Lipoxygenase Enzyme in Flaxseed†

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Lipoxygenase content of meal from eight flaxseed cultivars grown for 3 years at four locations in western Canada was evaluated to examine genotypic and environmental effects. Flaxseed, on average, contained 1.6−6.0 g of lipoxygenase/kg of meal. Lipoxygenase content in flaxseed was cultivar specific and strongly influenced by environmental conditions. Variation in lipoxygenase content was predominantly due to cultivar × location × year interaction. Seasonal differences in lipoxygenase enzyme content were significant at most locations. In flaxseed, lipoxygenase content was independent of oil and protein contents but was inversely related to the kinetic lag period. The lipoxygenase enzyme was expressed mainly in the embryo with only partial enzyme expression in the seed coat or hull of flaxseed.

Keywords: *Flaxseed; lipoxygenase; lag period; cultivar effects; seasonal variations; stability; environment; flaxseed hull; Linum usitatissimum*

INTRODUCTION

Lipoxygenase (linoleate:oxygen 13-oxidoreductase, EC 1.13.11.12) is widely distributed in plants. It catalyzes the hydroperoxidation of polyunsaturated fatty acids and esters containing *cis*,*cis*-1,4-pentadiene moieties (Eskin et al., 1977). The fatty acid hydroperoxides, which are highly reactive, are converted via a number of enzymatic reactions into compounds of significant physiological role in plant growth and development, senescence, wound and other stress responses, synthesis of regulator molecules, nitrogen partitioning, and lipid mobilization during germination (Siedow, 1991; Feussner et al., 1995). Because many products of the lipoxygenase reaction (or derivatives thereof) are aromatic, the presence of lipoxygenase activity in many foods can affect their properties, such as color, texture, functionality, and nutritive value, particularly during long-term storage in both desirable and undesirable ways (Gardner, 1979; Eriksson, 1982; Start et al., 1986).

In soybeans, large variations in lipoxygenase content have been observed (Zougari et al., 1995). Quantitative differences in lipoxygenase activity among soybean cultivars were reported to be under genetic control and not influenced by environment (Chapman et al., 1976). Hildebrand and Hymowitz (1981) found two soybean genotypes lacking lipoxygenase-1, the major form of lipoxygenase present in soybean seeds, while screening the soybean germplasm collection of 6499 accessions. Later, Hildebrand and Hymowitz (1982) showed that the presence of lipoxygenase-1 was simply inherited with no maternal or cytoplasmic effects on the inheritance of this enzyme. Recently, soybean mutants completely lacking lipoxygenase (L0) have been developed and shown to produce the lowest levels of both 1,3-diethyl-2-thiobarbituric acid (DETBA value, an index for hydroperoxide value) and *n*-hexanal production (Furuta et al., 1996; Wilson, 1996).

Although the enzyme has been well studied in soybeans for several decades, investigations on flaxseed lipoxygenase have been limited. Zimmerman and Vick (1970) showed that flaxseed lipoxygenase possessed a high degree of specificity for attachment of oxygen to linoleic and linolenic acids. Thus, with lipoxygenase from flaxseed, 80% and 88% of 13-hydroxyoctadecadienoate (13-isomer) were formed when linoleate and linolenic acid served as the substrate, respectively. Later, Zimmerman and Feng (1978) characterized a prostaglandin-like metabolite (phytonoic acid) produced on incubation of linolenic acid with an extract of flaxseed acetone powder (presumed to contain flaxseed lipoxygenase). The predominant formation of 13-hydroperoxides due to the oxidation of linolenic acid by flaxseed lipoxygenase was later confirmed by Grosch et al. (1976), who described flaxseed lipoxygenase as a poor carotene "oxidase". Flaxseed lipoxygenase behaves similar to type 1 soybean lipoxygenase (L-1), but it produces only small amounts of volatile carbonyl compounds and aroma substances unlike in soybeans (Grosch et al., 1976). The absence of type II lipoxygenase in flaxseed was demonstrated by Vernooy-Gerritsen et al. (1982) who saw no cross-reactivity with extracts from flax in immunodiffusion assays using an antibody against highly purified soybean cotyledon lipoxygenase-2. Recent evidence obtained following purification of lipoxygenase from flaxseed has allowed the identification of its activity to an iron-containing protein of 130 kDa which, upon incubation with α-linolenic acid, forms 13-hydroperoxy-9,11,13(*Z*,*E*,*Z*,)-octadecatrienoic acid (Rabinovitch-Chable et al., 1992). The flaxseed lipoxygenase enzyme is very labile ($T_{1/2} = 4$ h at 4 °C), exhibits a broad pH activity around 6.2, and is reduced by *β*-mercaptoethanol to monomeric bands (23 and 20 kDa) (Rabinovitch-Chable et al., 1992).

The consumption of flaxseed and flaxseed products is being encouraged because of the metabolic effects of its major components, α-linolenic acid, soluble fiber, and lignans. The demonstration of clinical activity associated with consumption of flaxseed, its potential clinical

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application in hyperlipidemia, cancer, renal disease, and malaria and in human and animal nutrition and health, and its possible use in neutraceuticals have stimulated interest in exploring phytochemicals and other metabolites present in the seed (Caragay, 1992; Haumann, 1993; Cunnane and Thompson, 1995). The production of phytohormones such as prostaglandin, a potent mediator of inflammation and autoimmune disease, via lipoxygenase enzyme has been suggested (Zimmerman and Feng, 1978). Since lipoxygenase enzyme plays such diverse and important roles in plant physiology and food chemistry, our investigation focused on determinants of variability of lipoxygenase in flaxseed.

MATERIALS AND METHODS

Samples of eight oil-type flaxseed cultivars including a "Solin" type, Linola 947, were obtained from standardized cooperative tests conducted at four locations (Brandon and Portage la Prairie in Manitoba, Elrose and Melfort in Saskatchewan) during the 1991, 1992, and 1993 growing seasons. Brandon, Melfort, and Portage la Prairie are located in the black soil zone at 49°, 52°, and 49° latitude, respectively. Elrose is situated in the dry brown soil zone at 51° latitude. Experimental design was according to procedures established by the Western Expert Committee on Grain for official registration of flax in Canada (Anonymous, 1992). Two replicate samples were taken from each cultivar at each location and growing season.

Flaxseed was defatted by the multisequential method of Appelqvist (1967) with petroleum ether. The defatted meal was dried in a forced-air oven for at least 24 h at 50 °C. Lipoxygenase was quantitatively assayed essentially as described by Hildebrand and Hymowitz (1981). Briefly, defatted meal (50 mg) was added to 1.5 mL of Tris-HCl buffer (0.06 M Tris, 0.015 M $CaCl_2$, 13% sucrose, pH 8.3) and mixed for 10 s using a vortexer. The sample extract was then microfuged at 3000 rpm for 8 min (Biofuge, Baxter Diagnostics Corp., Ontario, Canada). An aliquot of the supernatant (10 μL) was mixed with 2 mL of an aerated 1:10 dilution of the L-1 substrate (aqueous linoleate stock solution containing 0.4% Tween-20, 0.4% linoleic acid, and 1.2% phosphate buffer, pH 7.2). The change in absorbance at 234 nm (conjugated diene formation) relative to the control was monitored with a Beckman (DU-640B, Beckman Instruments Inc., Fullerton, CA) spectrophotometer at intervals of 2 min for 30 min. Concentration of lipoxygenase enzyme was calculated from a similarly prepared standard curve obtained with soybean lipoxygenase type 1-S (Cat. L-8383, Sigma Chemical Co., St. Louis, MO). The lag period, defined as the time in minutes prior to the sudden linear increase in formation of the conjugated dienes and the slope, i.e., the rate of formation of conjugated dienes, was also noted.

Protein content (N × 5.41) of defatted meal was determined by the Kjeldahl method with a Tecator digester and a Kjeltec (System 1002) distillation unit (Tecator AB, Höganäs, Sweden). Oil content was measured gravimetrically after defatting the seed. Carbohydrate content was determined by the phenol-sulfuric acid method (Dubois et al., 1956) with rhamnose as standard and expressed as percentage of seed weight. HPLC methods were used for the determination of cyanogenic compounds (Oomah et al., 1992) and tocopherol contents (Oomah et al., 1997) of flaxseed. Phenolic acids were determined as described previously by Oomah et al. (1995). Dehulling of flaxseed was performed as described by Oomah et al. (1996) using the tangential dehulling device except that the sample was not heat-treated prior to dehulling.

Analyses of variance by the general linear models (GLM) procedure, means comparison by Duncan's test, and Pearson correlation were performed according to the Statistical Analysis System (SAS Institute, 1990). The variance components were estimated with PROC VARCOMP of SAS with the Maximum-Likelihood method.

Table 1. Lipoxygenase Content, Lag Period, and Slope of Meal from Flaxseed Cultivars Grown at Four Locations for 3 Years[a]

cultivar	lipoxygenase content (g/kg)	lag period (min)	slope (value/100)
AC Emerson	3.62[b]	4.92[c]	11.46[b]
AC Linora	1.86[f]	6.88[b]	6.24[f]
Flanders	2.36[d]	6.00[b]	7.72[d]
Linola 947	5.98[a]	2.71[d]	18.44[a]
McGregor	2.25[e]	4.88[c]	7.39[e]
NorLin	1.63[g]	10.13[a]	5.54[g]
Somme	3.57[b]	4.13[c]	11.29[b]
Vimy	3.14[c]	6.20[b]	8.91[c]

[a] Means within the same column followed by the same superscript are not significantly different by Duncan's multiple range test at the 5% level.

RESULTS AND DISCUSSION

The lipoxygenase content of defatted flaxseed meal differed significantly among cultivars (Table 1). The mean lipoxygenase content ranged from 1.6 g/kg for the cultivar NorLin to 6 g/kg for the low-linolenic yellow-seeded Linola 947, the Solin-type flax. The high lipoxygenase content of Linola 947, with high linoleic acid (~71% of total fatty acid; Dribnenki and Green, 1995), is not surprising since the preferred substrate for lipoxygenase enzyme is linoleic acid (Zimmerman and Vick, 1970). The lag period and slope ranged from 2.7 to 10.1 min and from 0.055 to 0.184, respectively. The trend for variation in lipoxygenase content of flaxseed cultivars was similar to that of the slope but contrary to that of the lag period. The variation, expressed in terms of ratio of the poorest to the best cultivar, was 3.67 for lipoxygenase content, 3.74 for lag period, and 3.33 for the slope. This variation in lipoxygenase content of flaxseed is higher than that reported for five soybean genotypes (ratio of 2.47 for LOX1 and 2.10 for all lipoxygenases; Zougari et al., 1995). Our data on lipoxygenase content are in accordance with those of Grosch et al. (1976) for diene formation from defatted flaxseed meal. The average lipoxygenase content of flaxseed (3 g/kg) was over 4-fold (≈4.7) lower than that of a soybean sample, cv. Maple Amber (14.1 g/kg), produced in Canada and assayed under the same conditions.

The lag period and slope, indicators of the rate of production of the conjugated dienes, also varied significantly among cultivars (Table 1). The range for lag period and slope may be grouped into three types depicted in Figure 1. The first type (A) has no lag period, high slope value, and hence high level of lipoxygenase content. The reverse, i.e., high lag period, low slope value, and therefore very low or undetectable level of lipoxygenase content, is represented by the second type (C). The third type (B) is intermediate between the two extremes, A and C.

To further elucidate the variability in flaxseed lipoxygenase, environmental effects (year and location) were studied in combination with cultivars. The variance analysis (Table 2) showed the occurrence of significant ($P < 0.0001$) cultivar, year, location, and their interaction effects on the lipoxygenase content, lag period, and slope of flaxseed grown at four locations for 3 years. The quantitative differences in lipoxygenase content and activity (slope) were primarily due to cultivar (i.e., under genetic control), which was 10 and 25 times larger than those due to location and years, respectively. Similar predominant cultivar effect on lipoxygenase activity has been reported for soybeans (Chapman et al., 1976;

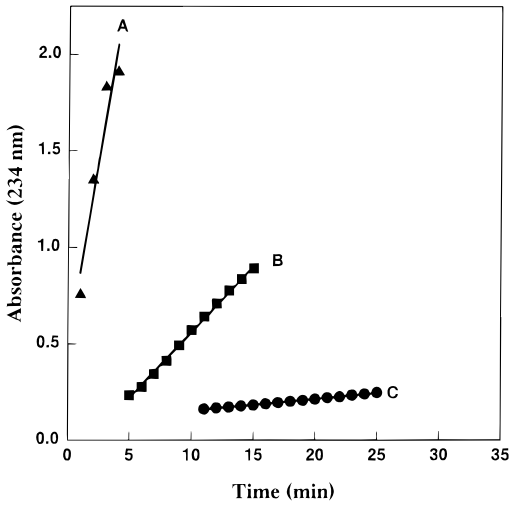

Figure 1. Lipoxygenase activities of (A) cultivar AC Emerson grown at Elrose in 1993, (B) AC Linora grown at Melfort in 1992, and (C) NorLin grown at Portage in 1991. The slopes for A−C were 0.3781, 0.0694, and 0.0055, respectively. The lag periods (in min) for A−C were 0, 6, and 14, respectively. The lipoxygenase contents (in g/kg) for A−C were 12.52, 2.10, and not detected, respectively.

Table 2. Analysis of Variance for Lipoxygenase of Flaxseed Grown at Four Locations for 3 Years

		mean squares[a]		
source	df	lipoxygenase content	lag period	slope
cultivar (C)	7	49653 (25.35)	816.67 (13.66)	0.29 (25.35)
location (L)	3	4831 (0)	243.54 (2.5)	0.03 (0)
year (Y)	2	1923 (0)	38.17 (0)	0.01 (0)
C × L	21	35594 (13.55)	1032.38 (20.86)	0.21 (13.55)
C × Y	14	17201 (3.01)	311.08 (0)	0.10 (3.02)
L × Y	6	6129 (0)	202.83 (2.05)	0.04 (0)
C × L × Y	42	41342 (57.58)	998.25 (50.36)	0.24 (57.59)
error	96	4.21 (0.49)	2.63 (12.79)	0.0 (0.49)
CV %		0.04	28.43	5.16

[a] All mean squares are significant at 0.0001 probability levels. Values in parentheses are variance components.

Hammond et al., 1972; Zougari et al., 1995). The variation in lipoxygenase content was parallel to that of the slope and was mainly due to cultivar, C × L, and C × L × Y interactions which explained 25%, 14%, and 58% of the total variation, respectively. Most of the variation for lag period was accounted for by cultivar, C × L, and C × L × Y interactions which explained 14%, 21%, and 51% of the total variability, respectively. Location, year, and L × Y (i.e., environment) differences in lipoxygenase content, lag period, and slope (Table 2) accounted for a very small proportion of the total variation, since their variance components were nil or generally smaller than that of the experimental error. The high variance of the C × L × Y interaction indicates that cultivar responded (adapted) differently to environment. The opposite effect, i.e., little or no influence of environment on the level of lipoxygenase activity, has been observed in soybeans (Chapman et al., 1976), probably due to the limited environment (two locations and one year) chosen for that study.

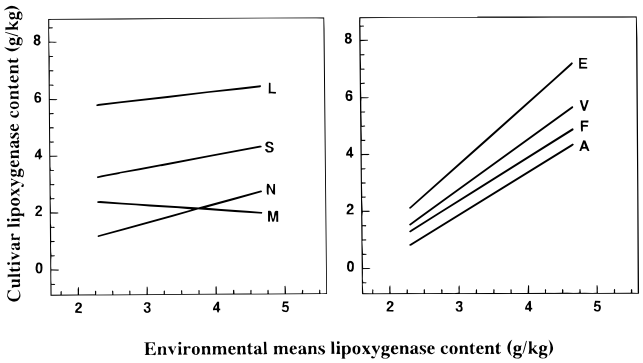

Figure 2. Influence of environment (L × Y) on cultivar performance (lipoxygenase content, g/kg). Cultivars: (L) Linola 947, (S) Somme, (N) NorLin, (M) McGregor, (E) AC Emerson, (V) Vimy, (F) Flanders, and (A) AC Linora.

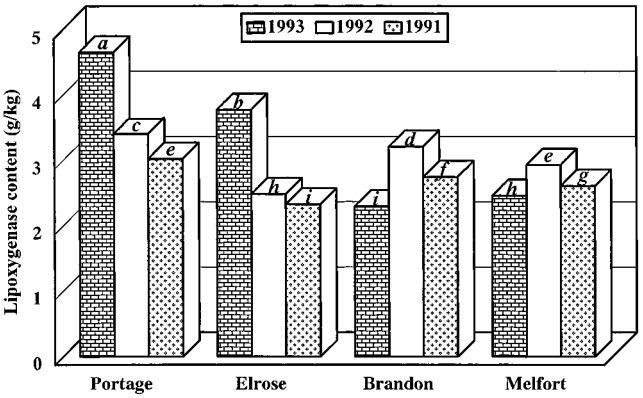

Figure 3. Lipoxygenase content of flaxseed grown in three different years at four locations. Bar graphs with the same letters are not significantly different by Duncan's multiple range test at 5% level.

The complexity of cultivar and environmental interaction is illustrated in Figure 2, where the performance of each cultivar, in regard to lipoxygenase content, was regressed in each environment against the 12 environmental (L × Y) means. This method was used to assess the effects of environment (climate, growing sites, etc.) on the ability of the cultivar to express lipoxygenase activity. Thus, cultivars Linola 947 and Somme show homogeneous regression which suggests limited interaction with environment. Cultivars AC Linora, AC Emerson, Flanders, and Vimy showed quantitative cultivar and environment interaction, although only Flanders showed a significant increase ($r = 0.56$; $P = 0.05$) with increasing environmental means. McGregor, with no response, and NorLin, with low response to the environment, showed qualitative cultivar and environment interaction with rank reversal (Romagosa and Fox, 1993).

Generally, flaxseed (mean of all varieties) grown at Portage and Elrose showed similar trend in lipoxygenase content (i.e., decrease in concentration due to seasonal effects) (Figure 3). Inconsistent seasonal effect was observed in mean lipoxygenase content of all flaxseed varieties grown at Brandon and Melfort, although the trend was consistent within each of these locations. This difference in lipoxygenase content in homogeneous subsets of locations further illustrates the strong cultivar and environment interaction and the specific adaptation of cultivars to these locations.

Comparison of lipoxygenase content and lag period with oil and protein contents (data for oil and protein

Table 3. Correlation Coefficients for Lipoxygenase Content and Lag Period of Flaxseed

component	lipoxygenase content	lag period
oil	0.153	−0.213[c]
protein	−0.106	−0.037
carbohydrate	−0.281[a]	0.052
total cyanogenic compounds	−0.312[c]	0.431[a]
neolinustatin	−0.364[a]	0.460[a]
phenolics esterified	−0.081	0.209[c]
α-tocopherol	−0.111	0.263[b]
lipoxygenase content		−0.662[a]

[a] $P < 0.0001$. [b] $P < 0.0002$. [c] $P < 0.005$ ($n = 194$).

not presented) showed poor correlation. The Pearson correlation coefficients for lipoxygenase contents were 0.153, −0.106, and −0.281 for oil, protein, and carbohydrate contents, respectively (Table 3). Similar insignificant association between protein content and lipoxygenase concentration has recently been observed in soybeans (Zougari et al., 1995). The weak association between lipoxygenase content and protein, oil, and carbohydrate contents suggests that changes in lipoxygenase content should have very little effect on these seed components. The concentrations of total cyanogenic compounds and neolinustatin were inversely correlated with lipoxygenase content but positively correlated with lag period. This moderate association of cyanogenic compounds with lipoxygenase enzyme may be due to the known inhibitory effect of cyanide on lipoxygenase (Eskin et al., 1977). When lag period was compared with esterified phenolic acid and α-tocopherol contents of flaxseed from previous studies (Oomah et al., 1995, 1997), a modest correlation was obtained ($r = 0.209$ and 0.263, respectively; $P < 0.005$). This association could probably be a reflection of the known inhibitory effects of these compounds on lipoxygenase enzyme (Eskin et al., 1977). The antioxidative effects of α-tocophenol and phenolic acids hinder oxidation resulting in lipoxygenase enzyme inhibition. Cyanide is known to have a pronounced inhibition of iron reactive compounds which form part of the lipoxygenase enzyme. A significant negative correlation ($r = -0.662$) was observed between lag period and lipoxygenase content such that the cultivar with the highest enzyme content had the lowest lag period, thereby suggesting lipoxygenase enzyme inhibition probably by hydroperoxide dehydrase present in flaxseed (Rabinovitch-Chable et al., 1992).

Dehulling and sieve separation of a flaxseed sample (Linola 947 grown at Morden, Manitoba, in 1993) by the tangential abrasive dehulling device (Oomah et al., 1996a) yielded a bran and a dehulled fraction enriched in dietary fiber and oil, respectively. The lipoxygenase content of dehulled flaxseed (13.4 ± 0.4 g/kg of solid) was higher than for unprocessed whole seed (12.7 ± 0.7 g/kg of solid), thereby confirming the localization of the lipoxygenase enzyme in the cotyledons as reported by Zimmerman and Vick (1970). The fractionated hull contained only 7.9 ± 0.5 g of lipoxygenase/kg solid which is not surprising since lipoxygenase is also present in rice bran (Orthoefer, 1996). In soybeans, lipoxygenase activity of dehulled seed (16.8 ± 1.25 LA/mg of solid) was lower than for unprocessed whole seeds (21.8 ± 0.55 LA/mg of solid) (Savage et al., 1995). However, this 23% loss of initial lipoxygenase activity was proposed to be a result of the dehulling process which incorporated heating whole soybeans for 15 min at 98.9 °C. Since no heat was involved in our dehulling process, it is logical to conclude that at least part of the total lipoxygenase enzyme of flaxseed is located in the hull fraction.

Large variability exists in lipoxygenase content of flaxseed cultivars, and this should be exploited in breeding programs to develop genotypes with altered level of lipoxygenase enzyme. The reduction in lipoxygenase content will help improve the storage stability, nutritive value, and utilization of flaxseed, especially for the genetically modified Solin-type flax varieties destined for the edible oil and food markets. Results also indicate that cultivars with high lipoxygenase content may have considerably low levels of cyanogenic compounds and carbohydrate contents. The high level of probability, in spite of the low magnitude of correlation between these traits, suggests that it may be possible to select low-lipoxygenase flaxseed lines with concomitant reduction in both carbohydrate and cyanogenic compounds.

Reduction of lipoxygenase content in regular flaxseed is also important to improve the well-recognized positive benefits of omega-3 fatty acids in human nutrition involved in the protective functions of blood cells and coronary heart disease. On the other hand, cultivars with high lipoxygenase content might be sought for crude preparation of the enzyme for industrial applications such as baking, aroma production, fine chemicals, production of dihydroxy acids and prostaglandins (potent mediators of inflammation), and pharmaceuticals. The stability of lipoxygenase content due to the strong C × L × Y interaction might be a concern for breeding improvement. In this regard, cultivars Linola 947 and McGregor indicate a high degree of environmental stability for this trait. The results suggest that effective selection can occur for lipoxygenase content even with widely differing environments encountered in this study.

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