) of a particular protein is also important but does not necessarily increase linearly with the increase in protein hydrophobicity (Nakai, 1983). Thus, acetylated proteins do not show better emulsifying properties although they exhibit higher solubility when compared with their unmodified counterparts. The unfolding of the protein structure due to succinylation may expose more hydrophobic groups buried in the molecule and could change its hydrophobicity and hence the HLB value. Halling (1981) has suggested that increased rheological strength of protein films could reduce mechanical deformation and desorption of the interfacial proteins to give more emulsified droplets. Therefore, emulsion formation could be favoured by protein-protein (hydrophobic) interaction as well as rheological properties of the interfacial protein film which encapsulates the oil droplets.

Fat binding capacity of proteins is important as it enhances flavour retention and improves mouthfeel. The mechanism of fat absorption of a protein was suggested as physical entrapment of oil by protein particles (Wang and Kinsella, 1976). However, the method used in this study was proposed by Voutsinas and Nakai (1983) in order to minimize any physical entrapment effect and to estimate only the absorbed fat by the protein. The protein-lipid interactions are affected by protein conformation, protein-protein interaction and the spatial arrangement of the lipid phase resulting from lipid-lipid interaction. Non-covalent interactions such as hydrophobic, electrostatic and hydrogen bonding forces are also involved in protein-lipid interactions. The aromatic hydrophobicity indicates the exposed aromatic (hydrophobic) residues of the protein.

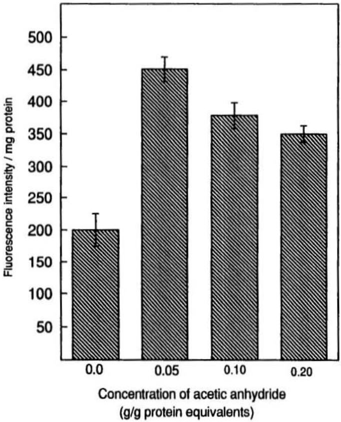
A good foam-forming protein should reduce the surface and interfacial tension of the liquid and form a structural, continuous, cohesive film around air bubbles (Kinsella, 1976). Increased negative charge of the protein could hinder protein-protein interactions due to changes in structure and hydrophobicity and may lead to low foaming ability of the modified protein products; however, solubility is positively related to foaming ability. Gueguen *et al.* (1990) have suggested that unfolding of the protein molecule due to succinylation may increase both the viscosity and interactions between polypeptide chains that may overcome the repulsive effects of the negative charges. Therefore, better foam forming and stabilizing effects of highly succinylated proteins could be related to electrostatic repulsive forces between the air bubbles which are due to the increasing charge of the adsorbed proteins. However, such behaviour was not observed for the succinylated flax protein isolates.

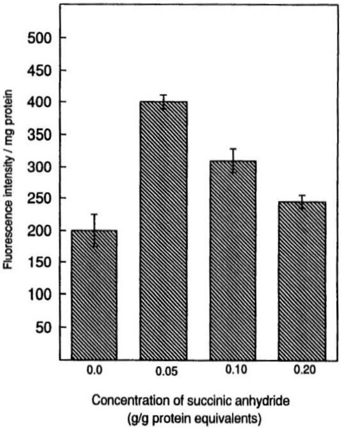
4.8 Surface (aromatic) hydrophobicity

Surface hydrophobicity of flax protein isolates measured as fluorescence intensity/mg protein is presented in Figure 4.17. Acetylation brought about an increase in surface hydrophobicity. Thus, as the degree of acetylation increased the hydrophobicity values also increased. Exposure of the hydrophobic interior and modification of positively charged lysine residues with uncharged acetyl groups results in a decrease in net charge of proteins and hence increased surface hydrophobicity (Kim and Rhee, 1989). Unfolding of the protein molecules may also make hydrophobic sites accessible for binding the fluorescence probe ANS. Schwenke *et al.* (1993) have reported an increased surface hydrophobicity of pea proteins at low and moderate levels of succinylation. According to these authors a drop in surface hydrophobicity was observed after passing the critical level (<70%) of N-succinylation, perhaps due to the effect of high negative charge density that inhibits the ANS molecule from approaching and binding to the protein surface.

Methods proposed for quantitative estimation of protein hydrophobicity included: i) calculation from hydrophobicity data for the individual amino acids and the amino

Figure 4.17 Surface (aromatic) hydrophobicity of modified and unmodified protein isolates.


Fluorescence intensity / mg protein
500
450
400
350
300
250
200
150
100
50
0.0
0.05
0.10
0.20
Concentration of acetic anhydride
(g/g protein equivalents)

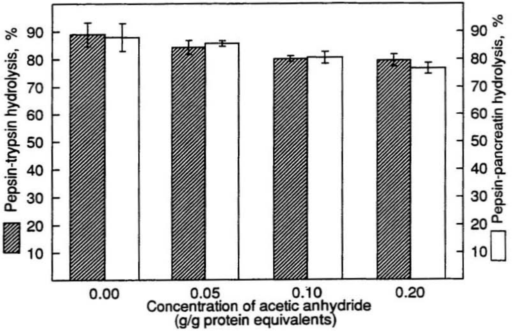

Fluorescence intensity / mg protein
500
450
400
350
300
250
200
150
100
50
0.0
0.05
0.10
0.20
Concentration of succinic anhydride
(g/g protein equivalents)

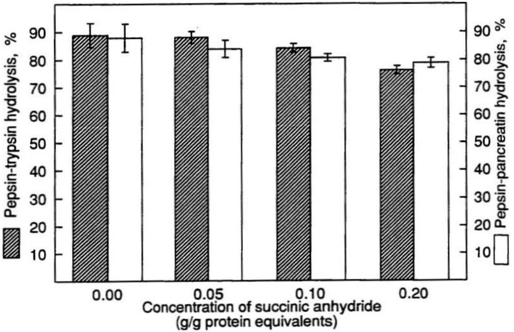
acid composition of the protein, ii) partition, reverse phase or hydrophobic interaction chromatography, iii) binding of aliphatic and aromatic hydrocarbons, triacylglycerols or corn oil, iv) spectroscopic methods and v) use of fluorescence probes (Nakai *et al.*, 1991). It is generally agreed that charged residues are located preferentially at the surface of protein molecules where they can interact with water; residues in the interior of the molecule are closely packed and burial of hydrophobic groups away from the surface can be a major source of stabilization of the protein structure. Therefore, it is likely that groups which can participate in functionality are hydrophobic residues present on the surface of the native protein molecules and those that become exposed during processing and referred as surface and available hydrophobicity, respectively. The hydrophobicity originating from the aromatic amino acid side chains is determined by using ANS and is sometimes regarded as aromatic or surface hydrophobicity (Nakai and Li-Chan, 1988).

4.9 *In-vitro* digestibility

In-vitro digestibility values of flax protein products with pepsin-trypsin and pepsin-pancreatin are given in Figure 4.18. Both acetylation and succinylation reduced the digestibility of proteins. At the highest degree of acetylation or succinylation, a significant ($p<0.05$) decrease in pepsin-pancreatin and pepsin-trypsin digestibility was observed from 90% to 78 and 80%, respectively. Pepsin-pancreatin and pepsin-trypsin digestion simulates gastrointestinal digestion of food proteins (Saunders *et al.*, 1973).

Figure 4.18 *In-vitro* digestibility of modified and unmodified protein isolates.


▨ Pepsin-trypsin hydrolysis, %
90
80
70
60
50
40
30
20
10
☐ Pepsin-pancreatin hydrolysis, %
0.00
0.05
0.10
0.20
Concentration of acetic anhydride
(g/g protein equivalents)


▨ Pepsin-trypsin hydrolysis, %
90
80
70
60
50
40
30
20
10
☐ Pepsin-pancreatin hydrolysis, %
0.00
0.05
0.10
0.20
Concentration of succinic anhydride
(g/g protein equivalents)

Ponnampalam *et al.* (1987) have reported that acetylation decreased tryptic hydrolysis but pepsin hydrolysed acylated canola proteins more effectively. *In-vitro* enzymic digestion of pea protein isolates was not impaired even when up to 95% of ε-amino groups were acylated (Johnson and Brekke, 1983). However, reduced availability of lysine is expected to be due to the susceptibility of ε-amino groups of proteins to chemical modification. This can be regarded nutritionally unfavourable but may be suitable to certain processing conditions by preventing Maillard browning.

4.10 Germination of flaxseed

4.10.1 Changes in ash and dry matter

Table 4.16 presents the content of dry matter, length of seedlings and ash content over an 8-day germination period. The content of dry matter decreased drastically as the seedlings grew to about 6.0 cm in height. A 35% loss of dry matter was observed on day-8 of the germination. At the termination stage of germination experiment two cotyledons opened up and most of them were green. Ash content which is a part of dry matter did not show any significant ($p<0.05$) change (Table 4.16). It is well known that during the initial stages of germination seed reserves are utilized to supply energy and other requirements for the growth of new cells. Since no external nutrients were added, only water and oxygen were consumed by the sprouting seeds. However, a net loss of dry matter occurred as a result of oxidation and breakdown of the stored macromolecules (Chavan *et al.*, 1981), as there was a decrease

Table 4.16 The contents of dry matter and ash and length of seedlings during germination of flaxseed[1]

Germination period (days)	Dry matter content (%)	Length of seedling (mm)	Ash content (%)[2]
0 (ungerminated)	73.5 ± 3.5^{c}	0.0	3.0 ± 0.1^{a}
2	67.6 ± 2.7^{c}	3.5 ± 1.1	2.9 ± 0.2^{a}
4	55.4 ± 1.2^{b}	20.0 ± 4.3	2.9 ± 0.2^{a}
6	53.8 ± 2.4^{b}	45.5 ± 3.9	2.8 ± 0.2^{a}
8	47.5 ± 2.0^{a}	59.2 ± 6.1	2.9 ± 0.1^{a}

[1] Mean ± SD (three replicates)
Values in the same column with different superscripts are significantly ($p>0.05$) different from one another

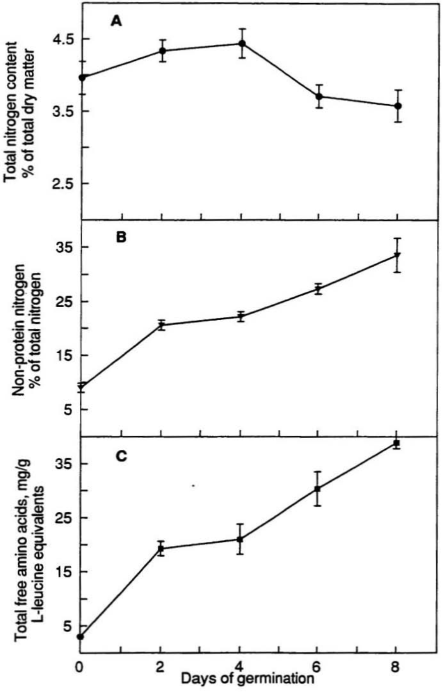
[2] On a dry weight basis

in the amount of majorseed components such as lipids and proteins.

4.10.2 Changes in nitrogenous components

Changes in the contents of total nitrogen, non-protein nitrogen (NPN) and free amino acids in flaxseed during 8 days of germination are summarised in Figure 4.19. Although a relatively small decrease was observed in total nitrogen content, the amount of NPN was increased from 9.0 to 33.5% of the total nitrogen content. Thus, the true protein content of seedlings was actually decreased during the germination. A parallel increase in the content of free amino acids was also observed as the NPN content of seedlings increased during the germination period, thus indicating increased proteolytic activity. The proteins of the cotyledons are hydrolysed to amino acids which are used for the synthesis of new proteins for the newly developing parts. However, utilization of liberated free amino acids after germination does not occur quantitatively since there is an increase in the free amino acid content (Finney, 1983; El-Mahdy and El-Sebaiy, 1985). The process of amino acid catabolism in germinating seeds involves removal of nitrogen from the carbon skeleton, which then undergoes breakdown or interconversion. The carbon skeleton may provide the basis for an alternate amino acid, a respiratory substrate or other non-nitrogenous metabolic components such as keto acid or sugar. Reactions involved in removal of nitrogen from amino acids are transamination and deamination. Transamination results in transfer of an amino group to an alternate keto acid, whereas deamination removes nitrogen from the compound to

Figure 4.19 Changes of the contents of total nitrogen (A), non-protein nitrogen (B) and free amino acids (C) during germination of flaxseed.


A
Total nitrogen content
% of total dry matter
4.5
3.5
2.5
B
Non-protein nitrogen
% of total nitrogen
35
25
15
5
C
Total free amino acids, mg/g
L-leucine equivalents
35
25
15
5
0
2
4
6
8
Days of germination

produce ammonia which is then reassimilated in amide synthesis (Lea and Joy, 1983). Examination of the free amino acid profile of the developing flax seedlings showed that the content of almost all the identified free amino acids was increased during germination (Table 4.17). The content of glutamine was 16, 23, 19 and 17% of total free amino acids on day 2, 4 6 and 8 of germination. At the same time, increased ammonia content in the samples suggests that deamination and synthesis of amide glutamine in the germinating seeds. Glutamine may be the favoured form of amide donor in developing flax seedlings. Small amounts of amides are directly incorporated into new proteins and most are used in nucleic acid or amino acid synthesis (Lea and Joy, 1983; El-Mahdy and El-Sebaiy, 1985).

Studies on the solubility of flax proteins indicated that water-soluble (albumins) and salt-soluble (globulins) proteins were predominant and present in nearly equal amounts in the ungerminated seeds (Table 4.18). However, during the germination an increase in the content of the water-soluble fraction and a decrease in the salt-soluble fraction was observed. It may be assumed that globulin breakdown products became part of the nitrogen determined as albumins (Balasubramaniam and Sadasivam, 1989). The alcohol- and alkali-soluble proteins comprised 7 to 8% of the total proteins of flaxseed. It was also noticeable that alkali-soluble proteins (glutelins) increased as germination proceeded while the content of alcohol-soluble proteins did not change.

Since albumins are mostly enzymic proteins (Bewley, 1978), synthesis of enzymes during germination might be responsible for the apparent increase in the

Table 4.17 Content of free amino acids (mg/g protein) of germinating flaxseed.

Amino acid	Duration of germination (days)				
	0	2	4	6	8
Alanine	0.24±0.01	2.46±0.11	2.43±0.10	3.97±0.20	4.89±0.01
Arginine	1.35±0.09	3.74±0.07	3.16±0.10	4.36±0.13	4.65±0.09
Asparagine	0.31±0.01	0.88±0.01	1.10±0.11	2.00±0.21	3.48±0.05
Aspartic acid	0.43±0.01	0.80±0.03	0.67±0.05	0.92±0.01	1.06±0.04
Cystine	0.06±0.00	0.14±0.10	0.25±0.01	0.33±0.00	0.42±0.03
Glycine	0.19±0.01	0.96±0.08	1.51±0.02	2.58±0.01	6.61±0.10
Glutamine	0.06±0.01	5.11±0.04	8.83±0.12	10.36±0.11	12.58±0.11
Glutamic acid	0.44±0.10	3.17±0.09	3.52±0.11	4.50±0.10	5.83±0.10
Histidine	0.15±0.08	1.24±0.03	1.38±0.08	2.00±0.08	2.47±0.10
Lysine	0.20±0.01	1.14±0.06	1.19±0.02	1.69±0.01	1.95±0.01
Leucine	0.01±0.00	1.84±0.01	1.69±0.10	2.57±0.02	2.63±0.12
Isoleucine	0.04±0.00	1.16±0.10	1.06±0.04	1.32±0.00	1.48±0.00
Methionine	0.01±0.00	0.57±0.02	0.48±0.05	0.55±0.01	0.52±0.00
Phenylalanine	0.13±0.01	1.38±0.06	1.03±0.01	1.57±0.01	1.58±0.01
Proline	0.27±0.02	1.04±0.04	2.35±0.30	4.38±0.10	4.60±0.07
Serine	0.08±0.01	1.92±0.01	1.92±0.01	3.42±0.11	5.08±0.10
Threonine	0.11±0.01	0.99±0.03	1.01±0.00	1.30±0.01	1.62±0.01
Tryptophan	0.37±0.08	0.93±0.05	1.21±0.06	1.79±0.06	1.95±0.08
Tyrosine	0.10±0.00	1.11±0.00	1.14±0.10	1.27±0.00	1.36±0.09
Valine	0.06±0.01	1.43±0.00	1.44±0.01	1.83±0.01	2.32±0.10
Total content	4.61±0.47	31.08±0.94	37.37±1.40	52.71±1.19	72.38±1.22
Ammonia	2.27±0.20	5.77±0.50	11.62±0.22	20.11±0.61	32.10±0.35

Table 4.18 Protein fractions of germinated flaxseed according to their solubility.

Duration of germination (days)	Fraction of total nitrogen (%)				
	Water-soluble	Salt-soluble	Alcohol-soluble	Alkali-soluble	Residue
0 (ungerminated)	36.9 ± 1.0	30.2 ± 0.9	3.5 ± 0.8	3.9 ± 0.9	27.5 ± 2.7
2	61.2 ± 2.1	17.3 ± 1.1	3.5 ± 1.0	3.9 ± 1.1	14.2 ± 2.3
4	61.0 ± 2.0	12.9 ± 1.1	3.5 ± 0.6	5.3 ± 1.2	17.3 ± 1.5
6	58.6 ± 1.5	8.7 ± 0.8	4.6 ± 1.2	9.8 ± 1.5	18.3 ± 2.4
8	55.9 ± 1.4	8.1 ± 1.0	4.1 ± 1.0	12.5 ± 2.6	19.4 ± 3.2

content of the albumin fraction. An increase in the content of free amino acids is another indication of the increased protease activity and proteolytic degradation during the germination process.

Amino acid composition of germinated and ungerminated flax proteins is presented in Table 4.19. The high content of non-essential amino acids, especially aspartic acid, glutamic acid and arginine may not be nutritionally important but may be useful in seed metabolism/enzyme synthesis or function (Finney, 1983). The amino acid score showed comparable values to FAO/WHO (1973) standard protein. On day 8 of germination, isoleucine and leucine were the first and second limiting amino acids, respectively. *In vitro* digestibility of proteins was lowered on day 8 of seed germination as compared to that of ungerminated seeds. *In vitro* digestibility and calculated protein efficiency ratio (C-PER) and BV values, based on the content of essential amino acids and digestibility data showed the highest values at the day-4.

The content of polyamines in flax seedlings during germination is shown in Table 4.20. The only polyamines present were agmatine and spermidine; putrescine, a diamine, was also present. The content of putrescine was higher than that of both polyamines present before germination started. As the germination proceeded the contents of agmatine and spermidine were increased by approximately 50 and 200 fold, respectively. Meanwhile the content of putrescine decreased by a factor of 3.

Polyamines of plants are fundamentally important in the control of cellular metabolism and growth, and their content may reach high levels in rapidly growing

Table 4.19 Amino acid composition (g/16 g N) and protein quality of germinated flaxseed.

Amino acid	Duration of germination (days)		
	0	4	8
Essential amino acids			
Histidine	2.50 ± 0.11	2.67 ± 0.08	2.53 ± 0.10
Isoleucine	4.54 ± 0.30	4.92 ± 0.09	3.74 ± 0.04[3]
Leucine	6.54 ± 0.26	7.06 ± 0.06	6.62 ± 0.20[3]
Lysine	4.55 ± 0.18	5.49 ± 0.10	5.20 ± 0.10
Methionine+Cysteine	6.09 ± 0.02	5.02 ± 0.10	3.84 ± 0.08
Phenylalanine+Tyrosine	7.50 ± 0.08	7.50 ± 0.13	6.01 ± 0.08
Threonine	4.37 ± 0.20	4.61 ± 0.20	4.03 ± 0.09
Tryptophan	0.70 ± 0.02	1.59 ± 0.01	1.28 ± 0.02
Valine	5.46 ± 0.06	5.85 ± 0.20	4.87 ± 0.06
Non-essential amino acids			
Alanine	5.02 ± 0.10	5.99 ± 0.09	5.88 ± 0.11
Arginine	10.03 ± 0.43	9.77 ± 0.15	6.48 ± 0.40
Aspartic acid+asparagine	10.65 ± 0.50	10.72 ± 0.58	8.87 ± 0.50
Glycine	7.44 ± 0.82	6.03 ± 0.08	7.19 ± 0.41
Glutamic acid+glutamine	20.76 ± 1.09	17.02 ± 0.90	15.04 ± 1.00
Proline	3.98 ± 0.23	4.96 ± 0.09	4.85 ± 0.03
Serine	5.02 ± 0.09	5.45 ± 0.11	5.31 ± 0.09
Total essential amino acids[1]	39.75	42.04	35.59
E/T %	37.80	40.17	38.79
Amino acid score	73.00	100	89.00
In vitro digestibility[2]	85.40	87.30	77.50
BV	85.50	98.30	87.90
C-PER	1.95	2.22	2.00

[1] Histidine not included

[2] *In vitro* digestibility with pepsin-trypsin

[3] Limiting amino acid

Table 4.20 Polyamine content of germinating flaxseeds[1].

Polyamine	Duration of germination (days)				
	0	2	4	6	8
Agmatine $NH_2(CH_2)_4NHCH(NH)NH_2$	0.08 ± 0.01	1.18 ± 0.10	2.44 ± 0.12	3.32 ± 0.11	4.21 ± 0.44
Putrescine $NH_2(CH_2)_4NH_2$	0.21 ± 0.02	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
Spermidine $NH_2(CH_2)_3NH(CH_2)_4NH_2$	0.03 ± 0.01	0.29 ± 0.05	2.54 ± 0.09	4.71 ± 0.23	5.86 ± 0.15

[1] μmol/g of dry matter
Mean ± SD of three replicates

tissues (Janne *et al.*, 1978; Smith, 1985). Decarboxylation of arginine serves as the basis for biosynthesis of agmatine, putrescine and spermidine in plants. Initially, arginine is decarboxylated to agmatine by arginine decarboxylase and then hydrolysed to putrescine by agmatinase (Bardocz, 1995). Spermidine is formed sequentially from putrescine by spermidine synthase (Smith, 1985). Polyamines are reported to enhance protease activity and to increase the mobilization of reserve proteins and the growth of radish seedlings (Srivastava *et al.*, 1985). Foods such as beer, cheese, chocolate, fish, sauerkraut and wine provide large quantities of polyamines (220-450 μmol putrescine, 100-180 μmol spermidine and 70 μmol spermine), in addition to their biosynthesis *in situ* (1-2 nmol putrescine/h/g of wet tissue in active organs). Polyamines are essential for the maintenance of the high metabolic activity of the normally functioning healthy gut and also play a role in repairing damage to gut tissues caused by deleterious components of food and/or by bacteria. However, concerns have been expressed about needs to minimize polyamine intake in order to slow down the growth of tumours (Bardocz, 1995). Therefore, it would be important to assess the polyamine content of rapidly growing tissues such as sprouting seeds especially those intended for human consumption. No previous studies have been carried out on identification and quantification of polyamines of germinated seeds intended for consumption.

4.10.3 Changes in lipid components of flaxseed during germination

Lipids comprised 43.5±1.0% of the dry matter of seeds and provided a major portion of the energy required for the growing of seedlings. Quantitative changes in the content of total, neutral, glyco- and phospholipids as well as free fatty acids of flaxseed occurred during an 8-day germination period (Table 4.21). A large decrease was observed in the total content of lipids after 4 days of germination. Lipid contents were lowered by 40 and 58% at 6 and 8 days of germination, respectively. A significant decrease was observed in the content of neutral lipids (storage lipid) fraction. Meanwhile, the proportion of the lipid fraction represented by structural lipids (glyco- and phospholipids) was increased, perhaps due to enhanced membrane formation activity and transformation during development of seedlings (Huang and Grunwald, 1990). The content of free fatty acids remained unchanged until the second day of germination and then increased by 13 fold by the end of the germination period. The content of free fatty acids on day 8 of the germination was 2.2% of total lipids; however, this is a rather low value compared to 10% reported by Zimmerman and Klosterman (1965) for flaxseed.

The initial step in lipid metabolism is the release of fatty acids from reserve triacylglycerols accomplished via hydrolysis due to increased lipase activity (Gilliard, 1980; Huang, 1987). The released fatty acids undergo β-oxidation (oxidation at β position to the carboxyl group and sequential removal of C units) to produce the required energy (Kindl, 1987) in the form of adenosine triphosphate (ATP). As the major energy reserve in the seed, lipids provide fatty acids that serve as an energy source to produce ATP and soluble carbohydrates for the growth of new cells during

Table 4.21 Changes in the content of total lipids, free fatty acids and major lipid classes of flaxseed during germination[1].

Germination period (days)	Total lipid content (%)	Free fatty acid content (µg/mg oil)[2]	Major lipid classes (% of total)		
			Neutral lipids	Glycolipids	Phospholipids
0 (ungerminated)	43.5 ± 1.0^{d}	1.5 ± 0.6^{a}	96.3 ± 1.9^{c}	2.3 ± 1.5^{a}	1.4 ± 0.9^{ab}
2	41.8 ± 0.9^{cd}	1.7 ± 0.8^{a}	95.0 ± 2.8^{c}	2.7 ± 1.7^{a}	2.3 ± 1.1^{ab}
4	40.6 ± 1.1^{cd}	5.7 ± 1.9^{b}	94.3 ± 2.9^{c}	4.2 ± 1.3^{a}	1.5 ± 1.0^{ab}
6	26.2 ± 1.5^{b}	15.1 ± 4.1^{c}	90.2 ± 2.5^{bc}	8.7 ± 1.5^{bc}	1.1 ± 1.0^{a}
8	18.2 ± 1.1^{a}	21.9 ± 2.9^{c}	82.9 ± 3.0^{a}	13.6 ± 2.1^{c}	3.4 ± 1.0^{b}

[1] Mean ± SD (three replicates)
Means followed by different superscripts within a column are significantly ($p<0.05$) different from one another.
Values are on dry weight basis

[2] As linoleic acid equivalents

germination (Zimmerman, 1971).

Neutral lipids account for nearly 96% of total lipids of flaxseed before germination (Table 4.21). Separation of flaxseed lipids by TLC-FID showed that the neutral lipid fraction (Figure 4.20A and Table 4.22) was composed of triacylglycerols (TAG), monoacylglycerols (MAG) diacylglycerols (DAG) and free fatty acids (FFA). Sterols (ST) were detected in minute quantities in some samples. The content of triacylglycerols was decreased while that of other components increased during germination. Decreased level of TAG indicates that they are the major compounds involved in catabolism to provide substrate for oxidation during germination of flaxseed as previously reported by Zimmerman (1971). Degradation of plant storage lipids was in the sequence of TAG→DAG→MAG→FFA (Huang and Grunwald, 1990). True plant lipases hydrolyse, not only TAG, but also DAG and MAG; however, enzymes such as esterases and hydrolases degrade only DAG and MAG (Gilliard, 1980). The accumulation of DAG and/or MAG indicates the presence of various concentrations or types of hydrolytic enzymes in different tissues of seedlings (eg. cotyledons, roots, etc.). Since in this study seedlings as a whole were considered a net increase in the content of DAG and MAG was observed. The increase in FFA content also suggests possible fatty acid biosynthesis during the latter stages of germination, similar to that observed for soybean and alfalfa (Huang and Grunwald, 1990).

Linoleic and linolenic acids comprised 80% of the fatty acids of the total lipids of flax. Changes in fatty acid composition of total (Table 4.23) and neutral (Table 4.24)

Table 4.22 Neutral lipids of flaxseed and their changes during germination[1].

Neutral lipids	Germination period (days)				
	0	2	4	6	8
Monoacylglycerol (MAG)	1.10 ± 0.19	2.39 ± 0.34	3.62 ± 0.33	6.29 ± 0.66	4.77 ± 0.36
Diacylglycerol (DAG)	2.08 ± 0.26	3.85 ± 0.41	4.19 ± 0.20	5.22 ± 0.63	5.45 ± 0.41
Triacylglycerol (TAG)	96.40 ± 6.29	88.64 ± 4.57	85.93 ± 4.20	78.39 ± 7.29	79.89 ± 3.95
Free fatty acids (FFA)	1.37 ± 0.49	5.12 ± 0.40	6.25 ± 1.00	10.16 ± 0.38	9.89 ± 0.73
Sterols (ST)	trace	ND[2]	trace	trace	trace

[1] As a weight percentage of total neutral lipids
Mean ± SD of three replicates

[2] Not detected

Figure 4.20 TLC-FID chromatograms of flax lipids. (A) Neutral lipids; triacylglycerols (TAG), free fatty acids (FFA), diacylglycerols (DAG) sterols (ST) and monoacylglycerols (MAG): (B) Phospholipids; phosphatidic acid (PA), phophotidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), phosphatidylserine (PS) and lysophospha tidylcholine (LPC).

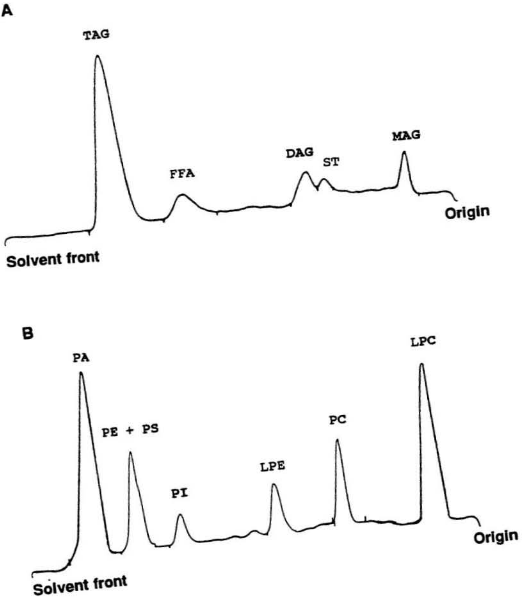

A
TAG
FFA
DAG
ST
MAG
Origin
Solvent front
B
PA
PE + PS
PI
LPE
PC
LPC
Origin
Solvent front

Table 4.23 Fatty acid composition of total lipids of flaxseed during germination[1].

Fatty acid	Germination period (days)				
	0	2	4	6	8
C14:0	0.6 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
C16:0	5.2 ± 0.5	5.1 ± 0.4	5.0 ± 0.3	5.5 ± 0.3	6.1 ± 0.3
C18:0	2.7 ± 0.3	2.7 ± 0.4	2.8 ± 0.4	3.0 ± 0.3	3.1 ± 0.4
C20:0	trace	trace	trace	0.3 ± 0.1	0.3 ± 0.1
C22:0	trace	trace	trace	0.3 ± 0.1	0.2 ± 0.1
C24:0	trace	trace	trace	trace	trace
C14:1	trace	trace	trace	trace	trace
C16:1	trace	0.1 ± 0.1	0.2 ± 0.0	trace	0.3 ± 0.1
C18:1	11.7 ± 0.5	11.9 ± 0.3	11.7 ± 0.4	12.0 ± 0.3	12.1 ± 0.3
C20:1	trace	trace	trace	trace	trace
C22:1	trace	trace	trace	trace	trace
C18:2	16.1 ± 0.9	16.1 ± 0.8	16.6 ± 0.8	18.0 ± 0.5	18.5 ± 0.3
C18:3	63.6 ± 1.0	63.7 ± 0.9	62.8 ± 0.5	59.9 ± 0.7	58.1 ± 0.7
C20:5	trace	trace	0.1 ± 0.1	0.5 ± 0.1	0.6 ±0.1
Total saturated fatty acids	8.5	8.2	8.6	9.6	10.3
Total monounsaturated fatty acids	11.7	12.0	11.9	12.0	12.4
Total polyunsaturated fatty acids	79.7	79.8	79.5	78.4	77.2

[1] As area percentage
Mean ± SD of three replicates

Table 4.24 Fatty acid composition of neutral lipids of flaxseed during germination[1].

Fatty acid	Germination period (days)				
	0	2	4	6	8
C14:0	0.0	0.05 ± 0.00	0.06 ± 0.00	0.10 ± 0.00	0.09 ± 0.00
C16:0	4.35 ± 0.03	4.91 ± 0.03	4.90 ± 0.05	5.15 ± 0.20	5.52 ± 0.04
C18:0	2.29 ± 0.04	2.70 ± 0.06	2.71 ± 0.02	2.88 ± 0.10	3.02 ± 0.00
C20:0	0.0	0.11 ± 0.00	0.14 ± 0.00	0.20 ± 0.00	0.28 ± 0.01
C24:0	0.0	0.0	0.33 ± 0.00	0.59 ± 0.01	0.78 ± 0.02
C14:1	9.96 ± 0.10	0.0	0.0	0.0	0.0
C16:1	0.0	0.02 ± 0.00	0.06 ± 0.00	0.08 ± 0.00	0.09 ± 0.00
C18:1	9.96 ± 0.11	11.59 ± 0.10	11.40 ± 0.10	11.57 ± 1.00	12.00 ± 0.10
C20:1	0.0	0.14 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.16 ± 0.02
C22:1	0.0	0.0	0.0	0.0	0.04 ± 0.00
C18:2	13.46 ± 0.12	15.47 ± 0.15	16.07 ± 1.00	17.17 ± 0.29	17.83 ± 0.17
C18:3	59.96 ± 0.59	64.94 ± 1.20	64.07 ± 1.19	61.86 ± 1.90	59.85 ± 1.10
C20:5	0.0	0.08 ± 0.0	0.12 ± 0.01	0.25 ± 0.02	0.39 ± 0.00
Total saturated fatty acids	6.64	7.77	8.14	8.92	9.69
Total monounsaturated fatty acids	19.92	11.75	11.61	11.80	12.29
Total polyunsaturated fatty acids	73.42	80.49	80.26	79.28	78.07

[1] As area percentage
Mean ± SD of three replicates

lipids followed a similar pattern. Approximately 60% of the fatty acids in the neutral lipid fraction was linolenic acid while the content of linoleic acid was about 13.5% (Table 4.24). The total content of polyunsaturated fatty acids of neutral lipids did not decrease, but there was a net decrease in total content of monounsaturated fatty acids (MUFA) and an increase in the total content of saturated fatty acids during the germination period. Saturated, monounsaturated and polyunsaturated fatty acids of C20 and longer appeared during the latter part of germination, indicating possible chain elongation and desaturation activities in the neutral lipid fraction.

Phospholipids comprised 1.4% of total lipids of flaxseed (Table 4.21). Their content fluctuated slightly during germination but this was not significant. TLC-FID separation (Figure 4.20B) of phospholipids of flaxseed revealed that phosphotidylcholine (PC) was the major phospholipid of flaxseed (Table 4.25). Phosphatidylserine and phosphatidylethanolamine were eluted together and their total comprised the next largest fraction. While the content of phosphatidylcholine (PC) decreased during germination, the content of lysophosphatidylcholine (LPC) increased and that of phosphatidic acid (PA) increased drastically from a negligible amount to 46% of the total phospholipid fraction after 8 days of germination. The content of phosphatidylinositol (PI) was also increased as germination proceeded.

Polyunsaturated fatty acids comprised a major fraction of fatty acids (67%) of phospholipids of flax which exhibited little change during germination (Table 4.26). Linolenic acid was the predominant fatty acid in the phospholipid fraction of flax,

Table 4.25 Changes in the contents of phospholipids of flaxseed during germination[1].

Phospholipid	Germination period (days)				
	0	2	4	6	8
Phosphatidic acid (PA)	ND[2]	20.4 ± 1.1	38.6 ± 3.1	40.6 ± 2.9	46.2 ± 2.2
Phosphatidylcholine (PC)	34.9 ± 2.0	53.7 ± 5.0	14.8 ± 1.6	16.9 ± 1.1	9.9 ± 1.1
Phosphatidylinositol (PI)	0.5 ± 0.1	0.8 ± 0.1	13.1 ± 1.9	5.7 ± 1.1	4.6 ± 0.7
Phosphatidylserine + phosphatidylethanolamine (PS+PE)	49.5 ± 2.5	11.4 ± 1.0	17.5 ± 1.6	14.6 ± 1.1	9.9 ± 0.2
Lysophophatidylcholine (LPC)	15.1 ± 1.5	14.6 ± 2.2	15.4 ± 1.1	13.0 ± 0.9	25.1 ± 1.1
Lysophosphatidylethanolamine (LPE)	ND[2]	ND[2]	0.6 ± 0.1	9.7 ± 0.8	4.2 ± 0.4

[1] As weight percentage of total phospholipids
Mean ± SD of three replicates

[2] Not detected

Table 4.26 Fatty acid composition of phospholipids of flaxseed during germination[1].

Fatty acid	Germination period (days)				
	0	2	4	6	8
C14:0	1.82 ± 0.04	3.61 ± 0.09	0.47 ± 0.01	0.33 ± 0.00	0.55 ± 0.00
C16:0	11.78 ± 0.10	7.98 ± 0.10	10.13 ± 0.50	15.77 ± 0.10	15.21 ± 0.90
C18:0	3.71 ± 0.04	2.66 ± 0.02	2.83 ± 0.08	4.99 ± .09	4.89 ± 0.10
C20:0	0.69 ± 0.00	0.0	0.0	0.0	0.0
C22:0	0.68 ± 0.00	0.0	0.0	1.00 ± 0.00	1.23 ± 0.09
C14:1	0.0	0.0	0.0	0.0	0.23 ± 0.00
C16:1	0.20 ± 0.00	0.67 ± 0.01	0.0	1.31 ± 0.07	1.42 ± 0.07
C18:1	13.76 ± 0.69	10.74 ± 0.90	2.99 ± 0.09	5.39 ± 0.10	5.48 ± 0.50
C22:1	0.69 ± 0.05	1.33 ± 0.05	0.0	0.0	0.59 ± 0.01
C18:2	29.86 ± 0.90	17.59 ± 0.99	10.37 ± 0.89	15.43 ± 0.99	15.62 ± 1.00
C18:3	36.80 ± 0.89	55.42 ± 1.80	73.21 ± 2.10	55.95 ± 1.50	54.82 ± 1.50
Total saturated fatty acids	18.68	14.25	13.43	22.09	21.88
Total monounsaturated fatty acids	14.65	12.74	2.99	6.7	7.72
Total polyunsaturated fatty acids	66.66	73.01	83.58	71.38	70.43

[1] As area percentage
Mean ± SD of three replicates

similar to other lipid fractions of flax and its content was increased during germination. The only other polyunsaturated fatty acid found in the phospholipid fraction of flax was linoleic acid, the content of which was decreased during germination. Oleic acid which was the predominant monounsaturated fatty acid of flax showed a decrease and then an increase during the germination period.

The content of glycolipids of flax was significantly ($p<0.05$) increased during seed germination (Table 4.21). Huang and Grunwald (1990) have suggested that glycolipids are important components of photosynthetic membranes and their increase during germination reflects chloroplast development and tissue greening. The content of saturated fatty acids of glycolipids was higher (Table 4.27) and the total polyunsaturated fatty acids (PUFA) contents were lower compared to neutral or phospholipid fractions (Figure 4.21). The total content of PUFA increased but that of monounsaturated fatty acids of glycolipids decreased as germination proceeded. The total saturated fatty acid content of flax showed only a small overall change during the germination process. Linolenic, oleic and palmitic acids were found predominantly among the polyunsaturated, monounsaturated and saturated fatty acids of the glycolipid fraction of flax lipids, respectively; their content did not change during germination (Table 4.27).

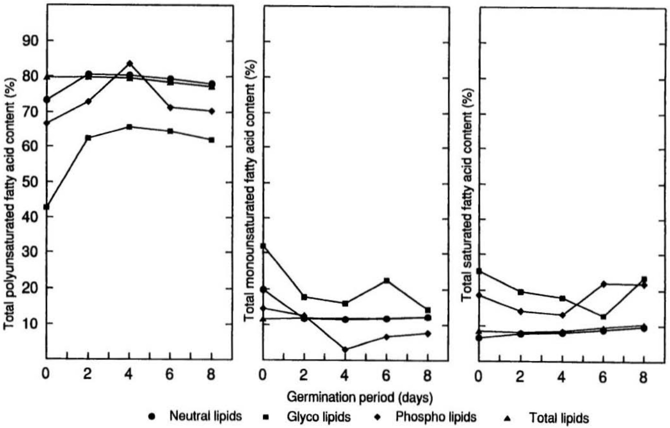
At the end of the germination period, flax seedlings contained 58% less lipid than the amounts present in the original seeds (approximately 18%), on a dry weight basis. Flax lipids after 8 days of germination were rich in PUFA (77%) and MUFA (12%) and contained only 10% of saturated fatty acids.

Table 4.27 Fatty acid composition of glycolipids of flaxseed during germination[1].

Fatty acid	Germination period (days)				
	0	2	4	6	8
C14:0	7.48 ± 0.10	0.80 ± 0.01	1.71 ± 0.10	2.87 ± 0.20	3.21 ± 0.20
C16:0	12.27 ± 0.20	8.44 ± 0.20	10.71 ± 0.90	7.44 ± 0.40	13.36 ± 0.98
C18:0	4.32 ± 0.04	2.94 ± 0.10	3.55 ± 0.20	2.73 ± 0.02	3.97 ± 0.09
C20:0	0.73 ± 0.00	0.77 ± 0.00	0.0	0.0	0.36 ± 0.00
C22:0	0.0	0.0	0.0	0.0	1.74 ± 0.02
C24:0	0.0	6.76 ± 0.10	2.09 ± 0.10	0.0	0.94 ± 0.00
C14:1	4.97 ± 0.20	0.37 ± 0.00	0.0	2.43 ± 0.02	2.50 ± 0.20
C16:1	8.81 ± 0.39	0.57 ± 0.00	0.57 ± 0.03	2.06 ± 0.20	1.25 ± 0.01
C18:1	13.82 ± 0.98	13.03 ± 0.90	12.54 ± 1.29	14.00 ± 0.60	10.76 ± 0.45
C20:1	0.77 ± 0.00	0.60 ± 0.00	0.0	0.0	0.0
C22:1	3.52 ± 0.00	3.34 ± 0.20	2.92 ± 0.10	3.98 ± 0.08	0.0
C18:2	13.34 ± 0.90	16.57 ± 1.00	17.55 ± 1.09	17.10 ± 0.98	13.62 ± 0.56
C18:3	29.18 ± 1.00	45.00 ± 2.06	47.08 ± 1.25	47.39 ± 1.00	48.39 ± 1.08
C20:5	0.28 ± 0.00	0.80 ± 0.08	1.08 ± 0.30	0.0	0.0
Total saturated fatty acids	25.30	19.71	18.06	13.04	23.48
Total monounsaturated fatty acids	31.89	17.91	16.23	22.47	14.51
Total polyunsaturated fatty acids	42.80	62.37	65.71	64.49	62.01

[1] As area percentage
Mean ± SD of three replicates

Figure 4.21 Changes in total contents of polyunsaturated, monounsaturated and saturated fatty acids of total, neutral, glyco and phospholipid fractions of flaxseed during germination.


Total polyunsaturated fatty acid content (%)
90
80
70
60
50
40
30
20
10
0
2
4
6
8
Total monounsaturated fatty acid content (%)
0
2
4
6
8
Total saturated fatty acid content (%)
0
2
4
6
8
Germination period (days)
● Neutral lipids
■ Glyco lipids
◆ Phospho lipids
▲ Total lipids

4.10.4 Changes in the soluble sugars of flaxseed during germination

The content of total soluble sugars of flaxseed was increased during the germination period. Fructose, glucose, sucrose and raffinose were the only sugars present. Sucrose was the major soluble sugar present and comprised 59% of the total amount in flaxseed prior to germination (Table 4.28). Raffinose was the second predominant sugar and comprised 38% of the total. The monosaccharides, fructose and glucose, were present at a 3% level. When germination proceeded, sucrose content was reduced to 5% of the total sugars present at the termination of germination. After day 4 of germination, raffinose was totally consumed. However, the content of monosaccharides, namely fructose and glucose continuously increased from 1.8 to 22.5% and 2.4 to 72.0%, respectively.

It has been documented that rapid hydrolysis of oligosaccharides such as raffinose and stachyose occur during the germination of legumes (Chavan and Kadam, 1989). There is also evidence to indicate increased activity of glycolytic enzymes during seed germination (Finney, 1983). The content of total soluble and reducing sugars of flax seedlings was also increased. The reduction in the content of flatulence causing sugars during germination is an important factor which may positively affect the nutritional value of seeds.

4.10.5 Changes in minerals during germination of flaxseed

There was little change in the ash content of flaxseed (Table 4.16) during the

Table 4.28 Changes in total soluble sugar contents and their composition during the germination of flaxseed[1].

Duration of germination (days)	Total soluble sugar content (%)[2]	Composition of soluble sugars (mg/g)[3]			
		Fructose	Glucose	Sucrose	Raffinose
0 (ungerminated)	4.1 ± 0.9	0.60 ± 0.08	0.80 ± 0.09	19.79 ± 1.00	12.18 ± 1.09
2	3.9 ± 0.6	3.50 ± 0.20	3.95 ± 0.60	15.80 ± 1.09	2.05 ± 0.60
4	5.6 ± 0.5	9.85 ± 1.18	23.65 ± 2.17	12.20 ± 1.08	ND[4]
6	7.3 ± 0.7	16.85 ± 1.91	39.25 ± 3.46	2.60 ± 0.17	ND[4]
8	11.9 ± 0.8	24.45 ± 0.78	78.40 ± 2.45	5.75 ± 0.90	ND[4]

[1] w/w, dry matter
Mean ± SD of three replicates
[2] As glucose equivalents; determined colorimetrically
[3] Determined by HPLC
[4] Not detected

germination process, thus indicating insignificant loss of soluble minerals by leaching. The major minerals of flaxseed were Mg and Na (Table 4.29). The content of Mg (5.4-6.5 mg/g) was lower but Na (0.4-0.9 mg/g) was higher than those reported for defatted flaxseed meal by Bhatty and Cherdkiatgumchai (1990). Iron was the predominant microelement and molybdenum was present in trace amounts. Little change was observed in the contents of these minerals during germination. Chavan and Kadam (1989) have suggested that any change in the content of minerals is most probably due to leaching losses; however, translocation of individual elements within the growing seedlings may also occur.

4.10.6 Changes in the contents of cyanogenic glycosides, phytic acid and trypsin inhibitors during germination of flaxseed

The cyanogenic glycosides of the flaxseed variety used in this study were linustatin and neolinustatin (Table 4.30). Their contents in the seeds decreased by 40 and 70% on day-8 of germination, respectively. There is little information available on the fate of cyanogenic glycosides during germination of seeds. However, it has been demonstrated that linamarin in seeds of *Hevea brasiliensis* is metabolized into non-cyanogenic compounds without any liberation of HCN (Selmar *et al.*, 1988). Linustatin is the other cyanogenic glycoside present in this seed. According to Selmar *et al.* (1988) linamarin is first converted to the diglycoside linustatin which is the translocation form of cyanogenic glycosides that may not be broken down by β-glycosidase (linamarase) during transportation. During the germination phase of leaf

Table 4.29 Changes in the content of minerals during germination of flaxseed (w/w, dry matter).

Mineral	Germination period (days)		
	0	4	8
mg/g			
Calcium	0.21 ± 0.01	0.21 ± 0.12	0.19 ± 0.08
Magnesium	3.41 ± 0.60	3.49 ± 0.11	3.37 ± 0.12
Potassium	0.55 ± 0.07	0.79 ± 0.08	0.71 ± 0.02
Sodium	6.61 ± 1.7	5.84 ± 0.24	5.90 ± 0.44
µg/g			
Copper	11.2 ± 2.3	12.4 ± 1.7	11.5 ± 2.9
Iron	87.2 ± 11.3	95.8 ± 11.3	108.3 ± 2.3
Manganese	43.5 ± 3.0	43.4 ± 0.8	43.9 ± 0.3
Molybdenum	0.03	0.0	0.0
Zinc	31.5 ± 1.0	35.1 ± 0.4	31.8 ± 2.6

Table 4.30 Changes in the content of cyanogenic glycosides, phytic acid and trypsin inhibitors during germination of flaxseed[1].

Component	Duration of germination (days)				
	0	2	4	6	8
Cyanogenic glycosides (mg/g dry matters)					
Linustatin	2.70 ± 0.50	3.50 ± 0.67	3.00 ± 0.55	2.79 ± 0.20	1.60 ± 0.10
Neolinustatin	3.09 ± 0.90	3.60 ± 0.80	2.75 ± 0.18	0.65 ± 0.10	0.28 ± 0.07
Phytic acid (%, w/w)	1.02 ± 0.17	1.01 ± 0.09	0.91 ± 0.03	0.90 ± 0.03	0.74 ± 0.09
Trypsin inhibitors[2] (mg TI/g crude protein)	13.3 ± 1.4	ND	ND	ND	ND

[1] Mean ± SD of three replicates

[2] Not detected

expansion linustatin is transported out to the growing parts of seedlings, where the enzyme diglucosidase splits off gentiobiose. The HCN produced by dissociation of the resulting acetone cyanohydrin is immediately fixed by β-cyanoalanine synthase to β-cyanoalanine. The β-cyanoalanine so produced may be hydrolysed to produce asparagine. This pathway of degradation of linustatin suggests that cyanogenic glycosides are not solely stored secondary metabolites and may function as a source of N when there is a great demand for nitrogen, such as in certain developmental stages. From a nutritional point of view the disappearance of cyanogenic glycosides during sprouting reduces the risk of HCN production, thus improving the nutritional quality of sprouts.

The content of phytic acid in flaxseed at different stages of germination was lowered (Table 4.30). Germination is mainly a catabolic process that supplies important nutrients to the growing plant through hydrolysis of reserve nutrients; therefore, loss of phytic acid is expected as it is the main form of stored P in the seeds. Reddy *et al.* (1982) have shown that phytase activity increases during the germination process. Since phytic acid may be one of the factors responsible for reducing mineral availability, its loss during germination may enhance nutritional value of flax seedlings with respect to mineral bioavailability.

The content of trypsin inhibitors in flaxseed was very low compared to those of legumes and only trace amounts were present in the germinated samples (Table 4.30). This is a very good indication that there are no commonly found protease (trypsin) inhibitors in germinated flax.

4.10.7 Effect of germination on overall chemical composition and nutritional value of flax

In this study 95-98% germination percentage was achieved for flaxseed under the conditions provided. Flaxseed required no additional pretreatment besides soaking which expedites emerging of hypocotyl. Therefore, preparation of flaxseed sprouts requires only low cost inputs such as ordinary trays for germination, a lining that retains moisture and ample supply of water. However, possible growth of fungi should be carefully monitored as for other seeds.

Although levels of lipids in the sprouts were reduced, after 8 days of sprouting, the remaining lipids were rich in PUFA. The content of protein was lowered and amino acid composition, *in vitro* digestibility, BV, C-PER and amino acid score were not adversely affected. Trypsin inhibitor activity of flaxseed was very weak, but had completely disappeared in the sprouts. Levels of nutritionally important minerals were not reduced and presence of lower levels of phytic acid in seedlings would increase their bioavailability. Only simple sugars were found in sprouted flax and no oligosaccharides remained after day-8 of the germination. The content of major cyanogenic glycosides in the seeds were also decreased. There was a great loss of dry matter at the termination of germination; therefore, it is important to find the optimum stage of germination to achieve both high yield and optimum nutritional value.

Since all results on the compositional characteristics of germinated flaxseeds are given on a dry weight basis, Appendix 3 provides an alternative representation of results on a fresh weight basis. This would permit other possible interpretation of data.

CHAPTER 5

CONCLUSIONS

Polysaccharides of the flaxseed coat (flax mucilage) may be removed by soaking of seeds in water, sodium bicarbonate solutions or by treatment with polysaccharide-degrading enzymes. The content of total sugars, pentoses and viscosity values provided indices for monitoring the content of polysaccharides extracted into the aqueous medium. Soaking in a sodium bicarbonate solution (0.10 M, 12 h) or treatment with Viscozyme® L (22.5 mg protein/100 g seeds, 3 h) was the most effective pre-treatment in removing polysaccharides of flaxseed coat. Nitrogen solubility and protein recovery from pre-treated flax meal were improved due to less interference from seed coat polysaccharides. About 57% of meal protein may be extracted into a 2.8% (w/v) sodium hexametaphosphate solution at pH 9.0 and a meal-to-solvent ratio of 1:33 (w/v). Flax protein isolates so prepared had 78% protein with a balanced amino acid composition, reasonable values of BV, C-PER and *in vitro* digestibility. Acetylation and succinylation improved colour and solubility of the protein isolate in the low pH range, thus indicating their potential use in acid foods. Emulsifying properties of isolates were improved due to acetylation and succinylation. In addition, improved fat binding capacity of the protein isolate could provide potential use in emulsified meat products. Acylation had a negative effect on foaming properties and *in vitro* digestibility of flax protein isolates.

Germination provides an alternative method for use of whole flaxseeds in foods.

After 8 days of germination, flax seedlings had unchanged content of crude protein and amino acid, fatty acid and mineral compositions but low content of lipids. Furthermore, the content of cyanogenic glycosides, phytic acid and trypsin inhibitors of flaxseed was reduced during germination while the amount of simple sugars increased.

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APPENDIX 1

Examples of standard lines used for different determinations as described in Materials and Methods.

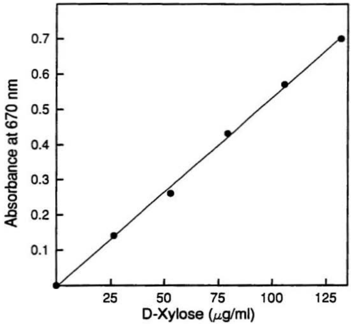

Figure A1. Standard line of concentration dependence of pentoses as reflected in the absorbance of xylose-orcinol complex

Regression coefficient (r) = 0.973
Equation of the line ($Y=aX+b$) where,

Y = absorbance at 670 nm (A_{670})
X = concentration of D-xylose in 0.1 ml solution, µg (C)
$a = 0.0057$
$b = 0.0$

$A_{670} = 0.0057 \times C$
Therefore, $C = 175.44 \times A_{670}$
Since extract solution has to be diluted, then
$C = k(175.44 \times A_{670})$
where, k = dilution factor

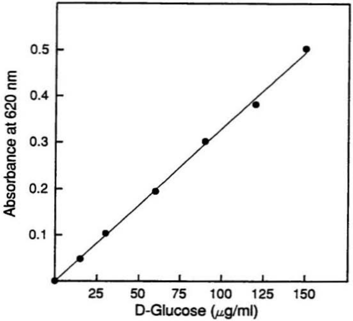

Figure A2. Standard line of concentration dependence of total sugars as reflected in the absorbance of glucose-anthrone complex

Regression coefficient (r) = 0.998
Equation of the line ($Y = aX + b$) where,

Y = absorbance at 620 nm (A_{620})
X = concentration of D-glucose in 0.1 ml solution, mg (C)
$a = 3.297$
$b = 0.0$

$A_{620} = 3.297 \times C$
Therefore, $C = 0.303 \times A_{620}$
Since extract solution has to be diluted, then
$C = k(0.303 \times A_{620})$
where, k = dilution factor

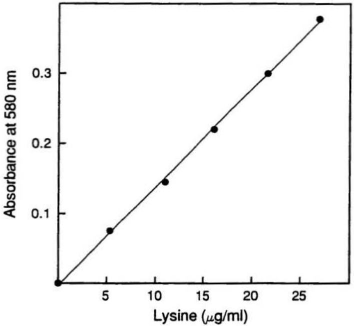

Figure A3. Standard line of concentration dependence of free amino groups as reflected in the absorbance of lysine-ninhydrin complex

Regression coefficient (*r*) = 0.997
Equation of the line (Y=*a*X+*b*) where,

Y = absorbance at 580 nm (A_{580})
X = concentration of L-lysine in 1 ml solution, mg (C)
a = 0.0139
b = 0.0

$A_{580} = 0.0139 \times C$
Therefore, $C = 71.94 \times A_{580}$
Since extract solution has to be diluted, then
$C = k(71.94 \times A_{580})$
where, k = dilution factor

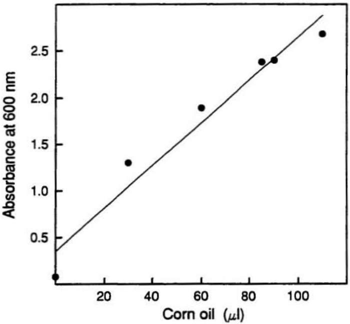

Figure A4. Standard line for determination of fat binding capacity

Regression coefficient (r) = 0.948
Equation of the line ($Y=aX+b$) where,

Y = absorbance at 600 nm (A_{600})
X = content of corn oil in 20.3 ml mixture μl (C)
$a = 0.0229$
$b = 0.3621$

$A_{600} = 0.0229 \times C + 0.3621$
Therefore, $C = (43.67 \times A_{580}) - 15.81$

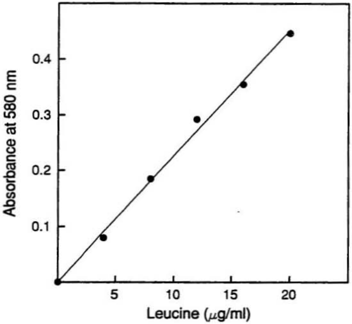

Figure A5. Standard line of concentration dependence of free amino groups as reflected in the absorbance of leucine-ninhydrin complex

Regression coefficient (*r*) = 0.988
Equation of the line ($Y=aX+b$) where,

Y = absorbance at 580 nm (A_{580})
X = concentration of leucine in 1 ml solution, mg (C)
a = 0.0227
b = 0.0

$A_{580} = 0.0227 \times C$
Therefore, $C = 44.05 \times A_{580}$
Since extract solution has to be diluted, then
$C = k(44.05 \times A_{580})$
where, k = dilution factor

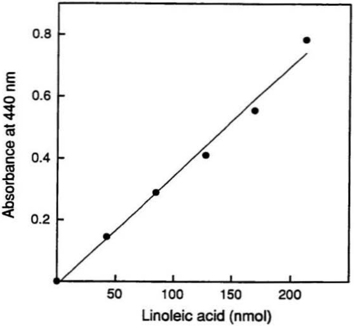

Figure A6. Standard line of concentration dependence of free fatty acids as reflected in the absorbance of linoleic acid-cupric nitrate complex

Regression coefficient (r) = 0.937
Equation of the line ($Y=aX+b$) where,

Y = absorbance at 440 nm (A_{440})
X = concentration of linoleic acid in 5 ml solution, nmol (C)
$a = 0.0034$
$b = 0.0$

$A_{440} = 0.0034 \times C$
Therefore, $C = 294 \times A_{440}$

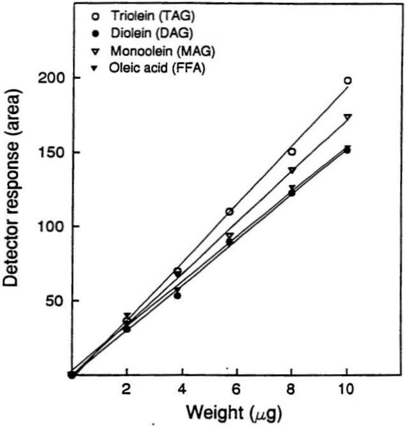

Figure A7. Correlation between areas of the peaks and concentration of triacylglycerol (TAG, as triolein), diacylglycerol (DAG, as diolein), monoacylglycerol (MAG, as monoolein), and free fatty acids (FFA, as oleic acid).

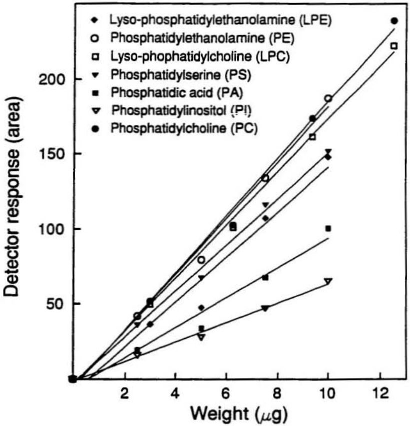

Figure A8. Correlation between areas of the peaks and concentration of lyso-phospatidylethanolamine (LPE), phosphotidylehanolamine (PE), lysophophatidylcholine (LPC), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylcholine (PC).

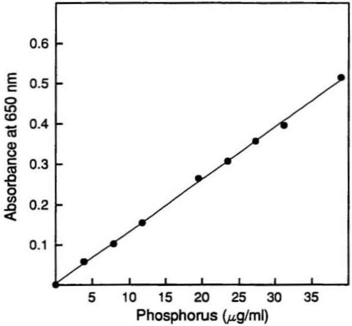

Figure A9. Standard line of concentration dependence of phosphorus as reflected in the absorbance of phosphorus-ammonium molybdate complex

Regression coefficient (r) = 0.998
Equation of the line ($Y=aX+b$) where,

Y = absorbance at 650 nm (A_{650})
X = concentration of phosphorus in 1 ml solution, μg (C)
$a = 0.013$
$b = 0.0$

$A_{650} = 0.013 \times C$
Therefore, $C = 76.92 \times A_{650}$
Since extract solution has to be diluted, then
$C = k(76.92 \times A_{650})$
where, k = dilution factor

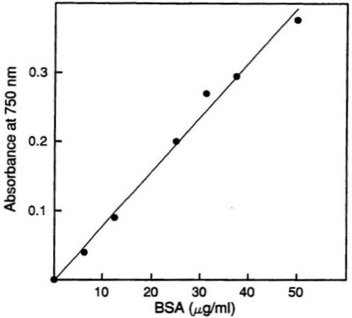

Figure A10. Standard line of concentration dependence of protein as reflected in the absorbance of bovine serum albumin (BSA)-Folin Ciocalteu complex

Regression coefficient (r) = 0.988
Equation of the line ($Y=aX+b$) where,

Y = absorbance at 750 nm (A_{750})
X = concentration of BSA in 0.5 ml solution, μg (C)
$a = 0.007$
$b = 0.0$

$A_{750} = 0.007 \times C$
Therefore, $C = 142.8 \times A_{750}$
Since extract solution has to be diluted, then
$C = k(142.8 \times A_{750})$
where, k = dilution factor

APPENDIX 2

Details of data analysis obtained by SAS programme for optimization of % nitrogen extracted and % protein recovered from low-mucilage flaxseed meal.

```
Y    =  Nitrogen (%)
X1   =  pH
X2   =  R
X3   =  SHMP (%)

OBS       Y        X3        X1        X2

 1     36.99     2.00      4.98      30.0
 2     44.59     0.50      6.00      20.0
 3     51.34     3.50      6.00      20.0
 4     50.20     0.50      6.00      40.0
 5     60.37     3.50      6.00      40.0
 6     60.89     2.00      7.50      13.2
 7     54.10     0.00      7.50      30.0
 8     69.93     2.00      7.50      30.0
 9     70.25     2.00      7.50      30.0
10     69.27     2.00      7.50      30.0
11     73.10     2.00      7.50      30.0
12     73.97     2.00      7.50      30.0
13     74.39     2.00      7.50      30.0
14     68.70     4.52      7.50      30.0
15     64.11     2.00      7.50      46.8
16     61.99     0.50      9.00      20.0
17     70.27     3.5       9.00      20.0
18     70.27     0.5       9.00      40.0
19     74.14     3.5       9.00      40.0
20     75.20     2.0      10.02      30.0
```

```
Coding Coefficients for the Independent Variables

    Factor        Subtracted off     Divided by

    X1                  7.500000       2.520000
    X2                 30.000000      16.800000
    X3                  2.260000       2.260000
```

```
Response Surface for Variable Y: % Nitrogen

     Response Mean  ·          63.703500
     Root MSE                   3.311321
     R-Square                     0.9524
     Coef. of Variation           5.1980
```

```
                     Degrees
                       of        Type I Sum
Regression           Freedom     of Squares      R-Square    F-Ratio    Prob > F

Linear                     3    1599.725210       0.6951     48.632     0.0000
Quadratic                  3     588.180165       0.2556     17.881     0.0002
Crossproduct               3       3.741637       0.0016      0.114     0.9500
Total Regress              9    2191.647012       0.9524     22.209     0.0000


                     Degrees
                       of         Sum of
Residual             Freedom      Squares       Mean Square    F-Ratio    Prob > F

Lack of Fit                5     84.243159       16.848632      3.316     0.1072
Pure Error                 5     25.405283        5.081057
Total Error               10    109.648443       10.964844


                        Degrees
                          of      Parameter      Standard    T for H0:
Parameter               Freedom   Estimate         Error     Parameter=0 Prob > |T|

INTERCEPT                  1    -159.434164     30.885871      -5.162     0.0004
X1                         1      41.619911      6.372541       6.531     0.0001
X2                         1       2.097738      0.804933       2.606     0.0262
X3                         1      12.212493      4.973600       2.455     0.0339
X1*X1                      1      -2.260175      0.386935      -5.841     0.0002
X2*X1                      1      -0.020750      0.078049      -0.266     0.7957
X2*X2                      1      -0.028160      0.008706      -3.235     0.0090
X3*X1                      1      -0.265000      0.520324      -0.509     0.6216
X3*X2                      1      -0.008250      0.078049      -0.106     0.9179
X3*X3                      1      -1.742186      0.448369      -3.886     0.0030

                          Parameter
                          Estimate
                         from Coded
Parameter                   Data

INTERCEPT                 72.150039
X1                        16.369634
X2                         3.928502
X3                         4.752357
X1*X1                    -14.353015
X2*X1                     -0.878472
X2*X2                     -7.948015
X3*X1                     -1.509228
X3*X2                     -0.313236
X3*X3                     -8.898388
```

```
             Degrees
               of           Sum of
Factor       Freedom        Squares        Mean Square    F-Ratio    Prob > F

X1              4        1700.832831      425.208208      38.779     0.0000     pH
X2              4         191.604023       47.901006       4.369     0.0267     R
X3              4         354.988496       88.747124       8.094     0.0035     SHMP

                    Canonical Analysis of Response Surface
                           (based on coded data)

                                  Critical Value
                Factor          Coded          Uncoded

                X1             0.552372        8.891978    pH
                X2             0.212346       33.567412    R
                X3             0.216454        2.749186    SHMP

                Predicted value at stationary point        77.602539

                    Canonical Analysis of Response Surface
                           (based on coded data)

                                                Eigenvectors
             Eigenvalues            X1              X2              X3

               -7.905849        -0.054066        0.991846       -0.115403
               -8.806233        -0.142327        0.106738        0.984048
              -14.487336         0.988342        0.069628        0.135396

                        Stationary point is a maximum.

        Estimated Ridge of Maximum Response for Variable Y: % Nitrogen

 Coded      Estimated        Standard                   Uncoded Factor Values
Radius      Response          Error            X1               X2               X3

  0.0      72.150039        1.343632        7.500000        30.000000        2.260000
  0.1      73.757322        1.340253        7.734467        30.396912        2.323317
  0.2      75.081588        1.334149        7.965699        30.838892        2.390907
  0.3      76.124478        1.334667        8.192715        31.336501        2.463506
  0.4      76.888164        1.356318        8.414186        31.903162        2.541940
  0.5      77.375535        1.417019        8.628318        32.555696        2.627082
  0.6      77.590459        1.534029        8.832737        33.314520        2.719722
  0.7      77.538082        1.719186        9.024424        34.202913        2.820337
  0.8      77.225142        1.976805        9.199831        35.244449        2.928713
  0.9      76.660167        2.305362        9.355319        36.457897        3.043511
  1.0      75.853376        2.700699        9.487991        37.850458        3.162023
```

```
Forward Selection Procedure for Dependent Variable Y

Step 1   Variable X1 Entered       R-square = 0.57493472   C(p) =  6.30883198

                DF        Sum of Squares      Mean Square          F   Prob>F

Regression       1         1323.09466052    1323.09466052      24.35   0.0001
Error           18          978.20079448      54.34448858
Total           19         2301.29545500

                Parameter       Standard          Type II
Variable         Estimate          Error   Sum of Squares          F   Prob>F

INTERCEP      14.46760061    10.11371278     111.20576361       2.05   0.1697
X1             6.56478659     1.33046332    1323.09466052      24.35   0.0001

Bounds on condition number:             1,            1
------------------------------------------------------------------------------

Step 2   Variable X3 Entered       R-square = 0.66212236   C(p) =  3.73293631

                DF        Sum of Squares      Mean Square          F   Prob>F

Regression       2         1523.73917587     761.86958793      16.66   0.0001
Error           17          777.55627913      45.73860465
Total           19         2301.29545500

                Parameter       Standard          Type II
Variable         Estimate          Error   Sum of Squares          F   Prob>F

INTERCEP       9.07650453     9.62883988      40.64173499       0.89   0.3591
X1             6.56478659     1.22058101    1323.09466052      28.93   0.0001
X3             2.66095562     1.27047300     200.64451535       4.39   0.0515

Bounds on condition number:             1,            4
------------------------------------------------------------------------------

Step 3   Variable X2 Entered       R-square = 0.69514117   C(p) =  4.00000000

                DF        Sum of Squares      Mean Square          F   Prob>F

Regression       3         1599.72521012     533.24173671      12.16   0.0002
Error           16          701.57024488      43.84814031
Total           19         2301.29545500

                Parameter       Standard          Type II
Variable         Estimate          Error   Sum of Squares          F   Prob>F

INTERCEP       1.99697240    10.85377199       1.48434180       0.03   0.8563
X1             6.56478659     1.19509036    1323.09466052      30.17   0.0001
X2             0.23598440     0.17926355      75.98603425       1.73   0.2066
X3             2.66095562     1.24394040     200.64451535       4.58   0.0482

Bounds on condition number:             1,            9
------------------------------------------------------------------------------
No other variable met the 0.5000 significance level for entry into the model.
```

```
Summary of Forward Selection Procedure for Dependent Variable Y

       Variable    Number   Partial     Model
Step   Entered         In      R**2      R**2         C(p)            F    Prob>F

   1   X1               1    0.5749    0.5749       6.3088      24.3464    0.0001
   2   X3               2    0.0872    0.6621       3.7329       4.3868    0.0515
   3   X2               3    0.0330    0.6951       4.0000       1.7329    0.2066


Maximum R-square Improvement for Dependent Variable Y

Step 1   Variable X1 Entered        R-square = 0.57493472   C(p) =   6.30883198

                 DF          Sum of Squares       Mean Square            F    Prob>F

Regression        1           1323.09466052     1323.09466052        24.35    0.0001
Error            18            978.20079448       54.34448858
Total            19           2301.29545500

                 Parameter         Standard           Type II
Variable          Estimate            Error    Sum of Squares            F    Prob>F

INTERCEP       14.46760061      10.11371278      111.20576361         2.05    0.1697
X1              6.56478659       1.33046332     1323.09466052        24.35    0.0001

Bounds on condition number:               1,             1
------------------------------------------------------------------------------------

The above model is the best  1-variable model found.

Step 2   Variable X3 Entered        R-square = 0.66212236   C(p) =   3.73293631

                 DF          Sum of Squares       Mean Square            F    Prob>F

Regression        2           1523.73917587      761.86958793        16.66    0.0001
Error            17            777.55627913       45.73860465
Total            19           2301.29545500

                 Parameter         Standard           Type II
Variable          Estimate            Error    Sum of Squares            F    Prob>F

INTERCEP        9.07650453       9.62883988       40.64173499         0.89    0.3591
X1              6.56478659       1.22058101     1323.09466052        28.93    0.0001
X3              2.66095562       1.27047300      200.64451535         4.39    0.0515

Bounds on condition number:               1,             4

------------------------------------------------------------------------------------
The above model is the best  2-variable model found.
```

```
Step 3   Variable X2 Entered        R-square = 0.69514117   C(p) =   4.0000000

                DF          Sum of Squares       Mean Square          F    Prob>F

Regression       3           1599.72521012      533.24173671      12.16    0.0002
Error           16            701.57024488       43.84814031
Total           19           2301.29545500

                Parameter        Standard           Type II
Variable         Estimate           Error    Sum of Squares          F    Prob>F

INTERCEP       1.99697240     10.85377199        1.48434180       0.03    0.8563
X1             6.56478659      1.19509036     1323.09466052      30.17    0.0001
X2             0.23598440      0.17926355       75.98603425       1.73    0.2066
X3             2.66095562      1.24394040      200.64451535       4.58    0.0482

Bounds on condition number:            1,           9
------------------------------------------------------------------------------
The above model is the best  3-variable model found.

No further improvement in R-square is possible.
```

```
Y    =  Protein nitrogen (%)
X1   =  pH
X2   =  R
X3   =  SHMP (%)

 OBS        Y          X3         X1          X2

   1      15.79      2.00       4.98       30.0
   2      23.00      0.50       6.00       20.0
   3      30.40      3.50       6.00       20.0
   4      29.90      0.50       6.00       40.0
   5      38.20      3.50       6.00       40.0
   6      39.98      2.00       7.50       13.2
   7      33.70      0.00       7.50       30.0
   8      48.91      2.00       7.50       30.0
   9      49.25      2.00       7.50       30.0
  10      48.27      2.00       7.50       30.0
  11      52.60      2.00       7.50       30.0
  12      52.20      2.00       7.50       30.0
  13      53.10      2.00       7.50       30.0
  14      49.50      4.52       7.50       30.0
  15      43.98      2.00       7.50       46.8
  16      41.60      0.50       9.00       20.0
  17      49.50      3.5        9.00       20.0
  18      48.92      0.5        9.00       40.0
  19      53.82      3.5        9.00       40.0
  20      56.70      2.0       10.02       30.0


Coding Coefficients for the Independent Variables

     Factor        Subtracted off     Divided by

     X1                  7.500000       2.520000
     X2                 30.000000      16.800000
     X3                  2.260000       2.260000


      Response Surface for Variable Y: % Protein

          Response Mean              42.966000
          Root MSE                    3.197839
          R-Square                      0.9573
          Coef. of Variation            7.4427
```

```
                Degrees
                  of        Type I Sum
Regression      Freedom     of Squares      R-Square      F-Ratio     Prob > F

Linear               3      1751.877651       0.7308       57.104       0.0000
Quadratic            3       540.271757       0.2254       17.611       0.0003
Crossproduct         3         2.772950       0.0012        0.0904      0.9637
Total Regress        9      2294.922357       0.9573       24.935       0.0000


                Degrees
                  of          Sum of
Residual        Freedom       Squares       Mean Square      F-Ratio     Prob > F

Lack of Fit          5       79.433040       15.886608        3.480       0.0987
Pure Error           5       22.828683        4.565737
Total Error         10      102.261723       10.226172


                  Degrees
                    of       Parameter      Standard       T for H0:
Parameter         Freedom    Estimate         Error       Parameter=0  Prob > |T|

INTERCEPT              1    -177.445563     29.827384       -5.949       0.0001
X1                     1      40.407107      6.154149        6.566       0.0001
X2                     1       2.167836      0.777347        2.789       0.0192
X3                     1      11.296751      4.803151        2.352       0.0405
X1*X1                  1      -2.161830      0.373675       -5.785       0.0002
X2*X1                  1      -0.025500      0.075374       -0.338       0.7421
X2*X2                  1      -0.028322      0.008408       -3.369       0.0071
X3*X1                  1      -0.161111      0.502492       -0.321       0.7551
X3*X2                  1      -0.017500      0.075374       -0.232       0.8211
X3*X3                  1      -1.624861      0.433003       -3.753       0.0038


                    Parameter
                    Estimate
                   from Coded
Parameter             Data

INTERCEPT           51.127110
X1                  17.263371
X2                   3.994034
X3                   5.015038
X1*X1              -13.728486
X2*X1               -1.079568
X2*X2               -7.993486
X3*X1               -0.917560
X3*X2               -0.664440
X3*X3               -8.299142
```

```
            Degrees
               of          Sum of
Factor      Freedom       Squares        Mean Square    F-Ratio    Prob > F

X1              4       1802.952531      450.738133     44.077     0.0000     pH
X2              4        197.859673       49.464918      4.837     0.0197     R
X3              4        344.640731       86.160183      8.425     0.0030     SHMP


                      Canonical Analysis of Response Surface
                              (based on coded data)


                                Critical Value
                  Factor      Coded          Uncoded

                  X1         0.612269        9.042919      pH
                  X2         0.197663       33.320744      R
                  X3         0.260383        2.848465      SHMP

                  Predicted value at stationary point      57.459679


                      Canonical Analysis of Response Surface
                              (based on coded data)

                                              Eigenvectors
               Eigenvalues          X1             X2              X3

                -7.772597       -0.038745       0.859987       -0.508844
                -8.426499       -0.125108       0.501044        0.856331
               -13.822018        0.991386       0.096839        0.088179

                         Stationary point is a maximum.

             Estimated Ridge of Maximum Response for Variable Y: % Protein

  Coded    Estimated      Standard                 Uncoded Factor Values
  Radius   Response        Error           X1              X2               X3

   0.0     51.127110      1.297584      7.500000       30.000000        2.260000
   0.1     52.833320      1.294320      7.735029       30.376057        2.323942
   0.2     54.269585      1.288435      7.967265       30.778065        2.393197
   0.3     55.437174      1.288993      8.196019       31.210418        2.468652
   0.4     56.337674      1.310054      8.420409       31.678295        2.551345
   0.5     56.973075      1.368938      8.639313       32.187702        2.642472
   0.6     57.345880      1.482268      8.851318       32.745417        2.743366
   0.7     57.459230      1.661345      9.054687       33.358752        2.855439
   0.8     57.317029      1.910121      9.247363       34.035022        2.980061
   0.9     56.924058      2.226792      9.427057       34.780653        3.118354
   1.0     56.286024      2.606816      9.591451       35.599955        3.270927
```

```
Forward Selection Procedure for Dependent Variable Y

Step 1   Variable X1 Entered        R-square = 0.60840586   C(p) =  7.27510050

                 DF        Sum of Squares      Mean Square          F    Prob>F

Regression        1        1458.46083002     1458.46083002      27.97    0.0001
Error            18         938.72324998       52.15129167
Total            19        2397.18408000

                 Parameter        Standard          Type II
Variable          Estimate           Error   Sum of Squares          F    Prob>F

INTERCEP       -8.72724578      9.90753003      40.46585191       0.78    0.3900
X1              6.89243277      1.30333989    1458.46083002      27.97    0.0001

Bounds on condition number:             1,            1
------------------------------------------------------------------------------

Step 2   Variable X3 Entered        R-square = 0.69739181   C(p) =  3.98606069

                 DF        Sum of Squares      Mean Square          F    Prob>F

Regression        2        1671.77654245      835.88827123      19.59    0.0001
Error            17         725.40753755       42.67103162
Total            19        2397.18408000

                 Parameter        Standard          Type II
Variable          Estimate           Error   Sum of Squares          F    Prob>F

INTERCEP      -14.28596642      9.30034545     100.68255588       2.36    0.1429
X1              6.89243277      1.17894006    1458.46083002      34.18    0.0001
X3              2.74369232      1.22712995     213.31571243       5.00    0.0391

Bounds on condition number:             1,            4
------------------------------------------------------------------------------

Step 3   Variable X2 Entered        R-square = 0.73080648   C(p) =  4.00000000

                 DF        Sum of Squares      Mean Square          F    Prob>F

Regression        3        1751.87765057      583.95921686      14.48    0.0001
Error            16         645.30642943       40.33165184
Total            19        2397.18408000

                 Parameter        Standard          Type II
Variable          Estimate           Error   Sum of Squares          F    Prob>F

INTERCEP      -21.55466951     10.40945781     172.93087066       4.29    0.0549
X1              6.89243277      1.14616768    1458.46083002      36.16    0.0001
X2              0.24229010      0.17192515      80.10110811       1.99    0.1779
X3              2.74369232      1.19301798     213.31571243       5.29    0.0353

Bounds on condition number:             1,            9
------------------------------------------------------------------------------
No other variable met the 0.5000 significance level for entry into the model.
```

```
Summary of Forward Selection Procedure for Dependent Variable Y

        Variable    Number    Partial      Model
Step    Entered         In       R**2       R**2          C(p)             F    Prob>F

   1    X1               1     0.6084     0.6084        7.2751       27.9660    0.0001
   2    X3               2     0.0890     0.6974        3.9861        4.9991    0.0391
   3    X2               3     0.0334     0.7308        4.0000        1.9861    0.1779

         Maximum R-square Improvement for Dependent Variable Y

Step 1   Variable X1 Entered          R-square = 0.60840586    C(p) =   7.27510050

                  DF          Sum of Squares       Mean Square            F    Prob>F

Regression         1           1458.46083002     1458.46083002        27.97    0.0001
Error             18            938.72324998       52.15129167
Total             19           2397.18408000

                  Parameter         Standard           Type II
Variable           Estimate            Error    Sum of Squares            F    Prob>F

INTERCEP        -8.72724578       9.90753003       40.46585191         0.78    0.3900
X1               6.89243277       1.30333989     1458.46083002        27.97    0.0001

Bounds on condition number:                 1,            1
------------------------------------------------------------------------------------

The above model is the best  1-variable model found.

Step 2   Variable X3 Entered          R-square = 0.69739181    C(p) =   3.98606069

                  DF          Sum of Squares       Mean Square            F    Prob>F

Regression         2           1671.77654245      835.88827123        19.59    0.0001
Error             17            725.40753755       42.67103162
Total             19           2397.18408000

                  Parameter         Standard           Type II
Variable           Estimate            Error    Sum of Squares            F    Prob>F

INTERCEP       -14.28596642       9.30034545      100.68255588         2.36    0.1429
X1               6.89243277       1.17894006     1458.46083002        34.18    0.0001
X3               2.74369232       1.22712995      213.31571243         5.00    0.0391

Bounds on condition number:                 1,            4
------------------------------------------------------------------------------------
The above model is the best  2-variable model found.
```

```
Step 3   Variable X2 Entered        R-square = 0.73080648   C(p) =   4.00000000

                DF        Sum of Squares       Mean Square           F    Prob>F

Regression       3         1751.87765057      583.95921686       14.48    0.0001
Error           16          645.30642943       40.33165184
Total           19         2397.18408000

                Parameter        Standard          Type II
Variable         Estimate           Error   Sum of Squares           F    Prob>F

INTERCEP     -21.55466951     10.40945781     172.93087066        4.29    0.0549
X1             6.89243277      1.14616768    1458.46083002       36.16    0.0001
X2             0.24229010      0.17192515      80.10110811        1.99    0.1779
X3             2.74369232      1.19301798     213.31571243        5.29    0.0353

Bounds on condition number:              1,              9
------------------------------------------------------------------------------

The above model is the best  3-variable model found.

No further improvement in R-square is possible.
```

APPENDIX 3

Composition of germinated flaxseed calculated on fresh weight basis (g/100g seedlings; mean values are provided).

Germination period (days)	Moisture	Ash	Crude protein[1]	Total lipids	Lipid classes		
					Neutral lipids	Glyco lipids	Phospho lipids
0	26.5	2.20	18.14	31.97	30.78	0.74	0.45
2	32.4	1.96	18.34	28.26	26.85	0.76	0.65
4	44.6	1.61	15.37	22.49	21.21	0.94	0.34
6	46.2	1.51	12.47	14.10	12.62	1.23	0.16
8	52.5	1.38	10.63	8.65	7.17	1.18	0.29

[1] %N × 6.25

Germination period (days)	Total soluble sugars	Fructose	Glucose	Sucrose	Raffinose	Phytic acid	Cyanogenic glycosides	
							Linustatin	Neolinustatin
0	3.01	0.04	0.06	1.45	0.89	0.75	0.20	0.23
2	2.64	0.24	0.27	1.07	0.14	0.68	0.23	0.24
4	3.10	0.55	1.31	0.67	ND	0.50	0.17	0.15
6	3.93	0.90	2.11	0.14	ND	0.48	0.15	0.36
8	5.65	1.16	3.72	0.27	ND	0.35	0.07	0.01

Mineral	Germination period (days)		
	0	4	8
Ca	0.015	0.012	0.009
Mg	0.250	0.193	0.160
K	0.040	0.044	0.034
Na	0.480	0.324	0.280
Cu[1]	0.823	0.687	0.546
Fe[1]	6.409	5.307	5.144
Mn[1]	3.197	2.460	2.085
Mo[1]	0.022	-	-
Zn[1]	2.315	1.944	1.510

[1] mg/100g seedlings

BIO DATA

Janitha Priyakanthi Deepani Wanasundara
(Nee Panduwawala)

1963	Born on April 3, Colombo, Sri Lanka
1981	Completed school education from Devi Balika Maha Vidyalaya, Colombo, Sri Lanka
1982-1986	Undergraduate student, Faculty of Agriculture, University of Peradeniya, Kandy, Sri Lanka
1986	B.Sc. in Agriculture, Honours, Second Class Upper Division, University of Peradeniya, Sri Lanka
1987	Joined Dept. of Food Science and Technology, Faculty of Agriculture, University of Peradeniya, Sri Lanka as a Lecturer
1990	M.Phil. in Food Science, Postgraduate Institute of Agriculture, University of Peradeniya, Sri Lanka
1990-1996	Graduate student, Dept. of Biochemistry, Memorial University of Newfoundland, Canada
1992	M.Sc. in Food Science, Memorial University of Newfoundland, Canada
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