The Major Component Phospholipids of Rapeseed Gum[1]

R. O. WEENINK[2] and A. P. TULLOCH, National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan

Abstract

The phospholipids from a commercial rapeseed gum have been fractionated on DEAE-cellulose and silicic acid columns. The molar percentages of the major components were phosphatidyl choline (22), phosphatidyl inositol (18) and phosphatidyl ethanolamine (15). Other acidic phospholipids (16) were also observed but were not further investigated. The fatty acids from the phospholipid fractions showed little variation in composition. The chief components were palmitic, oleic and linoleic acids.

Introduction

THE USE OF RAPESEED oil in the edible oil industry has resulted in the production of a highly refined oil comparable in quality with soybean or peanut oil. The degumming operation of the refining process produces a dark-brown gum which consists chiefly of an aqueous emulsion of the crude phospholipids together with smaller amounts of triglycerides and nonlipid material. Since appreciable quantities of the gum might become available it seemed desirable to undertake an analysis of the component phospholipids. Much of the work to date on seed phospholipids has been performed using more abundant commercial preparations such as those from soybean, corn or groundnut phospholipids while those from rapeseed have received little attention.

Rewald (1) separated rapeseed phospholipids into two classes according to their solubility in ethanol and reported approximately 60% cephalin and 20% lecithin. In 1954, however, Sulser (2) using quantitative paper chromatographic methods showed that the products of the hydrolysis of rapeseed phospholipids contained inositol, serine, ethanolamine and choline. Recently Carter et al. (3) have isolated "phytoglycolipid" from corn phospholipids. The presence of this complex phospholipid which on acid hydrolysis yields inositol, carbohydrates and ninhydrin reacting compounds may interfere with the determination of other phospholipid species by solely hydrolytic methods.

In the present work the chief component phospholipids were isolated and purified by chromatography on DEAE-cellulose (4) and silicic acid columns, and their constituent fatty acids analyzed by gas-liquid chromatography.

Experimental

Isolation of the Phospholipids

Rapeseed gum was obtained from the Saskatchewan Wheat Pool, Saskatoon, Canada. Preliminary experiments showed that the gum was an emulsion comprising 51% phospholipid, 16% triglyceride, 9% nonlipid and 24% water. A sample of the gum (40 g) was dried in vacuo at 40C and separated from solid, nonlipid contaminants by filtering a chloroform suspension. Evaporation of the solvent in vacuo yielded 26 g of crude lipid which was dissolved in benzene (100 ml) and washed twice with 50 ml of water. Emulsions which formed were broken by the addition of a little ethanol followed by centrifugation. The benzene solution was evaporated and the lipid (25.0 g) was dissolved in the minimum volume of chloroform (ca. 10 ml) and the phospholipids precipitated by the addition of 350 ml of acetone. After standing overnight at 4C the precipitate was filtered and washed with cold acetone. The yield of phospholipid was 18.2 g (3.4% P) or 45.5% of the wet gum.

Chromatographic Methods

Phospholipids were chromatographed on DEAE-cellulose (Selectacel, type 20 Brown Co., Oregon) and silicic acid (Biorad, minus 325 mesh) columns. The DEAE-cellulose was treated with hydrochloric acid and sodium hydroxide before conversion to the acetate form prior to use (4). A column of 42 g of DEAE-cellulose (33 cm × 3.4 cm I.D.) was used with a load of approximately 1 g of phospholipid. Two hundred-milliliter volumes of solvent were collected and each of these was analyzed by running an aliquot on thin-layer chromatograms (TLC). Fractions showing similar patterns on TLC were pooled and run on columns of silicic acid which had been activated at 100C for 18 hr prior to use. Column loading was approximately 25 mg of phospholipid per gram of silicic acid. The details of the purification of individual fractions are given in the results section.

The solvent systems used in TLC were chloroform-methanol-acetic acid-water, 65:25:8:4 (v/v); chloroform-methanol-water, 65:25:4 (v/v); and chloroform-methanol, 2:1 (v/v) containing 40 ml per litre of concentrated aqueous ammonium hydroxide. The dried plates were developed by exposure to iodine vapours followed by spraying with either 40% phosphoric acid or 10% ethanolic phosphomolybdic acid (5).

Hydrolysis of Phospholipids

Phospholipid samples (ca. 30 mg) were deacylated as outlined by Shimojo (6). The water-soluble phosphate esters were chromatographed on Whatman No. 1 paper in phenol-water 2.5:1 (w/v) and *n*-butanol-acetic acid-water 5:3:1 (v/v). Spots were located by spraying with the Hanes-Isherwood spray (7) followed by exposure to sunlight. The spots were identified by comparison with those given by the deacylation products of mixed egg phospholipids and beef heart phosphophatidyl inositol, and with results in the literature (8).

Acid hydrolysis for the detection of inositol and glycerol was carried out by heating lipid (5 mg) to 100C for 2 hr with 2 N sulfuric acid. Fatty acids were extracted with light petroleum and the aqueous phase neutralized with barium hydroxide. The polyols were then detected by paper chromatography which were developed in butanol-pyridine-water, 2:1:1 v/v. The papers were developed by spraying with acetone-silver nitrate (9).

Isolation and Analysis of Fatty Acids

Samples of phospholipids (ca. 500 mg) were refluxed for 2 hr in 6% methanolic hydrogen chloride. An equal volume of water was then added to the

[1] Issued as NRC No. 8947.
[2] National Research Council of Canada Postdoctorate Fellow 1964–65.

cooled reaction mixture and the methyl esters extracted with Skellysolve B. Analysis of the methyl esters was carried out by gas chromatography using thermal conductivity detectors. Each sample was run on both SE30 and polyester packings at 200C (cf. Tulloch 10).

Analytical Methods

Phosphorus was determined by the method of Allen (11) after digestion of the samples in 70% perchloric acid. The content of acyl ester groups was measured by the hydroxamic acid method of Morgan and Kingsbury (12). Infrared spectra were recorded using films or KBr discs. The determination of the position of the double bonds in the unsaturated fatty acid methyl esters obtained by methanolysis of the total phospholipids was carried out by permanganate-periodate oxidation followed by GLC analysis of the fragments (13,14).

Results

Thin-layer chromatograms of the total gum phospholipids revealed a complex array of spots, one of which gave a strong reaction with ninhydrin and ran in the same position as egg phosphatidyl ethanolamine. Another spot which appeared to be present in major proportions corresponded to phosphatidyl choline. Using the neutral and basic solvent systems, however, this spot could be further resolved into two components which had TLC properties similar to phosphatidyl choline and phosphatidyl inositol.

Chromatography of the total phospholipids on DEAE-cellulose resulted in the separation shown in Table I.

Phosphatidyl Choline

Fraction 2 contained 3.43% of phosphorous and had a molar ratio of ester to phosphorus of 1.8. Chloroform-methanol (1:1 v/v) eluted 70% of this fraction from a silicic acid column as a homogenous material. The infrared spectra showed absorption at 10.3 μ which, in the absence of *trans* double bonds, is characteristic of lecithins (15), while on TLC the substance moved in a manner identical with phosphatidyl choline prepared from egg yolk phospholipids. An indication that this fraction contained sterol glycosides was obtained from the early fraction of the silicic acid separation. The material eluted in chloroform-methanol 4:1 (v/v), when run on TLC plates, showed red spots when the plates were sprayed with 50% perchloric acid and slowly heated. This behaviour has been observed by Lepage (16) for the sterol glycoside from potato tubers.

TABLE I

Chromatographic Separation of Rape-Gum Phospholipids on DEAE-Cellulose

Fraction number	Solvent	Weight of lipid eluted (mg)	Main component (TLC)
1		44	Unknown
2	Chloroform-methanol	259	Phosphatidyl choline
3	9:1 v/v	2	.....
4		9	.....
5		65	
6	Chloroform-methanol	60	Phosphatidyl ethanolamine
7	7:3 v/v	10	
8		98	
9	Methanol	30	Water solubles and
10		34	unknown
11	Chloroform-acetic acid	129	Mixture of unknowns
12	3:1 vv/	11	.....
13	Acetic acid	316	Phosphatidyl inositol

Phosphatidyl Ethanolamine

Fraction 5, 6 and 7 combined contained 3.50% phosphorus and a molar ratio of ester to phosphorus of 1.9. This fraction was chromatographed on silicic acid and chloroform eluted 11% of unknown material while chloroform-methanol 4:1 (v/v) eluted the major part of the fraction (65%) which on TLC produced one major ninhydrin positive spot corresponding to that given by egg yolk phosphatidyl ethanolamine. A small spot was found, however, which did not react with ninhydrin and ran just ahead of the phosphatidyl ethanolamine fraction. When the fraction was rechromatographed on silicic acid it was found, by collecting small volumes of eluant, that the leading edge of the phosphatidyl ethanolamine peak was rich in the contaminating substance while the following portion of the peak was almost free. By combining the latter fractions phosphatidyl ethanolamine which was almost pure according to TLC, was obtained. The early fractions containing the contaminant and phosphatidyl ethanolamine were bulked and again run on silicic acid. In this manner most of the contaminant was removed after a total of three passes through silicic acid.

Phosphatidyl Inositol

Fraction 13 contained 2.54% phosphorus and a molar ratio of ester to phosphorus of 1.6, these low values may be caused by the presence of "phytoglycolipid." This fraction could not be purified by column chromatography on silicic acid. Chloroform-methanol 4:1 (v/v) eluted 90% of the fraction which still showed a similar pattern of spots on TLC as the parent fraction. This is in contrast to the expected chromatographic behaviour of phosphatidyl inositol isolated by other workers (17). In order to obtain the fatty acids from the inositide the sample was streaked along the origin of two large thin-layer plates and developed in chloroform-methanol-water to which was added a trace of antioxidant, nordihydroguaiaritic acid. The plates were dried in an atmosphere of nitrogen, exposed briefly to iodine vapours and the inositide removed from the plate. The absorbent and sample were then refluxed for 2 hr in 6% methanolic hydrochloric acid and the methyl esters isolated. To confirm the identity of the inositide, a further sample of the total gum phospholipid was run on a DEAE column, the inositide being eluted with chloroform-methanol 3:1 (v/v) containing 10 ml/litre of concentrated aqueous ammonium hydroxide. In contrast to the previous sample this inositide could be chromatographed on silicic acid as expected. This behaviour may be due to the different salt forms of the inositide which could give rise to different chromatographic properties. This phosphatidyl inositol contained 3.6% P with an acyl ester:P molar ratio of 2.1. The quantity of inositide obtained in this latter separation was used to estimate the percentage inositide in the gum.

Acid hydrolysis showed the presence of inositol and glycerol while deacylation confirmed the presence of glycerophosphoryl inositol R_F 0.08 in phenol-water.

Fraction 11

This fraction contained 3.93% phosphorus and had a molar ratio of ester to phosphorus of 2.1. Thin-layer chromatograms showed that it consisted of at least four different components. Since none of them appeared to exist in major concentration the fraction was not further investigated.

Phytoglycolipid

The procedure devised by Carter et al. (3) was used to detect the presence of "phytoglycolipid." Approximately 9% by weight of a brown pyridine soluble material was isolated from the total rape gum phospholipids by this procedure. Hydrolysis with methanolic hydrochloric acid (18) followed by extraction with chloroform yielded 51% of the lipid weight as methyl esters. TLC in chloroform showed that these methyl esters contained a considerable quantity of impurity. Chromatography on silicic acid, according to Gaver and Sweeley (18), resulted in the removal of 23% of contaminating material. When the purified esters were analyzed by GLC using a silicone column (10), two major components formed 24% and 61% of the volatile material and had carbon numbers of 23.1 and 25.3, respectively. The carbon numbers of these components increased by 1–2 units on acetylation indicating that they were hydroxy esters of C22 and C24 acids (10). Since this "phytoglycolipid" fraction appeared to be a fairly complex mixture no further attempt was made to resolve the components.

Fatty Acid Composition of the Phospholipids

The component fatty acids of the phospholipids are shown in Table II. Permanganate-periodate oxidation indicated that unusual isomeric unsaturated acids were not present in appreciable quantities. Only minor amounts of unexpected fission products in the range of 2–5% were found. Their presence could be accounted for either by overoxidation or, more likely by peroxidation of the sample resulting from exposure to air during the isolation of the gum during the refining process.

Discussion

The isolation of the component phosphatides of rapeseed had not been carried out before. The present work has shown that the phospholipids comprise phosphatidyl choline 22%, phosphatidyl ethanolamnie 15%, phosphatidyl inositol 18%, and unknown acidic lipids 16%; these components have also been found in other oil-bearing seeds. Recently Aylward and Showler (19,20) isolated phosphatidic acid, phosphatidyl choline and phosphatidyl inositol from barley, oats and rye, while the latter phosphatide has also been found in a number of other seed fats (21).

TABLE II

Fatty Acid Composition of Phospholipids [a]

Phospholipid	Component (weight %)						
	16:0	16:1	18:0	18:1	18:2	18:3	20:1
Phosphatidyl choline	8.8	1.3	0.8	34.5	44.9	6.2	2.4
Phosphatidyl ethanolamine	12.9	1.2	1.0	23.1	51.2	8.2	1.7
Phosphatidyl inositol	34.4	2.8	1.5	15.0	36.4	8.5	....
Fraction 11	14.9	1.1	0.8	26.1	46.2	8.7	1.8
Total phospholipid	16.2	1.0	0.9	24.8	45.2	9.3	1.9

[a] Small amounts of unidentified acids (< 1%) were observed, but were omitted from the table.

Minor components are also present in the gum which may be true component phospholipids or possibly artifacts derived from the conditions of preparation of the gum. One of these minor components, "phytoglycolipid," accounts for approximately 9% of the total phospholipids and appears to be a true constituent of the gum as it has been isolated from other oil-bearing seeds (3). It yields hydroxy esters on interesterification which appear to be mainly C22 and C24 compounds as found previously (3).

The content of the major phospholipids found in this work agree fairly well wtih those found by Sulser (2) who used quantitative paper chromatographic methods to estimate the amount of various hydrolysis products obtained from the total phospholipids.

The fatty acids from the phospholipids show an overall similarity in composition though the inositide (Table II) contains more palmitic and less oleic and linoleic acids than the others. The chief components are palmitic, oleic and linoleic acids, which is also largely the case for cereal phospholipids (22). There was no indication of any unsaturation higher than triene. Hilditch and Pedelty (23) reported that the cephalin phospholipids of rapeseed contained 22.7% of erucic acid. In this work erucic acid was not detected, although 21% was found in the glyceride fraction obtained after acetone treatment of the total lipid. The content of erucic acid in rape glycerides is known to vary considerably so that the content of this acid in the phospholipids could also be subject to wide fluctuations.

REFERENCES

1. Rewald, B. J., Soc. Chem. Ind. *56*, 403T (1937).
2. Sulser, H., Mitt. Lebensm. Hyg. *45*, 251 (1954).
3. Carter, H. E., W. D. Celmer, D. S. Galanos, R. H. Gigg, W. E. M. Lands, J. H. Law, K. L. Mueller, T. Nakayama, H. H. Tomizawa and E. Weber, JAOCS *35*, 335 (1958).
4. Rouser, G., A. J. Bauman, K. Kritchevsky, D. Heller and J. S. O'Brien, JAOCS *38*, 544 (1961).
5. Randerath, K., "Thin Layer Chromatography," Academic Press, New York, 1964, p 127.
6. Shimojo, T., and K. Ohno, J. Biochem. (Tokyo) *55*, 355 (1964).
7. Hanes, C. S., and F. A. Isherwood, Nature (Lond.) *164*, 1107 (1949).
8. Ferrari, R. A., and A. A. Benson, Arch. Biochem. Biophys. *93*, 185 (1961).
9. Trevelyan, W. E., D. P. Proctor and J. S. Harrison, Nature (Lond.) *166*, 444 (1950).
10. Tulloch, A. P., JAOCS *41*, 833 (1964).
11. Allen, R. J. L., Biochem. J. *34*, 858 (1940).
12. Morgan, D. M., and K. J. Kingsbury, Analyst *84*, 409 (1959).
13. Tulloch, A. P., and B. M. Craig, JAOCS *41*, 332 (1964).
14. Craig, B. M., A. P. Tulloch and N. L. Murty, Ibid. *40*, 61 (1963).
15. Jack, R. C. M., Contrib. Boyce Thomson Inst. *22*, 335 (1964).
16. Lepage, M., J. Lipid Res. *5*, 587 (1964).
17. Hanahan, D. J., and J. N. Olley, J. Biol. Chem. *231*, 813 (1958).
18. Gaver, R. C., and C. C. Sweeley, JAOCS *42*, 294 (1965).
19. Aylward, F., and A. J. Showler, J. Sci. Fd. Agric. *13*, 92 (1962).
20. Aylward, F., and B. W. Nichols, Ibid. *12*, 645 (1961).
21. Allen, C. F., and P. Good, JAOCS *42*, 610 (1965).
22. Wittcoff, H., "The Phosphatides," Am. Chem. Soc. Monograph No. 122. Reinhold, New York, 1951.
23. Hilditch, T. P., and W. H. Pedelty, Biochem. J. *31*, 1964 (1937).

[Received November 29, 1965]