THE PURIFICATION OF LIPID EXTRACTS USING SEPHADEX LH-20

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INTRODUCTION

It is known that during extraction of lipids from plant material non-lipid contaminants are also extracted. These contaminants have long been a problem in lipid analysis since they are similar to the lipid hydrolysis products used in lipid assays.

A number of methods have been used in the past to remove these contaminants. These include dialysis[1,2], electrophoresis[3], paper chromatography[4,5], chromatography on silica impregnated paper[6], and cellulose column chromatography.

The most frequently used method involves repeated washing with water[7–10] or various salt solutions[11,12]. However, this has been found to result in a proteolipid "fluff" formation at the interface[12]. The authors report losses of up to 10% of the total lipid phosphorus with each washing and that lipids containing carbohydrates are preferentially removed. Phosphatides have been found to promote the formation of stable emulsions which result in lipid losses[13]. MARINETTI also reports that some lipids are soluble both in water and organic solvents. We have found that both the "fluff" and the wash water, on analysis by thin-layer silica chromatography, contain significant amounts of lipid material.

More recently, methods using Sephadex G-25 fine[14] and G-25 coarse[15] have been developed. However, these methods did not give satisfactory separation with broad bean lipid extracts. This paper presents a method using a new quality of gel, Sephadex LH-20.

EXPERIMENTAL

Lipid extraction

Mature leaves from 3-week-old broad bean plants (*Vicia faba*, Giant Windsor) were harvested and frozen immediately with liquid air. The tissue was ground thoroughly in a mortar and extracted with 2:1 C/M (chloroform–methanol, 2:1 v/v). This was then made to approximately 4:1 C/M and water removed by thorough mixing with 75–100 g of a 1:1 mixture of anhydrous di-sodium hydrogen orthophosphate and sodium sulphate. The extract was filtered through a fritted glass buchner funnel and evaporated to dryness on a flash evaporator. The residue was flushed thoroughly with nitrogen and redissolved with 3:1 C/M.

Since it had previously been found that this method resulted in the extraction of very small amounts of amino acids, 2.5 mg each of aspartic acid, asparagine,

arginine, tryptophan, and serine were added to give an indication of the elution rates of differing amino acids.

Preparation of Sephadex column

The fines were removed from Sephadex LH-20 (Pharmacia, Uppsala, Sweden) by suspending several times in water, allowing to settle for 15 min and then decanting the liquid. This removes a chloroform-insoluble residue which has been found[14] with Sephadex G-25 to be carbohydrate in nature. The Sephadex was then filtered in a buchner funnel and air-dried overnight. Before use, the gel was soaked in 3:1 C/M for a minimum of 48 h.

A column 18 × 1 cm was prepared without packing. A filter paper disc was placed on top of the gel since there is a tendency for the gel to float in solutions containing this proportion of chloroform.

Treatment of lipid extracts on column

A 1 ml sample of lipid extract in 3:1 C/M was pipetted carefully onto the filter paper disc and washed into the column with very small portions of solvent. To ensure good separation of lipids and contaminants, the flow rate was kept very low, approximately 0.1 ml/min. When all the pigment had been washed from the column, the rate was increased to 0.3 ml/min.

Samples were collected in conical graduated centrifuge tubes. The total effluent preceding the pigment bands was collected in sample 1. The subsequent green and yellow pigmented bands were collected separately although some overlap occurred. One ml and later two ml samples of the eluate were then collected until a volume of liquid greater than the bed volume of the Sephadex had passed through the column. The column was then washed with 1:1 M/water (methanol–water, 1:1 v/v) to remove any remaining contaminants retained by the column. This causes shrinkage of the gel and precludes re-use of the column.

Analysis of samples from column

All samples were dried down, redissolved to 1 ml with chloroform and then washed thoroughly with 2 ml of water. The two layers were separated by centrifugation at 1000 × *g* for 2–3 min and 0.5 ml aliquots of the water layer were removed for analysis of water-soluble components. Since this washing also dissolves some lipids, unwashed samples from duplicate columns were used for lipid analysis.

Water-soluble sugars were determined using 0.2 % anthrone in 90 % "Analar" sulphuric acid[16]. Sugar-containing lipids were also assayed by the anthrone method, assuming the major sugar component to be galactose from galactolipids. The unwashed samples were hydrolyzed at 100° in 3 *N* sulphuric acid for 2 h. Estimations of lipid sugars were made by subtracting the amount of previously determined soluble sugar in each sample. Readings were compared to standard curves for both sucrose and galactose. Chlorophyll content of the extracts, as well as total sugar and phosphorus content, was assayed before and after being run through the column as an indication of lipid recovery. Chlorophyll was determined by the method of ARNON[17]. Water-soluble phosphorus was assayed using the method of ALLEN[18]. Lipid phosphorus was assayed in unwashed samples after incineration for 30 min in 60 % perchloric acid. Amino acids were analyzed by thin-layer chromatography on Silica Gel G.

The plates were developed in *n*-butanol–acetic acid–water (60:20:20, v/v) and sprayed with ninhydrin.

RESULTS AND DISCUSSION

A number of different combinations of C/M had been tried before settling on a 3:1 solvent mixture. This proved to give the best separation with only slight tendency of the gel to float. A 1:18 width to height ratio of the column resulted in a narrow elution band. Increases in diameter beyond 1 cm resulted in considerable spreading of the lipid and overlap of the sugar band.

Free sugars have been found to be the most significant contaminant of broad bean extracts and will, therefore, be the major point of discussion. The results of assays of both water-soluble and lipid sugars eluted from the column are presented in Table I. While it is realized that plant material generally contains a wide range of sugars, many of which react to varying degrees with anthrone[16], for simplicity the soluble sugars are presented in terms of the predominant sugar, sucrose. Similarly,

TABLE I

DISTRIBUTION OF LIPID AND NON-LIPID SUGARS, AND TOTAL PHOSPHORUS IN ELUATE FRACTIONS OF SEPHADEX LH-20 COLUMNS

Total leaf extracts were applied to columns and eluted with chloroform–methanol 3:1. The final fraction from each column was eluted with methanol–water, 1:1.

Sugars (1.14 mg chlorophyll applied)						*Total phosphorus/fraction (2.64 mg chlorophyll applied)*		
Fraction No.	*Volume (ml)*	*Water-soluble sugars/fraction*		*Total sugars as galactose (μg)*	*Lipid sugars* as galactose (μg)*	*Fraction No.*	*Volume (ml)*	*Phosphorus (μg)*
		As sucrose (μg)	*As galactose (μg)*					
1	6.5	18.0	40.0	40.0	0.0	1	7.0	0.0
2	1.0	3.6	6.0	6.0	0.0	2	1.25	1.7
3	2.25	4.0	8.0	760.0	752.0	3	2.25	46.8
4	1.5	5.2	12.0	196.8	184.8	4	1.75	13.0
5	1.0	3.6	6.0	7.0	1.0	5	1.0	0.3
6	1.0	3.6	6.0	6.5	0.5	6	1.0	0.2
7	1.0	8.8	20.0	24.0	4.0	7	1.0	0.0
8	1.0	14.0	30.0	37.5	7.5	8	1.0	0.0
9	1.0	28.8	62.0	67.0	5.0	9	1.0	1.0
10	1.0	22.0	44.0	44.8	0.8	10	1.0	0.0
11	1.0	58.0	122.0	124.5	2.5	11	1.0	0.0
12	1.0	16.0	32.0	40.4	8.4	12	1.0	0.0
13	1.0	8.0	16.0	21.0	5.0	13	1.0	0.0
14	1.0	7.2	14.0	22.0	8.0	14	1.0	0.0
15	2.0	4.0	8.0	8.0	0.0	15	2.0	0.0
16	2.0	0.0	0.0	0.0	0.0	16	1.0	0.6
17	2.0	4.0	8.0	8.3	0.3	17	4.0	0.5
18	1.0	4.0	8.0	10.5	2.5			
19	6.0	7.2	14.0	17.5	3.5			
Total	34.25	220.0	456.0	1441.8	985.8	Total	29.25	64.1

* Calculated by substracting water-soluble sugars (as galactose) from total sugars (as galactose).

as the sugar-containing lipids are predominantly mono- and digalactolipids, results are also expressed in terms of their sugar moiety, galactose.

In Fig. 1, the elution patterns are presented graphically. Fraction 1 was found to contain a high amount of free sugar relative to the subsequent sample. Since it is not possible for sample components to be eluted until a volume of liquid greater than the void volume of the gel has passed through the column, it is suspected this was due to residual fines. It was, therefore, excluded from subsequent calculations.

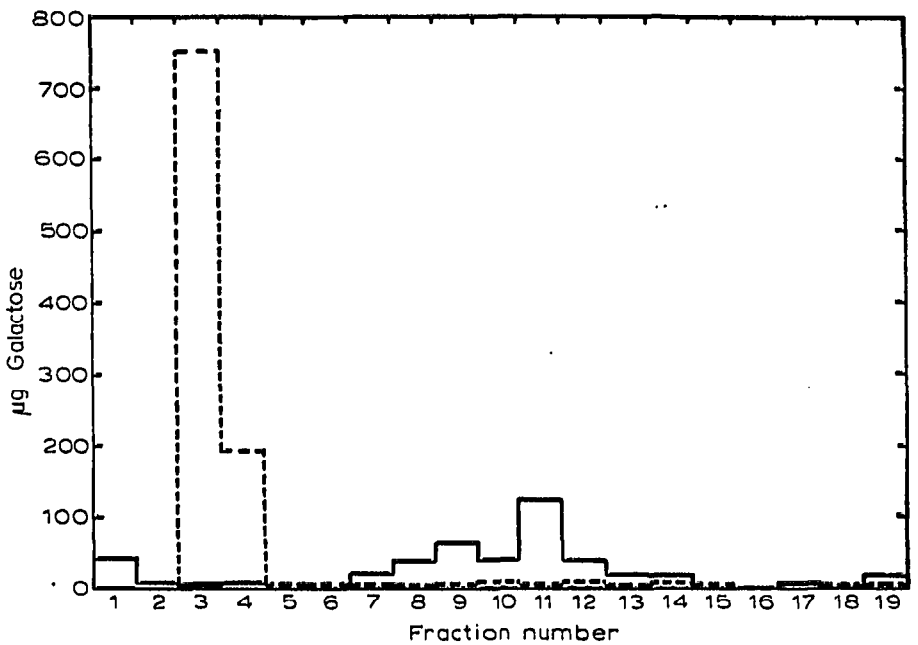

Fig. 1. Distribution of lipid sugars (– – –) and non-lipid sugars (———) expressed as galactose (µg)/eluate fraction. A whole leaf extract containing 1.14 mg chlorophyll was applied to the column. Fractions 1 to 18 were eluted with 3:1 C/M and fraction 19 with 1:1 M/water.

The sugar-containing lipids were found to be eluted in a narrow band closely corresponding to the visible pigment bands. The combined volume of lipid-containing fractions, 3 and 4, was less than 4 ml. Soluble sugars were eluted in a much wider band which lagged behind the lipid band by several millilitres, thus ensuring good separation. These water-soluble sugars originally comprised 29.7% of the total sugar in the extract. After the sample had passed through the column, only 4.8% of the total water-soluble sugars were left as contaminants in fractions 3 and 4. This is equivalent to 2.02% of the lipid sugars.

Inorganic phosphorus was assayed in washings of the whole extract as well as the column eluates. The readings obtained, however, were too low for accurate estimation. Since inorganic phosphorus is readily soluble in water, it is evident that the soluble phosphorus content of the lipid extracts is very low. The contribution to readings of lipid-phosphorus would be negligible.

The results of assays of total phosphorus eluted from the column are presented in Table I and Fig. 2. It was found that phosphorus-containing lipids were eluted in a narrow band corresponding to the pigment and galactolipid elution pattern. Approxi-

mately 96% of the phosphorus in the column eluate was contained in fractions 2, 3 and 4. Recovery of phosphorus applied to the column was 94%.

Amino acids, when assayed by thin-layer chromatography (Fig. 3), were found to be retained until 22 ml of solution had passed through the column and were well separated from lipid components. Arginine was detected first in the eluate and was detected in all subsequent fractions. Traces of the other amino acids were eluted in these fractions as well. However, the predominant amounts of each amino acid were found in the methanol–water fraction.

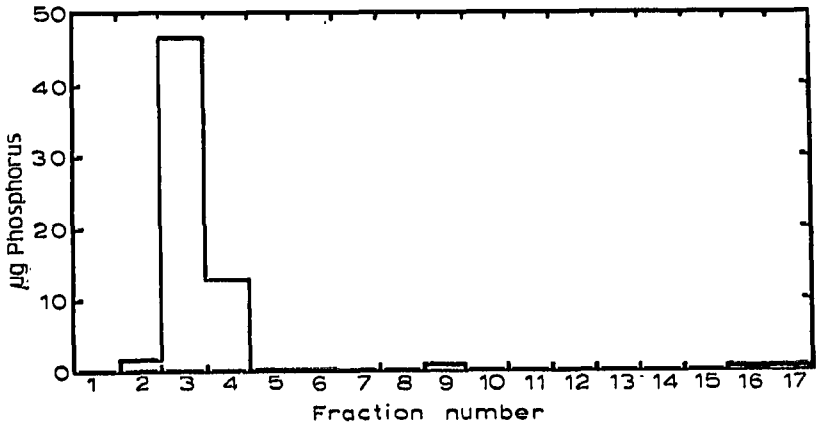

Fig. 2. Distribution of total phosphorus expressed as phosphorus (μg)/eluate fraction. A whole leaf extract containing 2.64 mg chlorophyll was applied to the column. Fractions 1 to 16 were eluted with 3:1 C/M and fraction 17 with 1:1 M/water.

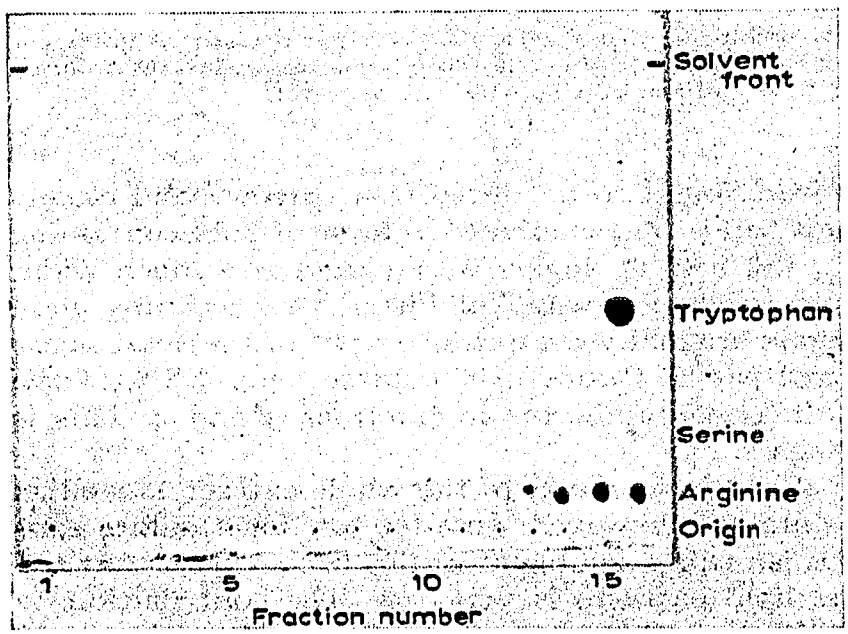

Fig. 3. Thin-layer Silica Gel G chromatogram showing the distribution of amino acids (added to a lipid extract) in fractions eluted from a Sephadex LH-20 column. The chromatogram was run in *n*-butanol–acetic acid–water (60:20:20, v/v) and developed with ninhydrin. Fractions 1 to 15 (2 ml) were eluted with 3:1 C/M and fraction 16 (4 ml) with 1:1 M/water.

Estimates of lipids recovered from the column are presented in Table II. An average recovery of greater than 96 % was found when assayed as chlorophyll. Total sugar recovery when assayed as galactose was 98.6%. Total phosphorus recovered was 94.0 %.

Since nearly all the lipids are removed in the first few millilitres, this presents a rapid and efficient method of removing unwanted contaminants. It was found that lipid concentrations short of saturation had no effect on the efficiency of separation so that the method can be used for large scale purification of lipid material. This method is now being used routinely in our laboratory to remove impurities from lipid extracts of whole leaves and chloroplasts.

TABLE II

ESTIMATES OF RECOVERY OF LIPID COMPONENTS FROM COLUMNS*

Compound	*Experiment*	*Quantity applied to column (mg)*	*Quantity in eluate (mg)*	*% recovery*
Chlorophyll	1	1.3458	1.2675	94.2
	2	1.3532	1.2909	95.4
	3	1.5312	1.4909	97.4
	4	2.5214	2.4470	97.0
	5	1.1407	1.1024	96.6
Average				96.1
Total sugars (as galactose)	5	1.4220	1.4018	98.6
Total phosphorus	6	0.0682	0.0641	94.0

* The data discussed in the text are taken from experiments 5 and 6.

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SUMMARY

A column chromatographic procedure for the removal of contaminants from lipid extracts has been developed using Sephadex LH-20, produced specifically for use with organic solvents. The method has been found to remove both sugars and amino acids from lipid extracts of *Vicia faba*. Lipid recovery from the columns of greater than 97 % has been obtained.

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