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New Methods of Chromatographic Separation of Gibberellins A_1 and A_3

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Nové způsoby chromatografického dělení giberelinů A_1 a A_3

K dělení fyziologicky aktivních giberelinů A_1 a A_3 byly použity některé nové chromatografické metody — chromatografie v odstředivém poli a chromatografie na tenké vrstvě kysličníku hlinitého. Rovněž byla zkoumána papírová elektroforesa, která se však pro dělení giberelinů A_1 a A_3 nehodí. Pro chromatografii v odstředivém poli se osvědčila soustava benzen-kyselina octová-voda (4 : 1 : 2). Vyvíjí se na přetečení za indikace bromfenolovou modří, až její skvrna dosáhne R_F 0,75. K dělení na tenké vrstvě kysličníku hlinitého byla vyzkoušena soustava benzen-kyselina octová (100 : 23). Chromatografuje se na přetečení průtokem asi 60 ml chromatografické směsi při rozměrech tenké vrstvy 10 × 23 cm. Detekci skvrn lze provést v obojím případě 0,5% manganistanem draselným. Detekce skvrny giberelinu A_3 70% kyselinou sírovou se v případě kysličníku hlinitého neosvědčuje. Lze ji nahradit 70% kyselinou chloristou, která reaguje i v případě tenké vrstvy. Obě analýzy trvají asi 6 hodin, což zkracuje dobu potřebnou k jejich provedení na jeden pracovní den.

Summary

New chromatographic methods, chromatography in centrifugal field and thin-layer chromatography on alumina, were used for separating physiologically active gibberellins A_1 and A_3. Paper electrophoresis was found unsuitable for the separation of the two gibberellins. For centrifugal chromatography the system benzene-acetic acid-water (4 : 1 : 2) was found convenient. In both the centrifugal and thin layer chromatography the overflowing

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*Addresses: Dr. Milan Kutáček, Výzkumný ústav rostlinné výroby ČSAZV, Praha-Ruzyně and Ing. Jan Rosmus, Ing. Zdeněk Deyl, Ústřední výzkumný ústav potravinářského průmyslu, Praha-Smíchov, Na bělidle 21 technique was applied with great success. In the case of centrifugal separation bromophenol blue was used to indicate the position of the solvent front. Development was stopped when the spot of the indicator reached 3/4 of the paper length. For separating on a thin layer of alumina the solvent system benzene-acetic acid (100 : 23) was used. Here again the solvent was left to overflow; for dimensions of the thin layer of 10 × 23 cm 60 ml of the solvent mixture were used. The spots were detected in both cases with 0·5% potassium permanganate. The detection of the spot of gibberellin A_3 using 70% sulphuric acid after thin layer chromatography was found unsufisfactory. Sulphuric acid may be replaced by 70% perchloric acid. Both analyses take about 6 hrs. which reduces the time necessary for their completion to one working day.

Introduction

The significance of various methods for the separation of gibberellins increases simultaneously with the the number of experimental results and the development of the industrial production of these compounds. TAKAHASHI and co-workers (1955) described several chromatographic systems for the separation of gibberellins by paper chromatography. PHINNEY and co-workers (1957), however, did not give a good account of these systems. The basic components of these systems were benzene, acetic acid and water. No reliable method of separation was described before BIRD and PUGH (1958). They also used benzene, acetic acid and de-ionised water (4 : 1 : 2). Using the overflowing technique a good separation of gibberellin A_1 from A_3 (gibberellic acid) was achieved in this solvent system. The separation process, however, lasts at least 36 hours.

The present communication summaries our attempts to reduce the time necessary for the separation of these physiologically active substances. Some new methods of chromatography were used, such as chromatography in the centrifugal field and chromatography on a thin layer of alumina. Attempts were also made to separate these substances by paper electrophoresis.

Methods

Centrifugal chromatography. One of the modern methods used for reducing the time required for separation by paper chromatography is chromatography in a centrifugal field. It was first described by CARONNA (1955) and MACDONALD (1957).

For the separation of gibberellins an apparatus for centrifugal chromatography with central point development described by PAVLÍČEK, ROSMUS and DEYL (1961) [see also PAVLÍČEK and DEYL 1959] was used. This apparatus ensures better quality of separation than the abovementioned devices. Whatman paper No. 3 was used throughout as it is better suited to the apparatus on account of its greater flui dcapacity. A 30—40 μl. sample (1·0 mg./ml.) was placed 1·5 cm. from the centre of rotation.

Chromatography on a thin layer of alumina. Chromatographic separation was carried out on an untreated layer of alumina 1,0 mm. thick which was prepared according to MOTTIER and POTTERAT (1955). Separation of the gibberellins was attempted in solvent systems containing benzene and acetic acid in different ratios. In view of the fact that after a single development with a mobile phase containing a higher proportion of acetic acid the desired resolution was not attained, a solvent containing a lower concentration was used. The migration of the gibberellins here was slower and in order to attain a good resolution it was necessery to run the chromatogram with overflow. For this purpose the alumina layer was surrounded longitudinally with thin glass strips slightly higher than the alumina layer. A thicker glass plate about 2—3 cm. shorter than the alumina layer was placed on the top. This formed a chamber with an open end. The solvent was brought in through a filter paper strip partly covered with polyethylene foil. One end of the filter paper strip reached into the layer of alumina, the other end was submerged into the mobile phase in a flask which also served as support for the whole chamber. At the side of solvent inlet the chamber was enclosed with another piece of glass. On the other side of the chamber the solvent was permitted to evaporate freely. The length of the chamber must be relatively greater so that the evaporation from the end of the layer does not interfere with the separation. A chamber about 22 cm. in length was used here. The apparatus used represents a simplified version of the apparatus for continuous-flow chromatography on a thin layer as described by BRENNER and NIEDERWIESER (1961).

Paper electrophoresis. Attempts at separating the gibberellins were carried out by low-voltage paper electrophoresis at a working voltage of 300 V using McIlwain's buffers diluted with water (1 : 2).

Detection. Detection techniques described by BIRD and PUGH (1958) were used. The spots of gibberellins A_1 and A_3 can be revealed by spraying with 0·5% aqueous potassium permanganate. After one or two minutes of treatment the unreacted permanganate is washed out with water. This produced brown spots on a white background. Gibberellin A_3 can be specificially detected by passing the chromatogram through 70% sulphuric acid. The chromatogram is then placed on a glass plate and the yellow-green fluorescence of gibberellin A_3 is observed (UV light from Philora 125 W). Perchloric acid (70%) was also used for this detection. Gibberellin A_3 produces blue fluorescence while giberellin A_1 does not react at all. Spray with 5% aqueous perchloric acid was also used for detecting gibberellin A_3 on paper. On applying this agent and heating, fluorescence is also produced and the paper remains intact. All the detection agents were also used on the thin layer of alumina.

Results

Chromatography in centrifugal field

For centrifugal chromatography the apparatus with central point development according to PAVLÍČEK, ROSMUS and DEYL (1961) was used. Benzene (thiophene-free)-acetic acid-water (4 : 1 : 2) which had been found suitable by BIRD and PUGH (1958) for descendent development were used for the centrifugal development, too. The solvent was left to overflow until the indicating spot of bromophenol blue reached 3/4 of the diameter of the paper which took about 6 hrs.; the time required could be still reduced by increasing the number of centrifuge revolutions. The chromatogram was detected by spraying with 0·5% aqueous potassium permanganate. The quality of separation of the gibberellins is satisfactory as follows from Fig. 1.

Thin-layer chromatography

For separating gibberellins in amounts of 60—70 μg, a mixture consisting of 100 parts of thiophene-free benzene and 23 parts of acetic acid was found to be suitable. The migration of gibberellins depends on acetic acid content in the mobile phase; with decreasing ratio of acetic acid their mobility decreases. After passage of about 60 ml. of the mobile phase through a chromatogram 10 × 23 cm. in size, the spot of gibberellin A_1 moves about 12·5 cm. from the start and that of gibberellin A_3 about 8·5 cm. The passage of 60 ml.

solvent is effected within about 6 hrs. Benzene can be replaced with ethanol in the solvent mixture. Somewhat moist chromatograms were detected by lightly spraying with 0·5% potassium permanganate. After a while yellow-brown spots appeared on a violet background. Contrary to expectation, sulphuric acid is not suitable for detecting gibberellin A_3. Blue fluorescence of gibberellin A_3 can be brought about by spraying with 70% perchloric acid and subsequent heating to 80—90° for 10 min.

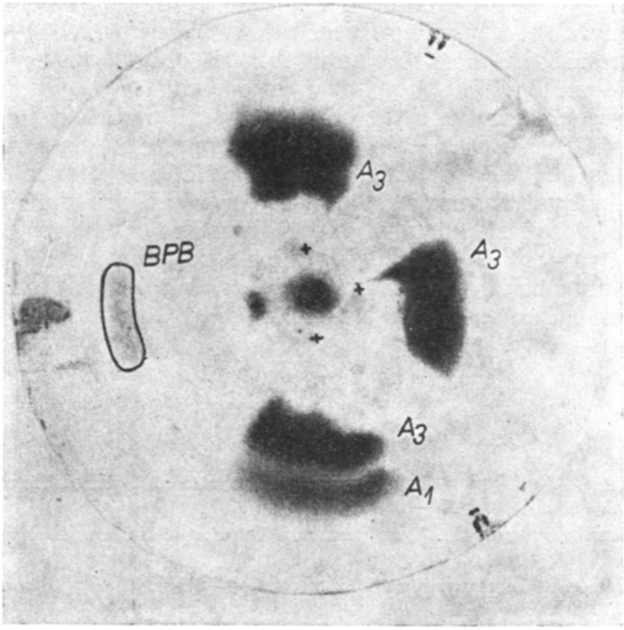

Fig. 1. Chromatographic separation of gibberellins A_1 and A_3
BPB — indicator spot of bromophenol blue.

Paper electrophoresis

Paper electrophoresis does not effect separation of gibberellins A_1 and A_3. Maximum mobility of the combined spot was observed at a pH of 5·5. Electrophoresis can be used for separating gibberellins from indoleacetic acid as pointed out earlier by KREKULE and ULLMANN (1958).

Discussion

Analytical separation of gibberellins presents certain difficulties. Among the gibberellins, greatest attention has been concentrated on the physiologically active gibberellins A_1 and A_3. On applying paper chromatography and different mobile phases the gibberellins are known to migrate but their spots cannot be resolved. This is well apparent from the table published by WEST (1958) in which gibberellins and gibberellin-like substances are listed according to their R_F values. Most suitable for the separation appear to be systems containing mostly benzene along with a smaller amount of acetic acid; in such systems the movement of the spots is quite slow and spots are resolved only on passage of a greater amount of solvent through the paper. The present work proceeds from this principle of letting the solvent overflow the paper. It is the advantage of the new chromatographic techniques that the mobile phase passes rapidly through the paper. It could therefore be assumed that their application will be suitable for separating the gibberellins A_1 and A_3 and that the time required for separation will be substantially reduced. Centrifugal chromatography was found to be suitable, the resolving power of which is better in this case than that of the thin-layer chromatography. It happened occasionally that in the thin layer the elongated spots of the gibberellins overlapped. This phenomenon can be prevented by reducing the acetic acid content in the mobile phase and by increasing the amount of passing solvent. Both methods represent a general time saving as a mixture of gibberellins A_1 and A_3 can be analyzed within a day.

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Новые методы хроматографического разделения гиббереллинов A_1 и A_3

Для разделения физиологически активных гиббереллинов A_1 и A_3 использованы нами некоторые новые хроматографические методы — хроматография в центробежном поле и хроматография на тонком слое окиси аллюминия. Также изучался электрофорез на бумаге, который, однако, для разделения гиббереллинов A_1 и A_3 не годится. Для хроматографии в центробежном поле больше всего подходит смесь бензен-уксусная кислота-вода (4 : 1 : 2). Хроматография проводится на перетечение при индикации движения мобильной фазы бромфеноловой синей до тех пор, пока ее пятно не достигнет 0,75 длины хроматографической бумаги. Для разделения на тонком слое окиси аллюминия испытывалась смесь бензен-уксусная кислота (100 : 23). Хроматографируется приблизительно 60 мл-ами хроматографической смеси при размерах слоя 10 × 23 см. Определение пятен возможно осуществить в обоих случаях 0,5% гиперманганатом. Определение пятен гиббереллина A_3 70% серной кислотой в случае окиси аллюминия не оправдало себя. Ее возможно заменить 70% хлорной кислотой которая реагирует и в случае тонкого слоя. Оба анализа длятся приблизительно 6 часов, что сокращает время необходимое для их проведения на один рабочий день.