CHROM. 6170

PURIFICATION OF INDOLE-3-ACETIC ACID MYOINOSITOL ESTERS ON POLYSTYRENE–DIVINYLBENZENE RESINS*

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(Received May 23rd, 1972)

SUMMARY

A method for the purification of indole-3-acetic acid and esters of indole-3-acetic acid and myoinositol on Dowex 50W-X2 and on partially sulfonated polystyrene resins is described. The method has been applied to the analysis of the indolylic compounds present in crude acetone–water extracts of the kernels of *Zea mays*. Sufficient purification and enrichment of these compounds is obtained by a single column chromatographic step so that the column eluates can be examined by thin-layer chromatography, gas–liquid chromatography or combined gas–liquid chromatography–mass spectrometry.

INTRODUCTION

Work in this laboratory has been concerned with the isolation and chemical characterization of esters of indole-3-acetic acid (IAA) and myoinositol and myoinositol glycosides[1–6]. The purification of these esters was accomplished by subjecting small samples of plant extracts to successive Sephadex G-10 column chromatography, silica gel column chromatography and preparative thin-layer chromatography (TLC). Only small amounts of these esters could be prepared by such methods, as they are present in low concentrations in plant tissues (35–45 mg/kg), are chemically diverse and undergo acyl migration during preparation. For preparative purposes, it was desirable to develop a technique that could be used to purify and concentrate these compounds as a group.

In the present communication, we present a column chromatographic technique involving the use of styrene–divinylbenzene copolymer resins capable of a large and relatively non-selective enrichment of the IAA esters from crude plant tissue extracts. To the best of our knowledge, there are no previously published single-step methods that permit qualitative and quantitative analysis of indolylic compounds in plant tissue extracts. A prior attempt had been made to use sulfonated polystyrene resin in the identification of growth-promoting substances in plant tissue extracts[7]. A brief report of our studies has been made[8], and subsequently there has been a study of the separation of some simple indole derivatives on neutral polystyrene resin[9].

* This work was supported, in part, by the National Science Foundation (GB-18353X). Journal Article No. 5931 from the Michigan Agricultural Experiment Station.

MATERIALS AND METHODS

Extraction

The extraction of ground sweet corn kernels of *Zea mays* L. (cultivar, Stowell's evergreen hybrid) was carried out as described previously[5,6], except that batches

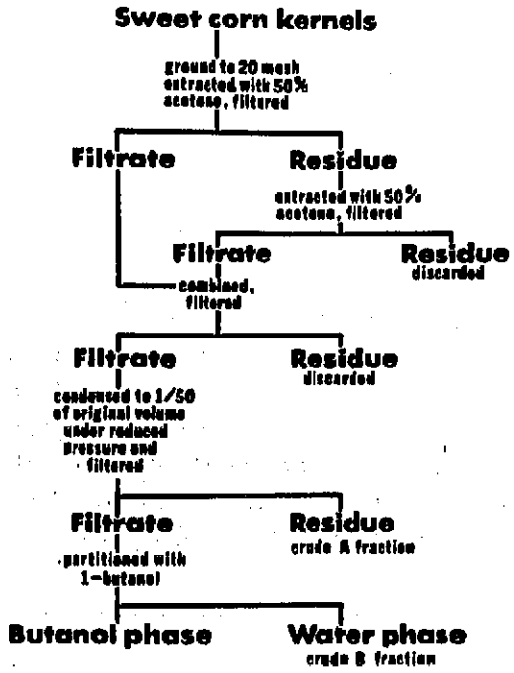

Fig. 1. Flow sheet of the extraction procedure of dry corn kernels.

of 15 kg were used and the filtered acetone–water extract was concentrated to 50 ml per kilogram of extracted corn meal[8]. Fig. 1 summarizes the extraction procedure used.

Column chromatography

Dowex 50W-X2 (H^+form), 200–400 mesh ('Bakers Analysed' reagent) was allowed to swell in distilled water, and washed exhaustively with water. The resin was then washed successively with 1, 2 and 5 *N* NaOH followed by washing with water until the pH of the eluate was stable at 5.5. The resin was then washed with 5 *N* HCl followed by water until the pH of the eluate was stable at 6.0. The resin was packed into a glass column of 1.8 cm I.D. The bed volume (V_B) was 66.5 ml and the void volume (V_0), determined with blue dextran, was 20.5 ml. When the resin was to be used at an acidic pH, the packed column was washed with 0.1 *M* sodium citrate buffer (pH 2.5), followed by washing with water until the pH of the eluate was stable at 4.2. The sample was then eluted with 1.0 m*M* sodium citrate buffer at pH 3.3. For use at a more neutral pH, the packed column was washed with 0.1 m*M* sodium citrate buffer (pH 5.6). Then the column was washed with water until the pH of the eluate was stable at 4.7. The sample was eluted with 1.0 m*M* sodium citrate buffer at pH 6.2.

The experimental "low capacity" resin, a styrene–divinylbenzene copolymer, 1% cross-linked, 100–200 mesh, and only 20% sulfonated (Dow Chemical Co.,

Midland Division, Midland, Mich. 48640, U.S.A.) was prepared in the same manner as the Dowex 50W-X2 resin. The resin was packed into a glass column of 0.55 cm I.D., V_B = 3.5 ml and V_0 = 1.8 ml. The sample was eluted with 1.0 m*M* sodium citrate buffer of pH 3.3, followed by water, and finally a linear acetone–water gradient from pure water to 50 % acetone.

Thin-layer chromatography

Crude extracts, at the stage of purity of fractions A and B (Fig. 1), contain too much dry matter to allow their examination by TLC. Following Dowex 50W-X2 or "low capacity" resin chromatography, TLC is possible. The developing solvents and TLC plates used were as described previously[5]. Indolylic compounds were made visible on the TLC plates with a modified Ehrlich reagent[10]. Within 5 min of spraying with the reagent, the IAA esters and free IAA spots developed a pink-red color that slowly changed to blue-purple, and reached a maximum intensity after 5–8 h. This color reaction is considerably slower than that with the Salkowski reagent[5], but is superior in that the color appears to be indefinitely stable. After full color development had been reached, other non-indolylic compounds on the same plates were made visible by spraying with concentrated (37 %) H_2SO_4 and charring for 20 min at 105°. After charring, the Ehrlich-positive spots lost their colors and intensities, and sometimes were no longer distinguishable from other charred matter. However, the initial blue-purple color of the indolylic compounds could be restored and intensified by simply submerging the TLC plate in water for 2–4 min. The plates were then dried at 35° and stored with no detectable changes in color with time.

Gas–liquid chromatography

Silylation with N-trimethylsilylimidazole (TSIM). A sample containing between 0.05 and 0.2 μmole of IAA esters from the appropriate column chromatographic eluant fraction was dried over anhydrous calcium sulfate in a 1.0–ml serum vial, and sealed with a silicone-rubber serum cap. Pure, dry N,N-dimethylformamide (10–20 μl) was added with a syringe through the serum cap to dissolve the dry residue. Then 20–40 μl of TSIM (Regis Chemical Co., Chicago, Ill. 60610, U.S.A.) were added. The vial was shaken for several seconds and allowed to stand at room temperature for 30 min. This silylating reagent completely derivatizes the free hydroxyl groups of the inositol and glycoside moieties of the IAA esters.

Silylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA). The preparation of the trimethylsilyl (TMS) derivative with BSTFA (Regis Chemical Co.) was the same as with TSIM except that the silylation was carried out at 50° for 1 h. Under these conditions, combined gas–liquid chromatography (GLC)–mass spectrometric analysis showed that all the hydroxyl groups were silylated as well as the nitrogen atom of the indole nucleus. The derivatized samples were analyzed on an F & M Model 402 gas chromatograph equipped with flame ionization detectors, with nitrogen as carrier gas at a flow-rate of 60 ml/min. Two columns were used, a 6 ft. × 3.0 mm I.D. U-shaped glass column packed with SE-30, 3 % on Supelcoport (Supelco Inc., Bellefonte, Pa. 16823, U.S.A.), and a 6 ft. × 6.0 mm I.D. U-shaped glass column packed with OV-1, 1 % on Gas-Chrom Z (Applied Science Lab. Inc., State College, Pa. 16801, U.S.A.). Combined GLC–mass spectrometry was carried out on a LKB-9000 instrument.

RESULTS AND DISCUSSION

Chromatography on Dowex 50W-X2 resin

Dowex 50W-X2 is a sulfonated polystyrene–divinylbenzene copolymer and, as a strong cation-exchange resin, would not retain IAA or its esters. However, the structure of the resin suggested that the salt of IAA would be excluded, whereas IAA, as the undissociated acid, would partition between the stationary resin phase and the moving solvent phase. Further myoinositol has a single axial and five equatorial groups and it is known that the sterically less hindered equatorial hydroxyl groups are more strongly absorbed to polar absorbants than axial hydroxyl groups[11]. Therefore, a non-polar stationary phase, such as a polystyrene–divinylbenzene copolymer, should have a greater affinity for equatorially acylated IAA–myoinositol esters. The axially acylated -2-O- esters should be eluted from the column first, followed by the equatorial esters, in order of decreasing polarity. That this does in fact occur is shown by the results in Figs. 2 and 3. The elution profiles were obtained when aliquot mixtures of partially purified, axially and equatorially acylated IAA–myoinositols, IAA–myoinositol glycosides, unesterified IAA and [1-^{14}C]IAA (only detectable radiologically) were chromatographed on Dowex 50W-X2. The samples were eluted with 1.0 m*M* sodium citrate buffer at pH 3.3 (Fig. 2) or at pH 6.2 (Fig. 3). IAA, as detected colorimetrically, or by radioactivity, was eluted between 7.5 and 12 bed volumes (0.5 and 0.8 l) from the column at pH 3.3, whereas at pH 6.2 IAA was eluted from the column between 0.3 and 1.2 bed volumes (0.02 and 0.08 l). Dissociated IAA was excluded from the column while the partially undissociated IAA was partitioning into the resin. The IAA esters were found to elute in three broad, overlapping peaks at 2.1–4 (0.14–0.27 l), 4.2–6.0 (0.28–0.40 l), and 6.1–8.3 (0.41–0.55 l) bed volumes

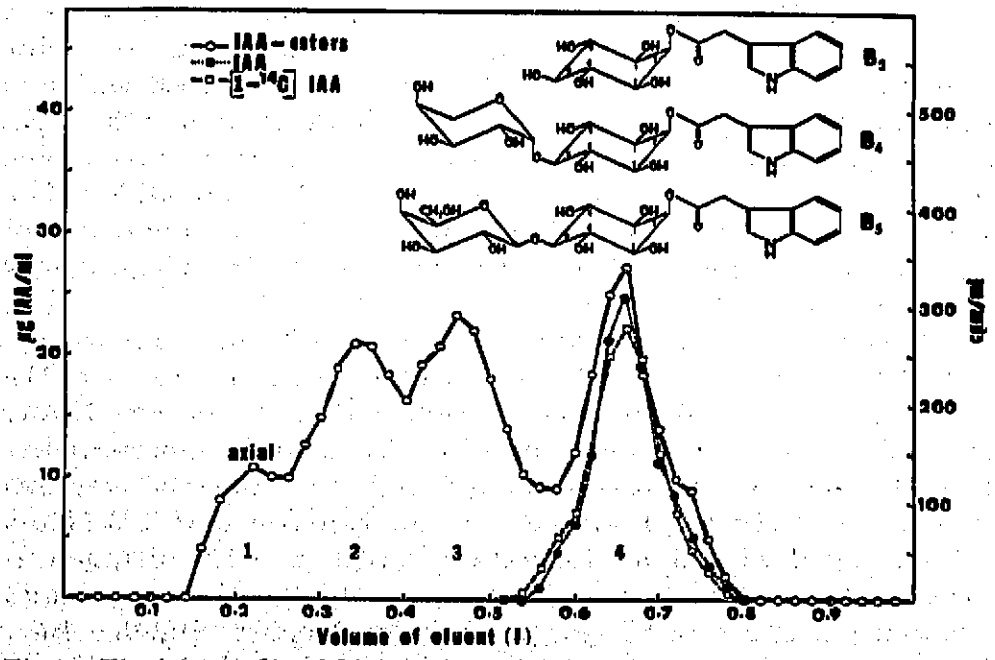

Fig. 2. Elution profile of IAA and partially purified IAA esters chromatographed on acidic Dowex 50W-X2 resin. For conditions of column chromatography, see text. The IAA of the IAA esters was measured as described previously[5]. The -2-O- esters of IAA and myoinositol and myoinositol glycosides (B_2, B_4 and B_5) are eluted ahead (see Fig. 4A) of the equatorially acylated IAA esters. The numbers under the peaks indicate the fractions used for examination by TLC (Fig. 4A).

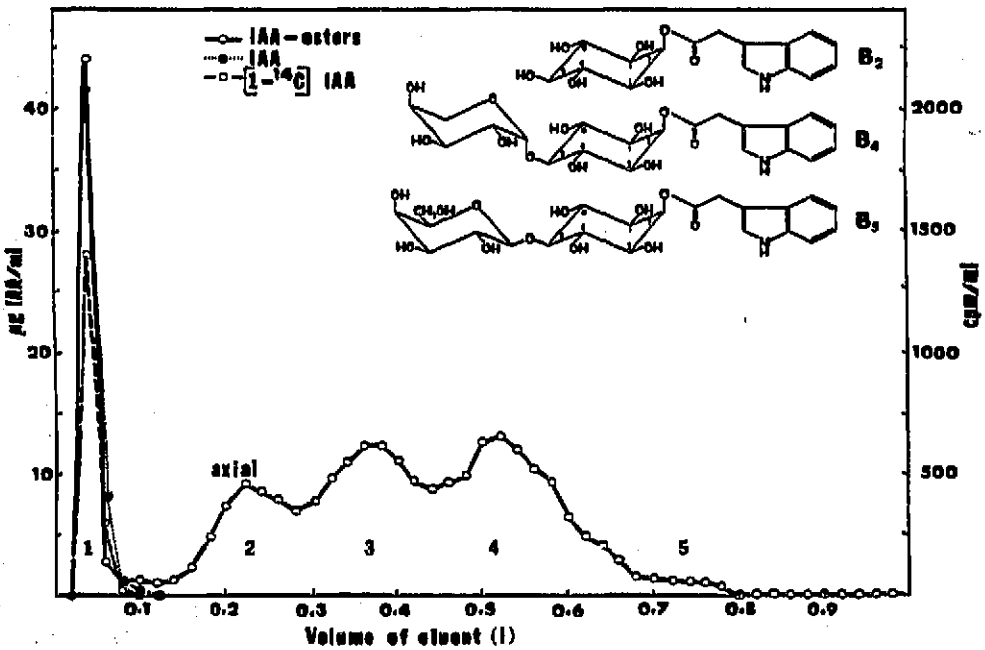

Fig. 3. Elution profile of IAA and partially purified IAA esters chromatographed on neutral Dowex 50W-X2 resin. For conditions of column chromatography and explanation of figure, see text and Fig. 2. The TLC pattern of the eluted fractions is shown in Fig. 4B.

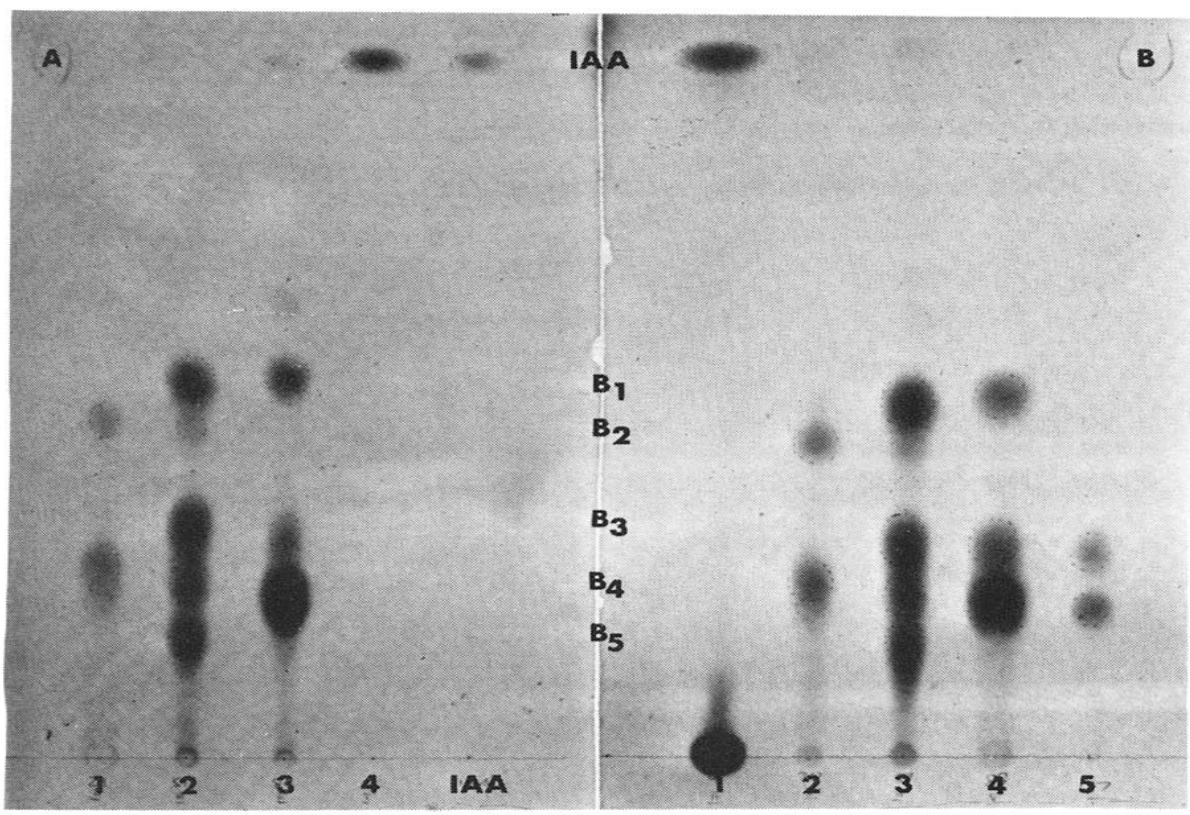

Fig. 4. TLC patterns of the distribution of IAA esters in eluent fractions from the acidic (A) and neutral (B) Dowex 50W-X2 column chromatography. Aliquots, containing between 2 and 20 μg of IAA from the respective peak areas (see Figs. 2 and 3), were spotted on Silica Gel F_{254} TLC plates. The plates were developed in ethyl acetate–methyl ethyl ketone–ethanol–water (5:3:1:1), made visible with Ehrlich reagent and charred with concentrated H_2SO_4.

from both the acidic and the neutral column. Therefore, pH had no effect on the elution profile or the elution volume of the IAA esters.

Fig. 4 shows the TLC profiles of the material eluted from the columns. It can be seen that the axially acylated -2-O- IAA–myoinositols and IAA–myoinositol glycosides occurred in the first peak (labeled axial). The equatorially acylated esters were in the second and third peaks. The position of the hydroxyl group of myoinositol to which IAA is esterified is not known for all the equatorial esters. The compounds to which we have assigned the -1- or -3-O- structures were present only in the second and third peaks[4,6]. Further characterization of these compounds will be presented elsewhere. Most important, however, in terms of the applicability of the Dowex 50W-X2 resin for the purification of the IAA–myoinositol esters is the fact that the esters are eluted essentially as a group. In contrast, Sephadex G-10 chromatography separates IAA–myoinositols from IAA–myoinositol glycosides[5].

Purification of crude extracts on Dowex 50W-X2 resin

The above behavior of IAA esters on Dowex 50W-X2 resin has been studied with extracts that had been partially purified by two successive Sephadex G-10 column chromatographic steps. It was therefore desirable to determine the behavior and degree of purification obtainable when a crude extract (crude B fraction, Fig. 1) is chromatographed on this resin. Provided that the IAA esters are retained sufficiently long, it should be possible to wash mono-, oligo- and polysaccharides and organic acids (present in large amounts in the crude extracts) through the column. Amino acids would be only partially retained and, if absorbed, would remain on the column while the neutral esters are eluted. We tested this theory by using the acidic column and found that the column behaved as expected. A sample of crude B fraction (Fig. 1) of 47.5 g dry weight containing 6.10 mg of esterified IAA (0.0123 % of the dry weight) dissolved in 60.0 ml of water was applied to the column (conditions as in Fig. 2). As before, when using pre-purified material, the esters were eluted in three poorly

TABLE I

QUANTITATIVE DETERMINATION OF IAA LIBERATED BY ALKALINE HYDROLYSIS IN ELUENT FRACTIONS FROM A SAMPLE OF CRUDE B FRACTION CHROMATOGRAPHED ON DOWEX 50W-X2 RESIN

Volume of eluent fraction (ml)	*Dry weight of fraction (mg)*	*Alkali-labile IAA (mg)*	*Dry weight of IAA (%)*	*Purification factor*[a]
244–336	386	1.26	0.33	26
377–480	113	1.80	1.59	124
481–640	94	1.58	1.68	131
641–800	103	0.80	0.78	61
801–860	153	0.43	0.28	22
Total	849	5.87	0.69	54

[a] The purification factor is the IAA content as a percentage of the dry weight of the fraction divided by the IAA content as a percentage of the dry weight in the crude fraction.

separated peaks within 13 bed volumes. Table I shows the IAA content of the pooled fractions. Recovery of the IAA esters was 96 %. The total dry weight of the material

recovered was 849 mg containing 5.87 mg of IAA (0.69 % of the dry weight). This represents an overall 54-fold purification of the IAA-esters in a single column-step.

Chromatography on "low capacity" resin

Owing to the limited porosity of the non-sulfonated divinylbenzene copolymer of which Dowex 50 is made, the native resin is probably not suitable for chromatography of IAA esters. Resins of low cation-exchange capacity, which show increased molecular sorption as a result of partial sulfonation[12], however, should be suitable for the purification of IAA esters. A small sample of a divinylbenzene copolymer with only 20 % sulfonation was made available to us. The elution profile of a mixture of pre-purified IAA–myoinositol glycosides (B_3–B_4 mixture and IAA, plus a small amount of [1-^{14}C]IAA) is shown in Fig. 5. Washing the column with 23 bed volumes

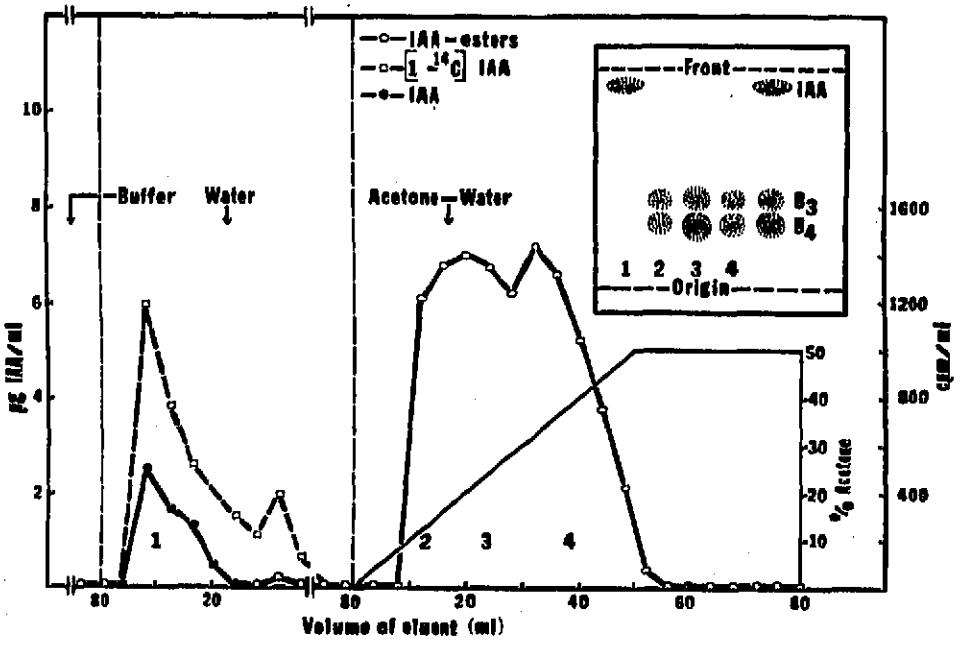

Fig. 5. Elution profile of IAA and partially purified IAA esters chromatographed on "low capacity" resin. For conditions of column chromatography, see text. Samples from the indicated eluent fractions (1–4) were chromatographed by TLC as shown in the insert. Conditions for TLC were the same as for Fig. 4.

(80.0 ml) did not elute IAA or the IAA esters. Free IAA, however, was eluted with water between 1.4 and 10 bed volumes (5.0 and 35.0 ml). Most interestingly, the IAA esters could be eluted only with an aqueous acetone gradient from 6 % (v/v) to 46 % acetone. The TLC profile of the eluted material is shown in the insert of Fig. 5. Although not as marked as for the Dowex 50W-X2 resin with aqueous eluant, there is nonetheless a tendency for the axial IAA esters to be eluted ahead of the equatorial esters. The advantage of this partially sulfonated divinylbenzene copolymer lies in the high sorption capacity for the IAA–myoinositol glycosides and presumably also the IAA–myoinositols.

Purification of crude extracts on "low capacity" resin

In view of the results obtained with the pre-purified IAA esters, the suitability of this resin for the purification of crude extracts was tested. A sample of crude B

fraction (Fig. 1) of 4.45 g dry weight containing 570 μg of esterified IAA together with free IAA and [1-^{14}C]IAA dissolved in 4.0 ml of water was applied to the column (conditions as for Fig. 5). The elution profile of this mixture of IAA and IAA esters is shown in Fig. 6. Again, the esters are not eluted with aqueous buffer or water, but

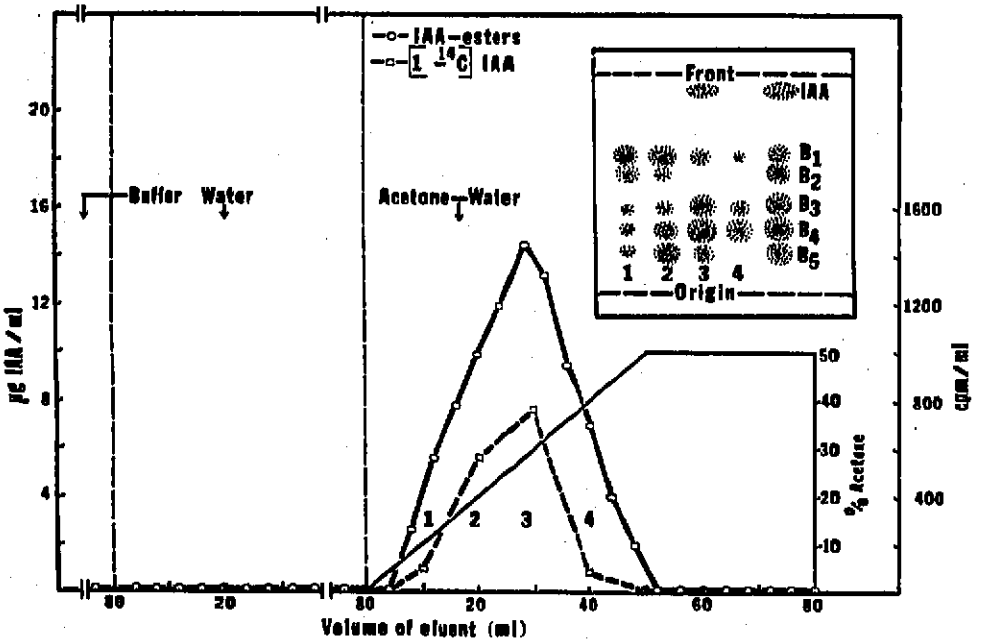

Fig. 6. Elution profile of IAA and IAA esters from crude plant extract chromatographed on "low capacity" resin. For conditions of column chromatography, see text and Fig. 5.

only with the aqueous acetone gradient. Surprisingly, IAA could not be eluted with water, but co-eluted with the IAA esters. This indicates that IAA in the crude extracts not only partitions between the resin and the mobile solvent phase, but also between a mobile phase and solute of the sample which apparently shows a high degree of solvation for IAA. Similar behavior of IAA upon column chromatography of crude B fraction has been observed before[5]. The insert in Fig. 6 shows the TLC profile of fractions taken from the respective regions of the single peak. The -2-O- esters are again eluted ahead of the equatorially acylated esters. Recovery of the indolylic compounds was 97 %. The dry weight of the pooled fractions (0.5–50.0 ml) was 34.4 mg containing 553 μg of IAA. Therefore the IAA content increased from 0.0128 to 1.60 %, which represents a 125-fold purification in this single column step.

Gas–liquid chromatography

It has been shown above that the material recovered from both column systems is suitable for direct TLC analysis. More important, however, is the fact that the same material may be used for GLC, and combined GLC–mass spectrometric analysis without additional purification steps. An example of the GLC behavior of a small sample taken from the pooled fraction of the 'low capacity' column is shown in Fig. 7. The GLC profile shows the complete series of IAA–myoinositols (B_1–B_2 peaks), IAA–myoinositol arabinosides (B_3–B_4 peaks) and IAA–myoinositol galactosides (B_5 peaks). The identity of these peaks was established by comparison of their mass

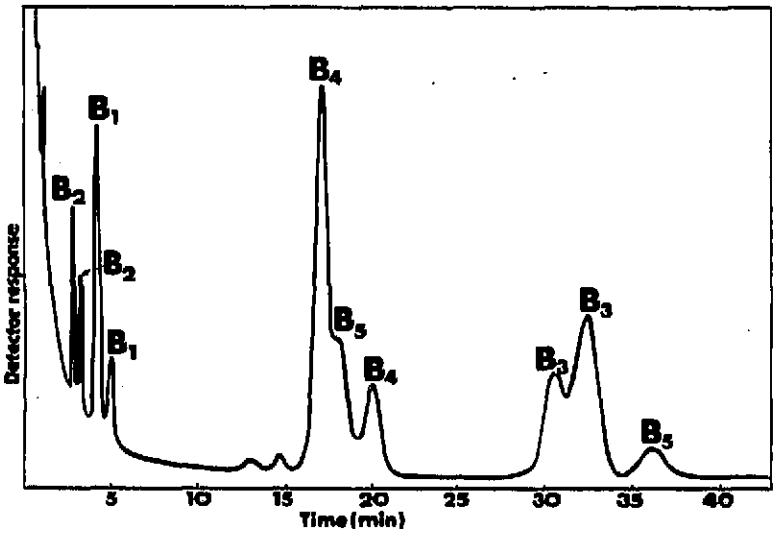

Fig. 7. Gas–liquid chromatogram of a mixture of TSIM–IAA ester derivatives. A pooled sample containing between 0.05 and 0.2 μM of IAA from the appropriate eluent fractions of crude plant extract chromatographed on "low capacity" resin was prepared for GLC (using BSTFA) as described in the text. The derivatized sample was run isothermally at 250° with a carrier gas flow-rate of 60 ml/min.

spectra with the previously published spectra for these compounds[6]. The results from the combined GLC–mass spectrometric analysis will be published elsewhere.

CONCLUSIONS

A sulfonated polystyrene–divinylbenzene copolymer (Dowex 50W-X2) column effectively purifies (54-fold) and concentrates indolylic compounds from extracts of kernels of *Zea mays* in a simple single column step.

After column chromatography, IAA and esters of IAA and myoinositol or myoinositol glycosides are of sufficient purity to be analyzed by TLC, GLC or combined GLC–mass spectrographic analysis.

A 20% sulfonated polystyrene–divinylbenzene copolymer resin was found to be even more efficacious than Dowex-50 in the purification (125-fold) of indolylic compounds.

Both Dowex-50 and the partially sulfonated resin show lower affinity for axially acylated than for equatorially acylated esters of IAA and myoinositol or myoinositol glycosides.

ACKNOWLEDGEMENT

We are deeply indepted to Dr. H. SMALL, Dow Chemical Co., Midland, Mich., for supplying the 20% sulfonated polystyrene–divinylbenzene copolymer.

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