14. M. Kates, Techniques of Lipidology, North-Holland, Amsterdam (1972).

ISOLATION OF PHOSPHATIDYLINOSITOL FROM SOYBEAN PHOSPHATIDES

É. Ya. Kostetskii and N. I. Gerasimenko

UDC 577.11+593.43+547.95

A method is described for isolating phosphatidylinositol (PI) from a lipid extract of soybean phosphatides. The method includes the separation of the extract with the aid of a Büchner funnel and alumina into two fractions — choline-containing phospholipids and a PI fraction — and the purification of the latter on a column of DEAE-cellulose. As a by-product, phosphatidylethanolamine with a chromatographic purity of 95-96% is obtained from the PI fraction.

Known methods for the isolation of phosphatidylinositol (PI) [1-9] are complex, laborious, and give a low yield of product, since they usually include a fairly large number of operations (complex extraction, solvent fractionation, dialysis, conversion into the salt form, and column chromatography using one or more columns) and in a number of cases [1, 3, 4] do not permit pure PI to be obtained, which makes additional rechromatography of its fractions necessary. The method which we propose is comparatively simple and permits chromatographically pure PI to be obtained in high yield (80% of its initial amount in soybean phosphatides). Preliminary results have been published in an Inventor's Certificate [10].

Brain lipids [1, 3, 5, 8] are most frequently used to obtain PI, but sometimes the lipids of the liver [7] and the kidneys [4]. All these sources contain a considerable amount of phosphatidylserine (PS), which seriously complicates the isolation of the PI in the pure form [1, 3, 4, 6, 8]. We have isolated the PI from commercial soybean phosphatides obtained from Far Eastern and American varieties of soybean which contain no PS and a fairly large amount of PI. The results of an analysis of the phospholipids (PLs) and the fatty acids (FAs) of the PI from soybean phosphatides are given in Table 1. They differ somewhat from those found in the literature [9, 11, 12]. Thus soybeans and soybean phosphatides (azolectin) contain PS, but no diphosphatidylglycerol (DPG), diphosphatidylinositol (DPI), or an unknown PL of low polarity at the zone of the front have been found. However, in these papers there are statements of the presence of an unknown PL in the zone of the start but similar in its chromatographic behavior to DPI. In our opinion, the appearance of DPI in a phosphatide extract is due to the presence in the soybean paste of lipoproteins which after their treatment with acetone (see the Experimental part) liberate DPI, since denaturation of the protein takes place. The fatty acid composition of the PI that we have isolated likewise differs somewhat from the composition given in the literature [9]. Thus, the amount of unsaturated acids was considerably higher in our material (70% as compared with 41%), although these differences are considerably less when they are compared with figures for the FAs of the PIs isolated from turnips and apples (70% as compared with 66 and 58%, respectively) [13, 14]. In our opinion, the most probable reason for such discrepancies is to be found in the procedure for treating the initial raw material — more severe in the case of the azolectin [9] and mild in the isolation of the phosphatides from the Far Eastern and American varieties of soybean.

The phosphatidic acid (PA) found in the soybean phosphatides in large amount (9-20%) complicates the isolation of the PI. With the aim of removing it, for chromatography we used Al_2O_3 (see the Experimental part), which has the property of strongly binding PA. As a result it was possible to obtain a fraction of choline-containing PLs (phosphatidylcholine (PC), lyso-PC, neutral lipids, glycolipids) and a PI fraction (PI, DPI, phosphatidylethanolamine (PE), N-acyl-PE, phosphatidylglycerol (PG), DPG, an unknown PL, glycolipids) uncontaminated by PA. To elute the first fraction we used the $CHCl_3$—CH_3OH (1:1) system, and for the second the $CHCl_3$—CH_3OH—1% aqueous CH_3COONH_4 (1:1:0.3) system.

Institute of Marine Biology, Far Eastern Scientific Center, Academy of the Sciences of the USSR, Vladivostok. Far Eastern State University, Vladivostok. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 35-38, January-February, 1984. Original article submitted January 26, 1983.

 0009-3130/84/2001-0032$08.50 © 1984 Plenum Publishing Corporation

TABLE 1. Phospholipid Composition of Soybean Phosphatide Concentrates

Phospholipid	Soybean phosphatides	
	far Eastern	American
PC	36,7	25,8
LPC	1,1	2,2
PE + N-acyl-PE	29,5	22,7
PI	12,0	17,0
DPI	1,7	2,0
PA	8,8	20,6
PG	2,3	1,7
DPG	2,2	2,0
X*	2,1	3,1
Start	3,6	2,8
Amount of lipids, % on the crude weight	80,0	85,0
Phospholipids, % on the total lipids	59,0	65,0

*Unknown saponifiable phospholipid migrating in the zone of the front on 2-dimensional MTLC.

To simplify and accelerate the procedure for obtaining the choline-containing PL fraction and the PI fraction, in contrast to what was done before [9], they were obtained on a Büchner funnel with the aid of a vacuum pump drawing into a Bunsen flask. The fraction of choline-containing PLs was used to obtain chromatographically pure soybean PC [15]. The PI fraction was further purified on a column with DEAE-cellulose prepared by the method of Rouser et al. [16], as described in the Experimental part.

The PI was obtained in the form of the ammonium salt. Its phosphorus content was 3.63%, nitrogen 1.69%. The fatty acid composition is given in Table 1. The molecular weight of the PI was 849. The preparation consisted of a white flocculent substance readily soluble in $CHCl_3$–CH_3OH (2:1, 1:1, and 1:4) and water, very sparingly soluble in $CHCl_3$, CH_3OH and benzene, and insoluble in acetone. The preparation was stored in $CHCl_3$–CH_3OH (1:1) at –(10-20)°C in a dark vessel. The PI contained the following fatty acids: 16:0 (14.0%), 16:1 (3.8), 17:0 (1.9), 18:0 (13.6), 18:1 (13.1), 18:2 (31.2), 18:3 (33.4).

EXPERIMENTAL

Preparation of the Lipid Extract. Commercial soybean phosphatides (50 g) were carefully stirred with a fivefold volume of acetone for 5-10 min. The acetone containing the neutral lipids and a very small amount of PLs (about 2%, mainly PC) was discarded. The residue was treated with acetone again. This procedure was repeated five times. The residue of phosphatides was stirred with 200 ml of $CHCl_3$–CH_3OH (2:1) and was washed with distilled water (20% of the volume of the mixture). The aqueous methanolic layer and an interlayer were discarded. The $CHCl_3$–CH_3OH layer was evaporated in vacuum to dryness. The yield of lipids was 24 g.

Fractionation of Lipids on Al_2O_3. A solution of 24 g of the lipids in 1000 ml of $CHCl_3$–CH_3OH (1:1) was treated with 200 g of Al_2O_3 (L 40/250 μm, alkaline, for chromatography), and the mixture was stirred for 1-2 min. Then it was poured into a Büchner funnel, and the solvent was sucked off by a vacuum pump into a Bunsen flask. The residue in the funnel was eluted additionally with 3.6 liters of $CHCl_3$–CH_3OH (1:1). The combined eluate consisted of a fraction of choline-containing PLs (PC, LPC, neutral lipids, glycolipids). The residue in the funnel was eluted with 12 liters of $CHCl_3$–CH_3OH–1% aqueous CH_3COONH_4 (1:1:0.3). The eluate was the PI fraction (PI, PE, PG, DPG, DPI, DPG, neutral lipids, and glycolipids). The PI fraction obtained was treated with distilled water (20% of the volume of the fraction). the mixture was stirred and it was left until it had separated completely into two phases (2 h). The upper, H_2O–CH_3OH, phase was discarded. The lower, $CHCl_3$–CH_3OH, phase was evaporated to dryness in a rotary evaporator, with benzene or propanol if traces of water were present, at 40-45°C. The yield of the PI fraction was 6.48 g.

Isolation of the PI on a Column of DEAE-Cellulose. DEAE-Cellulose (bead polymer, anion-exchange resin, Hungary; 300 g) was left in 1 N HCl for 20-30 min to swell and was then prepared by the method of Rouser et al. [16]. The DEAE-cellulose was transferred to a col-

umn (diameter 3 cm) in CH_3OH and was left to settle, a suspension of 50 g of silica gel (L 40/100 μm, Czechoslovakia) in CH_3OH was carefully poured in, the silica gel was allowed to settle, and a filter paper was placed on its surface. The CH_3OH was eliminated from the column by washing with two or three volumes of $CHCl_3$. Then 6.38 g of the PI fraction in 20 ml of $CHCl_3$ was transferred to the column with a pipette. The elution of the lipids was carried out by the following scheme:

1. $CHCl_3$ (0.8 liter) — eluted the neutral lipids;

2. $CHCl_3$–CH_3OH (80:20) (4.5 liters) in 50-ml portions: fractions 1-25 contained glycolipids and PE, and fractions 26-90 contained PE with traces of glycolipids (2 g, purity 95-96%);

3. $CHCl_3$–CH_3OH (20:80) (2.8 liters) — PE and LPE residues;

4. $CHCl_3$–CH_3OH–25% NH_3 (80:20:5) (2.4 liters) in 50-ml portions eluted PG, DPE, and an unknown PL; and

5. $CHCl_3$–CH_3OH–25% NH_3 (65:25:5) (1.5 liters) in 50-ml portions eluted the PI. The fractions containing the PI were combined and evaporated in a rotary evaporator to 1/10 of their initial volume, and then benzene or propanol was added and they were evaporated to dryness. The yield of PI was 1.3 g, with a chromatographic purity not less than 98-99%. Traces of DPI might be present.

The procedure for isolating PI was carried out for the soybean phosphatides from Far Eastern varieties. Similar results were obtained from the soybean phosphatide from American soybeans. The yields of PI and the amounts of solvents for eluting the lipids differed somewhat because of differences in the quantitative ratio of the lipids (see Table 1).

The Monitoring of the Lipid Fractions and the Yields of the Individual Substances. The micro-TLC of the lipids was carried out on KSK silica gel as described by Svetashev and Vas'kovskii [17] in the $CHCl_3$–CH_3OH–25% NH_3 (65:35:5) and $CHCl_3$–acetone–CH_3OH–CH_3COOH–H_2O (5:2:1:1:0.5) systems for two-dimensional chromatography or in the first system in the case of unidimensional chromatography. As standards we used PC, PE, LPC, LPE, PA, PG, DPG, and DPI obtained by methods described elsewhere [18]. Standard N-acyl-PE was kindly supplied by V. E. Vas'kovskii's laboratory (Institute of Marine Biology of the Far Eastern Scientific Center of the Academy of Sciences of the USSR). The substances were detected with the molybdate reagent for PLs [19], with 0.2% ninhydrin in acetone, with the Dragendorff reagent [20] and with 10% H_2SO_4 in CH_3OH followed by carbonization at 180°C. The phospholipids were determined quantitatively by the method of Vas'kovskii et al. [19]. The fatty acids of the PI were analyzed by the method of [21], and nitrogen by the Dumas method [22].

SUMMARY

1. A method is proposed for obtaining PI with a high chromatographic purity which includes two stages — the isolation of a PI fraction with the aid of alumina and its further purification on a column of DEAE-cellulose.

2. The possibility has been revealed of obtaining as a by-product PE with a purity of about 95-96%, and also a fraction of choline-containing phospholipids that can be used for the production of PC.

LITERATURE CITED

1. H. S. Hendrickson and C. E. Ballow, J. Biol. Chem., 239, 1369 (1964).
2. T. Shimojo and K. Ohno, J. Biol. Chem., 55, 355 (1964).
3. R. M. Saunders and C. E. Ballow, Biochemistry, 5, 352 (1966).
4. D. O. Tinker, D. J. Hanahan, Biochemistry, 5, 423 (1966).
5. C. Long and K. Owens, Biochem. J., 99, 617 (1966).
6. M. G. Luthra and A. Sheltawy, Biochem. J., 126, 251 (1972).
7. H. Nielson, J. Chromatogr., 89, 259 (1974).
8. G. V. Kiselev, Biokhimiya, 42, 2202 (1977).
9. G. Colacicco and M. M. Rapport, J. Lipid. Res., 8, 513 (1967).
10. É. Ya. Kostetskii, and N. I. Gerasimenko, USSR Inventor's Certificate, No. 950730, Byull. Izobret., No. 30 (1982).
11. H. Singh and O. S. Privett, Biochim. Biophys. Acta, 202, 200 (1970).
12. H. Singh and O. S. Privett, Lipids, 5, 692 (1970).

13. M. Lepage, Lipids, 2, 244 (1967).
14. T. Galliard, Phytochemistry, 7, 1915 (1968).
15. W. S. Singleton, M. S. Gray, M. Z. Brown, and J. White, J. Am. Oil Chem. Soc., 42, 53 (1965).
16. G. Rouser, G. Kritchevsky, D. Heler, and E. Lieber, J. Am. Oil Chem. Soc., 40, 425 (1963).
17. V. I. Svetashev, and V. E. Vaskovsky [Vas'kovskii], J. Chromatogr., 67, 376 (1972).
18. É. Ya. Kostetskii, and Yu. A. Shchipunov, Zh. Évol. Biokhim. Fiziol., 19, 11 (1983).
19. V. E. Vaskovsky [Vas'kovskii], E. Y. Kostetsky [É. Ya. Kostetskii], and I. M. Vasendin, J. Chromatogr., 114, 129 (1975).
20. H. Wagner, L. Hörhammer, and P. Wolff, Biochem. Z., 334, No. 2, 175 (1961).
21. É. Ya. Kostetskii, N. I. Gerasimenko, N. V. Naumenko, and V. E. Vas'kovskii, Bioorg. Khim., 7, 1069 (1981).
22. V. A. Klimova, Basic Micromethods for the Analysis of Organic Compounds [in Russian], Moscow (1975), p. 75.

SYNTHETIC ANTIGENS BASED ON POLYMERIC COUMARIN DERIVATIVES

A. Z. Abyshev and S. S. Krylov

UDC 678.447.42–134.26.615.717

The paper gives the results of the synthesis and a study of the structure of copolymers of 14 coumarin derivatives with N-vinylpyrrolidone. Using physicochemical methods, their characteristic viscosities, molecular masses, and chemical compositions have been determined. As the result of the immunological investigation performed, it has been shown that some of them possess antigenic properties. Thus, a new type of synthetic antigen has been created.

We set ourselves the aim of obtaining synthetic polymeric compounds possessing the properties of full-value antigens from chemical substances foreign to the organisms. Our attention was attracted by copolymers of certain vinyl derivatives and, in the first place, N-vinylpyrrolidone with coumarin derivatives. Such copolymers of suitable molecular mass are readily formed under the conditions of radical polymerization at a relatively low temperature — 70-80°C — in the presence of an initiator of the radical type — azoisobutyronitrile (AIBN). It should probably be pointed out that a number of the copolymers mentioned possess pronounced prolonged antiarrhythmic and hypotensive action [1, 2], and some of the coumarins composing them exhibit a photosensitizing effect [3, 4]. The latter can be explained by an allergization of the organism, which underlines the probability of the immunoactivity of such compounds.

The results given enabled us to suggest that some of these polymers would prove to be immunogenic.

To solve the problem we synthesized copolymers of the following coumarin derivatives — obtusifol acetate (I), obtusin (II), latilobinol (III), isosamarcandin (IV), grandivitin (V), the methyl ether of khellactone (VI), xanthyletin (VII), and synthetic analogs of them — 6-carboxy-7-(1'-carboxyisopropoxy)coumarin (VIII), oxypeucedanic acid (IX), 8-(3'-chloro-3'-methylbutyl)-7-methoxycoumarin (X)*, 7-(2'-bromoethoxy)coumarin (XI)*, 7-(2'-bromoethoxy)-4-methylcoumarin (XII), 7-(3'-chloropropoxy)coumarin (XIII), and 7-(3'-chloropropoxy)-4-methylcoumarin (XIV) with N-vinylpyrrolidone (scheme).

*These copolymers were obtained with the cooperation of I. V. Brodskii and V. A. Kropachev.

Institute of Toxicology, Ministry of Health of the USSR, Leningrad. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 38-43, January-February, 1984. Original article submitted February 11, 1983.

0009-3130/84/2001-0035$08.50 © 1984 Plenum Publishing Corporation