PHYTOSPHINGOSINE CONTENT OF THE SEED OF CORN AT VARIOUS STAGES OF MATURITY[1]

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ABSTRACT

Samples of grain from a single-cross hybrid corn were collected at 1, 3, 6, and 11 weeks after pollination. These samples were analyzed by two methods for phytosphingosine. In the dried material the concentration of long-chain base had decreased 16-fold at maturity. Germ, gluten, and crude fiber from mature corn were also analyzed. The highest concentration of phytosphingosine was found in the germ.

Only a small number of investigations have been made of the changes that occur in the lipids of the corn kernel during its development. Evans (1) noted changes in the characteristics such as iodine number and acid value of the ether-extracted fat. Brimhall and Sprague (2) found that the percentage of corn oil in the germ increased to a maximum at approximately 30 days after pollination, whereas the percent of germ in the kernel increased for at least 45 days. The changes in total phosphorus, which would include phospholipids, phytin, and other compounds, were analyzed by Earley and DeTurk (3).

The first sphingolipid of plant origin, phytoglycolipid, was isolated from corn and characterized by Carter *et al.* (4,5) in 1958. This complex lipid is composed of fatty acid, phosphate, inositol, D-glucosamine, D-glucuronic acid, galactose, arabinose, mannose, and the long-chain base (LCB), phytosphingosine (I).

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$$CH_3(CH_2)_{13}-\underset{OH}{CH}-\underset{OH}{CH}-\underset{NH_2}{CH}-CH_2OH$$

I

Phytoglycolipids of closely related structures also were isolated from the phosphatides of soybean, flax, peanut, wheat, cottonseed, and sunflower seed (4,6).

The following study was undertaken in the hope of obtaining some clues to the metabolism and function of this complex lipid in plant seeds. By determining the concentrations of phytoglycolipid in the seed of corn at various stages of maturity, it should be possible to determine if the lipid is a reserve storage material, as is starch, or if it has some other role.

Preliminary attempts were made to isolate phytoglycolipid from the grain by solvent extraction. The oils extracted by hexane, benzene, or acetone comprised 4.1 to 4.5% of the dry weight of mature corn and were composed almost entirely of triglycerides. Spectrophotometric determinations (7) of the long-chain base, phytosphingosine, in these oils revealed that only a trace of phytoglycolipid was present.

Analyses of the insoluble fractions for the LCB indicated that phytosphingosine also was present in these residues. These fractions were re-extracted with a variety of solvents, but in every case phytosphingosine was found in both the soluble and insoluble portions. Because of the difficulties experienced in trying to concentrate the lipid by solvent extraction and the very small amount of phytoglycolipid apparently present in the grain, attempts to determine the lipid by isolation procedures were abandoned. Instead, it was decided to analyze unfractionated samples for total LCB. The results would set a maximum limit on phytoglycolipid composition, although the possible presence of other LCB-containing lipids must be considered in evaluating the data. In this connection it should be noted that the major (and only established) phytosphingosine-containing constituent of corn phosphatides is phytoglycolipid.

The method of Lauter and Trams (7) was adapted to determine very small amounts of LCB in large samples, and the spectrophotometric determinations of phytosphingosine were run directly on the dried, ground corn samples. These results were checked by another independent measurement. The aldehyde formed by periodate oxidation of the LCB was determined with Feulgen reagent (8,9). Both analytical procedures were used on grain samples collected at four different stages of maturity.

An attempt was made to determine which portion of the kernel contained the highest concentration of phytosphingosine. Commer-

cial samples of germ, gluten, and crude fiber were analyzed for LCB. Viable corn embryos also were examined.

Materials and Methods

Materials. The samples of corn were obtained from a single-cross hybrid (R61 × 187-2). The corn was grown on the Agronomy farm of the University of Illinois during the summer of 1962. The plants were hand-pollinated to ensure samples of uniform development at specific time intervals.

After the husks and silks were stripped from the corn in the field, the ears were packed with dry ice in insulated packers and taken to the laboratory. The samples were kept frozen until the grain was removed from the ear and lyophilized. For the samples collected 1 week and 3 weeks after pollination, it was necessary to freeze the ears in liquid nitrogen in order to shell the small kernels from the cob with a sharp knife. All lyophilized samples were ground to a fine powder in a Raymond laboratory mill.

The fractions of the corn kernel that were analyzed were products of a commercial wet-milling process. The corn germ contained about 2% moisture and 50% oil.[3] There were some hulls in the germ fraction, because the germ was separated from the rest of the kernel by flotation. Most of the hull, however, went with the underflow to be finely ground. Then this crude fiber was separated from the starch-gluten slurry by screening. The slurry was centrifuged to separate the starch and gluten. The flash-dried gluten contained approximately 9% oil.

The viable corn embryos were prepared by a method developed by Johnston and Stern (10) for the isolation of viable wheat germ.

Total Nitrogen and Total Phosphorus Procedures. The nitrogen content of each sample was determined by the micro-Kjeldahl method and the phosphorus content by the procedure of Harris and Popat (11).

LCB-Methyl Orange Procedure. To determine the low levels of LCB present in the corn samples, the procedure of Lauter and Trams (7) was adapted in the following manner. One gram of finely ground corn was hydrolyzed overnight (12–14 hr.) with 40 ml. of approximately 8*N* anhydrous methanolic hydrochloric acid. The hydrolysis mixture was quantitatively transferred to a 100-ml. volumetric flask with water and methanol to give a final 1:1 ratio of methanol to water. The diluted mixture was centrifuged to remove a small amount of insoluble residue. A 75-ml. aliquot, containing 0.2 to 1.0 μmole of phytosphingosine, was made alkaline with 75 ml. of 3*N* sodium hy-

[3] Private communication from R. A. Reiners, Corn Products Company, Argo, Ill.

droxide and extracted three times with 75-ml. portions of ethyl acetate. The combined ethyl acetate phases were washed three times with 150 ml. of water and taken nearly to dryness on a rotary evaporator. The residue was transferred quantitatively to a 10-ml. volumetric flask with ethyl acetate (solution A). Aliquots of solution A were used for both the methyl orange determination of LCB and the aldehyde analysis.

Four milliliters of solution A were transferred to a 10-ml. volumetric flask and diluted to volume with ethyl acetate (solution B) for the methyl orange determination. A 5-ml. aliquot of solution B was transferred to a 10-ml. glass-stoppered centrifuge tube. The remaining solution was placed in a second centrifuge tube to be used as a compensating blank for the yellow color produced by the acid hydrolysis. No methyl orange was added to this second tube.

A phytosphingosine standard solution was prepared by dissolving 3.0 mg. of the LCB in 100 ml. of 0.01*N* hydrochloric acid. From this stock solution 0.25-ml., 0.5-ml., and 1-ml. portions were pipetted into graduated centrifuge tubes. The volumes of the standards were adjusted to 1 ml. by diluting with water, and 1 ml. of 1*N* sodium hydroxide was added. The standards and a reagent blank were extracted with 5 ml. of ethyl acetate. After centrifugation, the aqueous phase was removed. The ethyl acetate layer was washed twice with deionized water, and the volume of the ethyl acetate phase was adjusted to exactly 5 ml.

Two milliliters of 0.01*M* acetate buffer (pH 3.65) and 0.1 ml. of 0.5% methyl orange reagent were added to the reagent blank, standards, and corn sample. After thorough shaking and centrifuging, the phytosphingosine-methyl orange complex in the ethyl acetate layer was measured at 415 mμ on a Bausch & Lomb Spectronic 20 colorimeter.

LCB-Aldehyde Determination. A 5-ml. aliquot of the ethyl acetate solution A was transferred to a 10-ml. centrifuge tube, and the solvent was removed with a nitrogen stream. For the oxidation of phytosphingosine to aldehyde, the method of Sweeley and Moscatelli (12) was used. After addition of 1 ml. of methanol and 0.18 ml. of 0.2*M* aqueous sodium metaperiodate, the reaction mixture was allowed to stand in the dark for 60 min. Methylene chloride (1.8 ml.) and water (0.6 ml.) were added. The tube was shaken and centrifuged. The methylene chloride layer was removed to another 10-ml. centrifuge tube. The aqueous layer was re-extracted three times with 1.8-ml. portions of methylene chloride. The solvent was removed from the combined methylene chloride extracts with a nitrogen stream.

Immediately after the periodate oxidation, the aldehydes formed were measured by condensation with fuchsin reagent (8,9). The dimethyl acetal of myristaldehyde was used as a standard, because it was more stable than the aldehyde. Three standards, ranging from 0.04 to 0.2 μmole of the C_{14} dimethyl acetal, were prepared. A hydrolysis solution (0.5 ml.), consisting of 90% acetic acid, 0.015*N* mercuric chloride, and 0.5*N* hydrochloric acid, was added to the standards, reagent blank, and sample. The tubes were heated for 45 min. in a 50°C. water bath. After the tubes were removed from the bath, 2.0 ml. of fuchsin reagent were added with swirling, and the tubes were cooled in an ice bath for exactly 20 min. After addition of 2.0 ml. of 0.5% sodium metabisulfite in 0.1*N* hydrochloric acid and 5.0 ml. of capryl alcohol, the tubes were shaken vigorously for 1 min. and centrifuged. When the tubes had come to room temperature, the absorbance of the capryl alcohol layer was read at 550 mμ on a Bausch & Lomb Spectronic 20 colorimeter.

Results

The corn samples used were collected at intervals of 1, 3, 6, and 11 weeks after the hand-pollination. The percentages of moisture in the samples were determined from the weights of the grain before and after lyophilization. Total nitrogen and phosphorus values are expressed in Table I as percentages of the dry weights of the grain.

TABLE I

MOISTURE, TOTAL NITROGEN, AND PHOSPHORUS CONTENT OF GRAIN

TIME AFTER POLLINATION	MOISTURE	NITROGEN	PHOSPHORUS
weeks	%	%	%
1	87.5	3.08	0.46
3	73.1	2.20	0.40
6	43.5	1.55	0.32
11	12.8	1.46	0.27

The aldehyde method of determining phytosphingosine was used in addition to the methyl orange procedure to check if the methyl orange was complexed with other substances in addition to the LCB. The results of the two methods for the determination of phytosphingosine are compared in Table II; values are expressed as μmoles of LCB per g. of dry sample.

Analysis of variance of the data showed that there was no significant difference at the 1% level between the two methods of determining LCB, but that there was a significant difference in the amounts

TABLE II

THE PHYTOSPHINGOSINE CONTENT OF THE CORN SAMPLES AS DETERMINED BY TWO METHODS

TIME AFTER POLLINATION	METHYL ORANGE METHOD		ALDEHYDE METHOD	
	No. of Detns.	Content	No. of Detns.	Content
weeks		*μmol. LCB/g.*		*μmol. LCB/g.*
1	7	2.79 ± 0.24[a]	5	3.76 ± 0.39
3	4	0.56 ± 0.08	4	0.36 ± 0.06
6	4	0.22 ± 0.01	4	0.21 ± 0.04
11	10	0.22 ± 0.02	4	0.20 ± 0.04

[a] Mean ± standard error.

of phytosphingosine present at various stages of maturity. When the mean values of the two methods were compared by the Duncan Multiple Range test, only the 1-week corn was significantly higher in phytosphingosine. The 11-weeks, 6-weeks, and 3-weeks samples did not show a significant difference at the 1% level from each other.

The representative weight of a lyophilized kernel in each sample was determined by weighing 20 kernels and calculating the mean weight. These weights are given in Table III. The concentrations of LCB per kernel were calculated from the average of the results obtained by the two methods of measuring phytosphingosine.

TABLE III

CONCENTRATION OF LONG-CHAIN BASE PER KERNEL OF CORN

TIME AFTER POLLINATION	DRY WEIGHT OF KERNEL	LONG CHAIN BASE	
weeks	*mg.*	*μmol./kernel*	*%/wt.*
1	7.6 ± 0.6[a]	0.025	0.104
3	84.9 ± 2.6	0.039	0.015
6	257.8 ± 8.4	0.055	0.007
11	328.6 ± 12.0	0.069	0.007

[a] Mean ± standard error.

The micromoles of phytosphingosine per kernel increased gradually as the grain matured. Eleven weeks after pollination the amount of LCB per kernel was 2.8 times that present in the 1-week sample. However, if the percent by weight of phytosphingosine per kernel was calculated, the percentage at 1 week was 15 times that at 11 weeks. Other components of the kernel had increased at a much higher rate.

The cob of the 11-weeks corn also was analyzed for LCB by the methyl orange procedure. The value obtained was 0.031 μmole per g., indicating that the concentration of phytosphingosine in the cob was much lower than in the grain. The percentages of total nitrogen and phosphorus in the 11-weeks corn cobs were 0.41 and 0.059, respectively.

The fractions of the corn kernel obtained from the commercial wet-milling process were analyzed for total nitrogen and phosphorus. These values were given in Table IV.

TABLE IV

TOTAL NITROGEN AND PHOSPHORUS CONTENT OF FRACTIONS OF THE CORN KERNEL

FRACTION	NITROGEN	PHOSPHORUS
	%	%
Germ	2.59	0.20
Gluten	11.88	0.42
Fiber	1.51	0.07

The concentrations of phytosphingosine in germ, fiber, and gluten were determined by the methyl orange procedure and are shown in Table V.

TABLE V

PHYTOSPHINGOSINE CONTENT OF FRACTIONS OF THE CORN KERNEL

FRACTION	LCB		RECOVERY OF LCB IN WHOLE CORN
	% of dry wt. [a, b]	*μmol./g. fraction*	%
Germ	7.5	1.18	41
Gluten	6.5	0.72	23
Fiber	4.0	0.34	5

[a] See footnote 3, in text.
[b] Total dry weight of corn.

The germ contained the highest concentration of LCB. In wet-milling of the corn some of the germ was lost, for the germ normally makes up approximately 12% of the kernel.[4] If the value of 12% was used instead of the 7.5% obtained commercially, the recovery of LCB from the germ would be 64% of the total phytosphingosine present in the mature, whole kernel.

Commercial germ shows no development when it is tested for viability. Therefore, it was considered of interest to analyze embryos that had not undergone the rigorous wet-milling process. The viable germ had higher nitrogen (4.79%) and phosphorus (0.90%) values. This indicates that some loss may have occurred through leaching during the commercial process, and perhaps there is less contamination of the viable germ with starch and fiber. The LCB value of 1.50 μmoles per g. of dry weight was also higher than that obtained with commercial germ.

[4] Private communication (see footnote 3).

Discussion

If phytosphingosine is assumed to be an index of the phytoglycolipid present, this lipid does not appear to be used as a storage form by the developing seed. When the wet weight of the kernels was calculated, the percentage of LCB per kernel remained relatively constant after the third week. This is shown in Table VI.

TABLE VI
CONCENTRATION OF LONG-CHAIN BASE PER WET WEIGHT OF KERNEL

TIME AFTER POLLINATION	WET WEIGHT OF KERNEL	LCB BY WET WEIGHT
weeks	*mg.*	%
1	60.8	0.013
3	316	0.004
6	456	0.004
11	377	0.006

In the changes in composition of the corn grain during development listed by Evans (1), only crude fiber and reducing sugars showed this pattern of higher values in the immature seed and then a leveling off to a relatively constant value. The percentage of starch increased 13-fold, crude protein 2.5 times, and the ether extract 5.6 times in the period from 15 to 57 days following silking.

In flax and safflower seeds McKillican and Sims (13) found that phospholipids represented a larger fraction of the total lipids during the early development of the seed. At 10 days after fertilization, phospholipids comprised 24.5% of the total lipids of flax, but at the end of 40 days the percentage had fallen to 1.4. In the same period triglycerides had increased from 62.1 to 90.3%.

After studying the changes in total phosphorus and phytin during development, Earley and DeTurk (3) suggested that the metabolism of the corn grain phosphorus was divided into two periods. During the first 4 weeks after pollination the major portion of the phosphorus was used for formation of the cellular structure of the grain. In the 3rd and 4th weeks more phosphorus was diverted to the synthesis of phytin, and by the 5th week this was the dominant phosphorus compound formed. At maturity, 10 weeks after pollination, 88% of the total phosphorus of the kernel was found in phytin. From our work it appears that phytoglycolipid is one of the phosphorus compounds formed in the early period, although it would account for only a very small fraction of the total phosphorus present.

The phytosphingosine in corn may be a component of other lipids in addition to phytoglycolipid. Cerebrosides, which contain

the LCB, have been isolated from a plant source, wheat flour (14). Perhaps the phytosphingosine measured in the corn samples indicated changes in both cerebrosides and phytoglycolipid in the developing grain.

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