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USE OF INSOLUBLE POLYVINYLPYRROLIDONE FOR PURIFICATION OF PLANT EXTRACTS AND CHROMATOGRAPHY OF PLANT HORMONES

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Abstract—Polyclar AT, an insoluble form of poly-*N*-vinylpyrrolidone is highly effective (60-fold reduction in dry weight) in purification of gibberellin-like substances in plant extracts, presumably by selective removal of phenolic compounds and perhaps other organic acids Biological activity of extracts in several plant bioassays for gibberellins was invariably enhanced by the technique Quantitative elution of abscisic acid, indoleacetic acid, zeatin, gibberellins exhibiting a wide divergence of polarity and the glucoside of gibberellin A_3 was obtained from columns of Polyclar AT Separation of a number of gibberellins from each other and from abscisic acid was also possible The selective properties of Polyclar AT toward these plant hormones are discussed

INTRODUCTION

DETECTION, characterization, and quantitation of gibberellins in extracts of vegetative tissue is generally very difficult due to the presence of only microgram amounts of the hormones and excessive quantities of impurities which interfere with gibberellin (GA) bioassays.[1,2] A major source of impurities and possible inhibitory activity would seem to be phenolic compounds which in plants are widespread and frequently occur in high concentrations.[3]

Polyclar AT, an insoluble form of the polymer, poly-*N*-vinylpyrrolidone (PVP), has been shown to be reasonably specific in separating a phenolic fraction from plant tissue extracts by hydrogen bond formation.[3,4] It has been employed for column chromatography of nucleotides, purines, pyrimidines, riboflavin and vitamin B_{12}[5–7] and has been found effective for desalting nucleic acid components[8] and in separation of phenylalanine, tyrosine and tryptophan.[9] PVP has also been employed advantageously for purification of abscisic acid from woody plant tissue [10]

The present investigation was undertaken to determine whether or not PVP might be used to advantage for purification of certain major classes of hormones in plant extracts by selective removal of phenolic, and perhaps other organic acid impurities As well, the elution pattern of a number of plant hormones from PVP was investigated.

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RESULTS AND DISCUSSION

Preliminary Experiments

While the use of insoluble PVP to bind phenolic compounds and possibly various organic acids present as impurities in extracts seemed an attractive possibility, it was necessary to determine at the outset whether PVP would in fact bind irreversibly to gibberellins and other plant hormones and remove them from solution as well Andersen and Sowers[4] pointed out that under mildly acidic conditions not only compounds with aromatic hydroxyl groups, but also those with carboxyl and enolic groups may bond with the polymer

As a preliminary, PVP was slurried at pH 8 0 in buffer solution containing 200μg GA_3 Examination by GLC showed 97 per cent recovery of GA_3 in the experimental run as compared to the control (no PVP) An identical experiment with pH 6 5 buffer yielded similar results Extension and confirmation of the results obtained above were sought by employing (1,2-^{3}H)GA_1 in pH 8 0 buffer The results, presented in Table 1, indicate virtually complete recovery of (1,2-^{3}H)GA_1 from the PVP slurries

TABLE 1 RECOVERY OF (1,2-^{3}H)GA_1 FROM PVP SLURRIES

Treatment	Recovered (counts/min)	Percentage recovery of ^{3}H-GA_1*
Control 1	1 076 × 10^6	96 9
Control 1′	1 057 × 10^6	95 2
PVP 1	1 081 × 10^6	97 4
PVP 1′	1 087 × 10^6	97 9

* Given as percentage of calculated total counts/min originally present (1 11 × 10^6 counts/min)

Hence, despite the presence of hydroxyl and carboxyl groups, both GA_3 and GA_1 can be quantitatively recovered from PVP slurries in slightly alkaline or mildly acidic buffer solutions.

Column Chromatography with PVP

As obtained from the manufacturer, Polyclar AT particles range in diameter from greater than 60 mesh (250 nm) to less than 300 mesh (53 nm) [5] The effect of particle size on retention volumes and elution profiles was investigated with 100 μg abscisic acid (ABA) in pH 8 0 buffer on each of three PVP columns containing particles of diameters 60–120 mesh, 120–160 mesh and less than 160 mesh For all columns, fraction 9 (ml 81–90) contained the highest concentration of ABA (70–85 μg) The three elution profiles were very similar with a slight tendency for sharper peaks with the finer particle sizes Quantitative recovery (99%) of ABA was obtained from all columns

Since the presence of many smaller diameter particles imposes severe limitations on flow rates while apparently having little effect on retention volumes or elution profiles of compounds, particles of a size range from 60 to 120 mesh were selected for general use in the columns, and all data given below pertain to this size range

The elution volumes and band widths of a number of GA's, ABA, indole-3-acetic acid (IAA) and zeatin are given in Table 2 At both pH 8 0, and pH 5 0 some degree of selectivity is demonstrated among the various GA's and ABA, while considerable selectivity is appa-

rent between these compounds and IAA or zeatin which are retarded to a much greater extent on the PVP matrix. Gibberellins A_4, A_7 and A_9 are retarded to a greater degree than A_1, A_3, A_5 and A_8. This difference in retardation is not readily explicable, although it should be noted that A_1, A_3, A_5 and A_8 all posses a hydroxyl group at the C-13 position, whereas A_4, A_7 and A_9 do not.

A considerable influence of pH upon retention times of the various compounds is also evident In general the effect is in accordance with the results of Andersen and Sowers[4] who found increased tendency for PVP-plant phenolic bond formation in progressing from alkaline to acidic solutions, with optimum conditions for complex formation at pH 3 5 Among the GA's tested, this tendency is most evident with GA_{13} which possesses 3 carboxyl groups for bonding with the PVP matrix. At the alkaline pH, these groups would largely be dissociated and show relatively small affinity for bonding, whereas at pH 5 0 the compound is strongly retarded by the PVP matrix where a considerable proportion of these groups would be present in the acid form

TABLE 2 ELUTION VOLUMES OF PLANT HORMONES ON 1 9 × 30 cm PVP COLUMNS AND EFFECT OF pH ON BONDING

Compound	Elution volume* (ml)		Range of detection† (ml)	
	pH 8 0	pH 5 0	pH 8 0	pH 5 0
GA_1	90	100		
GA_3‡	90	100	70–120	75–125
GA_4	120	140		
GA_5	90	100	70–120	75–125
GA_7	120	140	90–160	110–170
GA_8	85	90	60–110	70–120
GA_9	110	125	80–140	100–160
GA_{13}	85	280	60–110	240–320
ABA	90	115	70–120	90–140
IAA	230	470	160–290	380–550
Zeatin	290	230	230–350	180–300

* Volume of eluate with maximum concentration of compound

† Each value representing average of at least 2 trials

‡ Dr G Sembdner, (Institut fur Biochemie der Pflanzen, 401 Halle/Saale, DDR) indicates that the glucoside of GA_3 (3-*O*-β-D-glucopyranosyl-GA_3) is quantitatively eluted one fraction before the acid from a 30 × 1 1 cm (10 g PVP, 10 ml fractions) column of PVP eluted with 0 1 M, pH 8 0, phosphate buffer

Also of interest is the behaviour of zeatin which, in contrast to the other compounds tested, exhibits a greater affinity for the PVP matrix at pH 8 0 than at pH 5 0. This situation might be explicable in terms of rupture of the hydrogen bond involving the zeatin amino group in acid solution.[7]

Differences in selectively at various pH's may be used to advantage for separation of components (e g. GA_3 and ABA) which have identical retention volumes at higher pH's but which exhibit different affinities for the PVP matrix in more acidic solutions. Thus, employing 1 9 × 120 cm PVP columns, and elution at pH 4 4, GA_3 is eluted in the range 390–480 ml and ABA in the range 490–600 ml

It is highly probable that in currently used procedures, ABA and many of the endogenous plant GA's are extracted simultaneously The ability to effect a separation of ABA from

GA's present in the same extract may be an important consideration since the former has been shown to be a potent inhibitor in several commonly used GA bioassays (see review by Addicott and Lyon[11]). Conversely, the occurrence of GA's might mask the presence of ABA in bioassays designed to detect inhibition of growth or induction of dormancy produced by this hormone We have found that chromatography in buffer on Sephadex G-10 or G-25 columns is impractical for this separation since ABA and GA_3 have nearly identical retention volumes (reaching maximum concentrations at ml 120 from a $1{\cdot}9 \times 40$ cm column) As well, ABA is eluted[12] in approximately the same position as GA_4 and GA_7 on the gradient elution partition column of Powell and Tautvydas,[13] and its complete separation from from GA's in the thin-layer or paper chromatographic procedures currently in use is unlikely.

Recovery of Hormones from PVP Columns

Essentially quantitative recovery (90–97%, as estimated by examination of GLC traces) of GA_1, GA_3, GA_4, GA_5, GA_7, GA_8, GA_9, GA_{13}, GA_{14} and GA_{17} was obtained from 60 to 120 mesh PVP columns

As an additional check, 10^3 μg GA_3 was chromatographed on a PVP column and a 200 ml fraction of eluate collected. Two subsequent 500 ml fractions were collected, extracted and taken to dryness in preparation for application on the 'Tanginbozu' dwarf rice microdrop bioassay[14] which is capable of detecting $1\,0 \times 10^{-4}$ μg GA_3 per plant. The residues of the two subsequent fractions were applied to rice seedlings at dilutions of 1/1000, 1/500 and 1/100, and failed to show biological activity above control levels In a previous test, the residue from a pure buffer eluate of the PVP columns had failed to show either inhibitory or growth promoting activity when tested on the rice microdrop bioassay, either alone or in combination with standard amounts of added GA_3 Only when assayed on the barley aleurone α-amylase assay[15] at 1/5 dilution was any activity evident, and even here only in the first 500 ml fraction It is possible then to calibrate a column with an easily detectable amount of the hormone, wash thoroughly, and subsequently use the same column for experimental procedures involving plant extracts. In fact, if the upper 10 cm of the PVP column is removed and replaced with fresh PVP, the column can be washed and re-used for purification of a number of plant extracts.

Complete recovery of ABA from PVP columns has been recorded above, trials with IAA and zeatin at pH 8 0 yielded recoveries of 93 and 96%, respectively.

Efficiency of Insoluble PVP in Purification and Separation of Gibberellin-like Substances from Plant Extracts

In order to assess the effectiveness of PVP in removing impurities present in GA extracts, crude acidic ethyl acetate-soluble fractions (ranging from 300–500 mg dry wt. in several trials) from 20 g freeze-dried vegetative shoots of the conifer *Cupressus arizonica* were chromatographed on PVP columns Residues (37–85 mg dry wt) recovered from column eluates represented dry wt reductions to 1/10 to 1/5 of original values In no instance was

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biological activity (as shown by the barley aleurone α-amylase and rice seedling bioassays), of the extracts diminished by this procedure, in fact, it was invariably enhanced

In trials where the eluates from the above-mentioned columns were re-chromatographed on 1·9 × 120 cm PVP columns, the fractions comprising the GA_3 elution zones (320–400 ml) represented further dry wt reductions to 1/50 to 1/60 of the original dry wt Other experiments using extracts from seedlings of *Phaseolus*, *Pisum*, pollen of *Pinus*, and foliage of *Pseudotsuga* have given 60–70-fold reductions in dry weight when the crude acidic ethyl acetate soluble fraction was chromatographed first on a 30 cm PVP column (ml 51–250 collected and bulked), then on a 120 cm PVP column (ml 261–710 collected and bulked). Levels of endogenous GA's as detected by bioassays after further purification and chromatography on silica gel partition columns in these tissues range from 100–300 μg/kg dry wt in vegetative shoots of Arizona cypress, to 100–200 μg/kg fr wt. in pollen of *Pinus*, 10–20 μg/kg fr wt in seedlings of *Pisum*, and 100–150 μg/kg dry wt in foliage of Douglas-fir

The dual column technique was also employed in a trial designed to determine if plant extracts might contain compounds which complex with GA's and subsequently bind to the PVP matrix Hence, crude extracts from 20 g of *C arizonica* tissue were 'spiked' with 100 μg of GA_4, GA_5, GA_7, GA_9 and GA_{13}, 200 μg of GA_3, and GLC used as a method of quantitative detection. The relatively large amounts of 'spike' were required for accurate quantitation by GLC because of the presence of a high background of impurities from the plant extract A bioassay method could not be used due to the sensitivity of all of the known assays to impurities (i.e. an accurate baseline for the 'control' extract which remained untreated with PVP could not be established accurately and those impurities remaining after PVP treatment still affected the assay, thus giving values which were too low) Quantitative recovery (90 + %) of all of the above GA's after chromatography on a 30-cm and a 120-cm column of PVP was obtained.

Based on the above experiments, we recommend the use of PVP in column chromatography as a safe and efficient method of purification of plant extracts containing a broad spectrum of GA's and/or ABA A 50–70-fold reduction of dry weight is possible with essentially no loss of gibberellins or ABA, and bioassay activity is invariably enhanced Also, PVP may be useful in purification of plant extracts containing IAA and phytokinins as is evidenced by the quantitative elution of IAA and zeatin in column chromatography. The glycosides of at least one gibberellin is eluted quantitatively from PVP (Table 2) and correspondence with G. Sembdner indicates that PVP is a useful tool for purification of crude butanol soluble extracts of the aqueous phase.

The possibility of chromatography of gibberellins using PVP exists, especially if pH and length of column are varied to affect elution pattern. As well, ABA can be separated from at least certain of the gibberellins by sequential chromatography on PVP columns at several pH's.

EXPERIMENTAL

Materials Insoluble PVP (trade name Polyclar AT Powder) was used in all experiments.

Lyophilized vegetative shoots of 8-month-old *Cupressus arizonica* Greene seedlings were extracted to yield the crude, acidic ethyl acetate-soluble fraction[2] which was tested by PVP chromatography.

Gibberellins A_1, A_3, A_4, A_5, A_7, A_9, A_{13}, A_{14}, A_{17}, ABA, IAA and zeatin solutions were prepared immediately prior to each run by dissolving in 0 1 M pH 8 0 phosphate buffer, and, unless otherwise noted, all buffers employed were of this composition Quantitation experiments were conducted using 200 μg quantities of gibberellins A_1, A_3, A_4, A_5 and A_{13} and 100 μg quantities of gibberellins A_8, A_9, A_{14}, A_{17}, IAA, ABA and zeatin.

Radioactive GA_1 [(1,2-^{3}H)GA_1] the radioactive composition of which consisted of approximately 35% of (1,2-^{3}H)GA_1, 50% tetrahydro derivatives, and 15% unknown compound was used in the slurry technique. One μCi of (1,2-^{3}H)GA_1 (sp act of 753 4 mCi/mM) was used in each duplicate run for experimental and control (no PVP) treatments

Use of PVP for slurries and chromatography columns For use in batch procedures, PVP at concentrations ranging from 50 to 100 mg ml^{-1} was added to buffer solutions of extracts or standards and slurried thoroughly with constant shaking for 30 min [4] The PVP was filtered off by vacuum filtration and the residue washed with successive aliquots of buffer Fresh PVP was then added to the filtrate and the shaking and filtration procedure repeated for a total of 3 times

In preparation for use in column chromatography, dry Polyclar AT powder was processed through a series of sieves (60 mesh/120 mesh/160 mesh) with constant shaking for at least 2 hr Particles of 60–120 mesh size range were mixed thoroughly with at least 5 times their volume of distilled H_2O and remaining fines decanted after a settling period of about 15 min Fine mesh size particles required longer settling times

Following 6–8 decantations, the slurry was poured into 1 9 × 45 cm (or on occasion 1 9 × 130 cm) columns, employing glass wool overlain by glass beads as a support The columns were packed to a height of 30 (or 120) cm with the aid of gravity flow and a disc of filter paper placed over the bed to prevent subsequent disruption Unless otherwise noted, all columns contained particles of 60–120 mesh packed to a height of 30 cm These columns yielded flow rates of approximate 250 ml hr^{-1} Columns of the same height but prepared with 120–160 mesh and particles of less than 160 mesh diameter had flow rates of 65 and 25 ml hr^{-1}, respectively

Columns were equilibrated with the appropriate eluent prior to use in chromatography Standards and extracts were applied to the columns singly or in mixtures, in solutions of from 50 μg to somewhat more than 1 mg in 5 ml or less of buffer Elution was carried out with 0 1 M phosphate buffer (except at pH 4 4 where a 0 1 M KH_2PO_4 solution acidified to pH 4 4 with HCl was employed) and delivered from reservoirs 20–30 cm above the column at room temp

Volume of eluate was measured from the first ml of test solution applied to the top of the PVP matrix Usually 10 ml fractions were taken for determination of retention volumes Otherwise appropriate bulked volumes (usually 200 ml for quantitation trials) of eluate were collected for subsequent extraction and analysis In a control run, the compound in question was left in 200 ml buffer solution for the length of time required to collect the equivalent amount of eluate from the columns, and then processed as per the PVP run

Recovery of gibberellins or ABA from filtrates or eluates was effected by acidifying the solutions to pH 2 8 with HCl and partitioning 5 times against EtOAc (buffer–EtOAc, 5 3 v/v) The combined extracts were dried, filtered and evaporated to dryness *in vacuo* Residues were taken up with successive aliquots of MeOH–EtOAc (1 1) and prepared for analysis by GLC or TLC

Detection and analytical methods Qualitative detection of GA's and ABA in column eluates was accomplished by addition of 3 ml conc H_2SO_4 to the buffer solutions and observing fluorescence under UV (225 nm) light This method served to detect less than 1 μg ABA, about 1μg GA_7, 2 5 μg GA_9, 10 μg GA_3 and GA_4, and 100 μg GA_5 and GA_1 in 10 ml buffer solution

Alternatively, the buffer solution was spotted on TLC plates directly, or extracted into EtOAc and the residue spotted on the plates Plates were sprayed with conc H_2SO_4–H_2O (7 3) and viewed in UV light [16]

ABA, IAA and zeatin in column eluates were monitored in phosphate buffer by their absorption maxima at 245 (ABA), 280 (IAA) and 268 nm (zeatin) Estimates of percentage recovery were made on the basis of comparisons of absorbance readings with standard curves in buffer, prepared for each hormone

A dual column F & M 402 gas chromatograph with heated injectors and flame ionization detectors was employed for the quantitative assay of all gibberellins Silanized 183 × 0 32 cm i d glass colums were packed with 2% QF-1 or 2% SE-30 on Gaschrom Q Columns were pretreated by baking for 24 hr at 250° with N_2 carrier gas flowing at 30 ml min^{-1} Chromatography was carried out at oven temp of 200°, injector and detector temp of 240° and 250° respectively, and N_2 flow rates of 60 ml min^{-1} and 70 ml min^{-1} on the 2% QF-1 and 2% SE-30 columns respectively Efficiency of the 2% QF-1 and 2% SE-30 columns were 1950 and 5090 theoretical plates respectively as calculated by α-cholestane The methyl esters and the methyl ester trimethylsilyl ethers were prepared for chromatography by the method of Cavell *et al* [17]

The methyl esters were chromatographed for all GA's except A_8 for which the methyl ester trimethylsilyl ether was used A known quantity of each methyl ester or methyl ester trimethylsilyl ether mixture was chromatographed on GLC, and the area under each peak was measured Dilution and reinjection of controls was necessary since it was found that GLC peak areas did not exactly correspond to gibberellin derivative concentrations The error in this method was estimated to be approximately 6%

Where (1,2-^{3}H)GA_1 was used, a Packard Liquid Scintillation Counter was employed to measure radioactivity present in experimental and control residues dissolved in a Brays (Modified) Counting Medium consisting of 100 g napthalene, 5 g PPO and 0 25 g POPOP dissolved to 1 l in dioxane–toluene–EtOH (78 12 10, v/v) solution

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